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Notice

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Meet the editor

Dr. Deniz Ekinci obtained a Bachelor's degree in Chemistry in 2004, a Master's degree in Biochemistry in 2006 and a Doctorate degree in Biochemistry in 2009 from Atatürk University, Turkey. He studied at Stetson University, USA, in 2007-2008, and at the Max Planck Institute of Molecular Cell Biology and Genetics, Germany, in 2009-2010. Dr. Ekinci currently works as Associate Professor of Biochemistry at the Faculty of Agriculture, and is the Head of the Enzyme and Microbial Biotechnology Division, Ondokuz Mayıs University, Turkey. He is a member of Turkish Biochemical Society, American Chemical Society and German Genetics society. Dr. Ekinci published over seventy scientific papers, reviews and book chapters and presented at several conferences. His research interests include enzyme inhibitors, drug design, protein dynamics, toxicology and lipidomics. His recent work has focused on antioxidant and metabolic enzyme systems. He has received numerous publication awards from several scientific councils. Dr. Ekinci serves as the Editor in Chief of four international books and is involved in the Editorial Board of several international journals.

Contents

Preface

Biotechnology is a discipline at the intersection of biology, chemistry, pharmacology and various other biological specialties, where they are involved with use of living systems and organisms to develop or make useful products. It often overlaps with the related fields of bioengineering and biomedical engineering depending on the tools and applications.

Biotechnology uses technological applications that utilize biological systems, living organisms or derivatives to make or modify products or processes for specific use. For many years biotechnology has been used by humans in agriculture, food production and medicine. In the late 20th and early 21st century, biotechnology has expanded to include new and diverse sciences such as genomics, recombinant gene technologies, applied immunology, and development of pharmaceutical therapies and diagnostic tests.

This book titled *"Biotechnology"* contains a selection of chapters focused on the research area of molecular biology, molecular aspects of biotechnology, synthetic biology and agricultural applications in relevant approaches. The book provides an overview on basic issues and some of the recent developments in biotechnological studies. Particular emphasis is devoted to both theoretical and experimental aspects of modern biotechnology. The primary target audience for the book includes students, researchers, biologists, chemists, chemical engineers and professionals who are interested in associated areas.

The book is written by international scientists with expertise in chemistry, protein biochemistry, enzymology, molecular biology and genetics, many of which are active in biochemical and biomedical research. I would like to acknowledge the authors for their contributions. We hope that the book will enhance the knowledge of scientists in the complexities of some medicinal approaches; it will stimulate both professionals and students to dedicate part of their future research in understanding relevant mechanisms and applications.

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Chapter 1

Current Concepts and Translational Uses of Platelet Rich Plasma Biotechnology

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Additional information is available at the end of the chapter

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1. Introduction

A two-decade long research has expedited knowledge about tissue repair mechanisms, and the field of Regenerative Medicine is gaining ground stimulated by novel insights and the development of therapeutic biotechnologies, intending to restore tissue architecture and functionality. Regenerative Medicine technologies concern not only traumatic tissue injuries but also involve the biological manipulation of pathological conditions aiming to drive tissue circumstances to normal, i.e. the recovery of tissue homeostasis.

Recent advances in biology and the new understanding of mechanisms such as angiogenesis, inflammation and main cell activities including proliferation, differentiation and metabolism have prompted researchers to seek how to manipulate these aspects of tissue and cell biology. Translation of this knowledge into the development of regenerative medicine technologies is imperative in order to address the current health care demand markedly boosted by demographic changes. Indeed the dramatic increase in the economic and social burden of chronic and degenerative diseases urges the development of novel therapies.

Biological interventions in Regenerative Medicine fall into four main categories including gene therapy, tissue engineering, cell-based therapies, and platelet rich plasma (PRP) therapies, with different success in clinical translation. For example, tissue engineering approaches, i.e. cells loaded within scaffolds, are in development but still several limitations of 3D tissue constructs are unresolved; these questions include biocompatibility, improvements in mechanical properties and/or the size of the 3D constructs [1]. Similarly, the efficacy of different categories of cell therapies, including mesenchymal stem cells, embryonic stem cells or induced pluripotent stem cells (iPSC), is being tested [2]. However, while registration of new clinical trials using MSCs derived from the bone marrow or from adipose tissue is growing rapidly supported by both public and private investments, the iPSC therapies are advancing at a slower

pace because reprogramming raises serious concerns about safety because of their genetic instability and potential to form tumors.

PRP, an autologous plasma fraction of peripheral blood, is the simplest regenerative medicine intervention that is rapidly extending to multiple medical fields mainly due to the easy use and biosafety that facilitates translation in humans. In fact, regulatory requirements for cell therapy involve multiple preclinical experiments to demonstrate their safety and non-teratogen effects in addition to GLP compliance in the preparation, and the use of adequate expensive installations [3]. In contrast, PRP therapies involve minimal manipulation, and in general, regulatory requirements are easy to comply thereby facilitating the widespread clinical use and commercial success of PRP kits and devices. In fact, PRP can be prepared by using any of the commercial systems available. PRPs can also be prepared by in house procedures, providing that basic rules of quality are implemented.

While regenerative medicine with cells is directed to inherent non-healing problems and a wide range of pathological conditions, PRP embrace normal healing conditions such as tissue repair during surgical invasion or traumatic injuries seeking to enhance and accelerate physiological repair. Alternatively, PRPs as occurs with cell therapies, seek to direct non-healing conditions, e.g. chronic conditions such as osteoarthritis (OA) or tendinopathy, towards healing and restoration of tissue homeostasis.

Due to the biosafety of these products, i.e. advantageous balance risk-benefit, clinical applications have preceded the basic research. Actually, in its very beginnings PRPs have been used with a vague idea of the biological mechanisms they were influencing. Thereafter, most studies were directed to examining clinical outcomes rather than identifying the precise biochemical mechanisms underlying PRP effects, which remain to be elucidated in the most part. In fact, PRP widespread use was not driven by the principles of the scientific methods instead patient demand has been boosted by sports news and propaganda reporting that outstanding elite athletes had been successfully treated with PRP. The need is clear, to investigate and describe main PRP targets and action mechanisms underlying their clinical effects. In fact, translational medicine addresses both, the biological and the clinical aspect of the novel biotechnologies.

In this book chapter, first, we will discuss recent progress on understanding the tissue regeneration process with a particular focus on the healing stages, and the role of PRP released signaling proteins in targeting different cells and inducing paracrine actions. Current biological interventions aiming tissue regeneration stem from two concepts, namely cells responsible for tissue homeostasis, and the signaling cytokines that control cell fate. Several cell phenotypes are involved in tissue repair and some processes such as inflammation and angiogenesis are commonly involved in the repair process in several conditions. Hence, several notions of tissue repair mechanisms are compatible with the biological hallmarks of regeneration in different tissues. Common mechanisms involved in healing can be modulated using PRP. This is the basic knowledge to drive clinical applications.

Second, from a practical point of view on PRP biotechnology we will discuss the main formulations, and summarize commercial systems to prepare PRP. Regulatory requirements will be briefly exposed.

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Lastly, we will focus on translational uses, that is to say current PRP interventions from the clinical investigation perspective. We will summarize PRP applications in surgery with special emphasis in novel developments, the current use of PRP in ulcers, ophthalmology and dermatology, as well as foremost conservative treatments in orthopedics and sports medicine.

We will discuss main obstacles for the advancement of PRP science and future perspectives.

2. Tissue repair and regeneration

Despite growing knowledge on tissue regeneration mechanisms currently we are incapable to fully regenerate human tissues. The only approximation to tissue regeneration in the human body is the so-called "compensatory regeneration" in the liver. In fact, after lobe removal the liver compensates the loss and recovers its former size by balanced proliferation of all the existing cell types, including hepatocytes, kupffer macrophages, endothelial cells, duct cells, and fat storing cells. Moreover, these cells retain their functional identity and are able to produce all the liver-specific enzymes necessary for liver function [4].

In contrast to the lack of regenerative mechanisms in humans where there is no return to the embryonic state and no recapitulation of differentiating mechanisms, some amphibians as the salamander, after amputation replace their body parts by recapitulating embryological events. In these amphibians regeneration involves reactivation of developmental mechanisms in the post-natal life to restore wounded tissues identically as they were before injury.

Research in this area of experimental biology has provided useful information to the field of Regenerative Medicine. For example, the study of amphibians offers important insights into the mechanisms involved in the regeneration of complex structures. Indeed, after limb amputation in the salamander, a mass of undifferentiated cells called blastema is formed, and the blastema is capable of growing into different body parts [5].

Nevertheless, dramatic differences between frogs and salamanders in tissue repair/regeneration exist. Indeed adult frogs, despite being amphibians, cannot recapitulate embryologic mechanisms in their adult life. These differences are mainly attributed to at least three broad dissimilarities, first in their immune systems, secondly in cell differentiation mechanisms, and lastly in their potential for nerve regeneration [6].

Therefore these three notions derived from studies on experimental biology will drive our exposition of potential layers of PRP control in healing mechanisms. We will focus firstly, on immune-modulatory mechanisms i.e. the pattern of leukocyte infiltration (PMNs, monocytes, lymphocytes), and macrophage polarization, second the importance of stem/progenitor cell activation, and adequate differentiation, and third the requirement of nerve participation, as regeneration is dependent on the presence of nerves. In fact a minimum number of nerve fibers is necessary for regeneration to take place. We will emphasize the importance of an adequate crosstalk between immune cells, progenitor cells as well as local differentiated cells and the paracrine actions.

All these regenerative events constitute different layers of biological control that can be influenced by PRP administration.

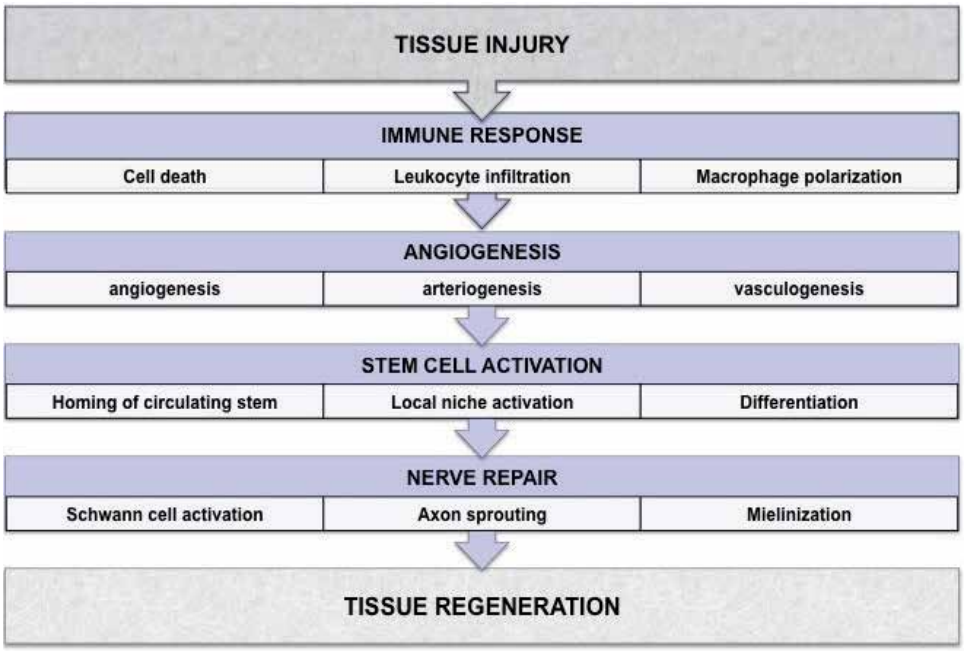

Figure 1. Potential layers for PRP influence in tissue regeneration

3. Outlook for the control of tissue healing using PRP

3.1. Inflammation

3.1.1. Cell death and DAMPs in the extracellular space

Injury in multicellular organisms is accompanied by cell damage and death, proportional to the magnitude of tissue injury that triggers a sophisticated sequence of reactions to cope with the insult. The degree of the inflammatory response depends on the severity of the injury that can induce different magnitudes of cell damage and death. Loss of cell integrity activates innate immune sensors by releasing to the extracellular space a myriad of intra-cytoplasmic molecules, known as DAMPs (Danger Activating Molecular Patterns). Among the DAMPs released by dying cells there is a growing list including cytosolic and nuclear proteins such as high mobility group box 1 (HMGB1), alarmins such as S100, and non-proteins including uric acid, DNA, RNA, and ATP. The inflammatory response triggered by the detection of DAMPs is an evolutionary conserved mechanism present in both vertebrates and invertebrates.

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DAMPs transmit stress signals to the organism, and stimulate innate immune responses, starting by leukocyte infiltration, following by macrophage polarization and closing with the resolution of inflammation. This set of mechanisms is known as the inflammatory response, and serves to minimize the insult, and repair the damaged tissue in doing so contributes to the recovery of tissue homeostasis.

Cell death can result from injury but can also occur physiologically as a component of tissue homeostasis, since all tissues in accordance with their physiologic turnover rate replace old cells by new ones. In tissue turnover cell death is not accompanied by any inflammatory reaction, probably because DAMPs in the extracellular space do not reach a threshold concentration. Importantly, errors in the control of immune homeostasis may be behind chronic diseases.

The administration of PRP during this phase can rescue damaged cells as PRP contains cytokines that can promote cell survival, as shown both in vivo and in vitro. For example, during cell auto-transplantation for the treatment of tissue defects in plastic surgery, the use of PRP increases the survival of pre-adipocytes and adipocytes. Pre-adipocytes treated with PRP showed anti-apoptotic activities and decreased the expression of molecular mediators of cell death including Bcl-2-interacting mediator of cell death [7]. Additionally PRP can protect human tenocytes against cell death induced by ciprofloxacin and dexamethasone [8]. Furthermore, PRP could alleviate BMSC death under hostile conditions increasing the levels of paracrine interactions via stimulation of PDGFR/PI K/AKT/NF-kB signaling pathway [9]. PRP also promoted rejuvenation of aged and senescent MSC in vitro [10].

TLR receptors and DAMP-TLR activation is thought to be important in restoring homeostasis after cell death. Recent research has added layers of complexity to our understanding of PRP, and information about how molecular components of PRP interfere with DAMP signaling through NF-kb illustrates the anti-inflammatory effect of PRP in several tissues [11].

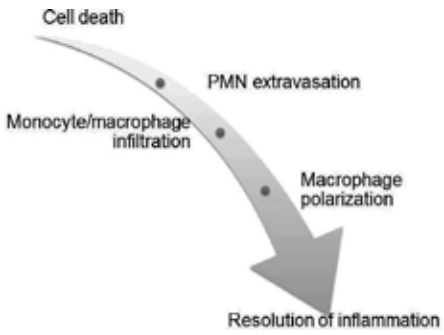
3.1.2. Pattern of leukocyte infiltration

The magnitude, pattern and timing of leukocyte infiltration are better described when tissue stress is induced by pathogens. However, in the case of sterile injuries, the extravasation of leukocytes in response to tissue damage is less understood. Actually, it is uncertain how PRP influences these three parameters: first, the magnitude of leukocyte infiltration, second, the pattern, and lastly, the timing.

The way PRP influences infiltrating immune cells is important because the latter play a major role in determining the outcome of tissue repair along with the secretory phenotype of local cells

3.1.2.1. Polimorphonuclear cell (PMNs) infiltration

The increase in vessel permeability and chemotactic signals from the injured tissues facilitates extravasation and movement of leukocytes within tissues by diapedesis. The use of PRP in this stage of healing modifies several aspects, first PRP increases vessel permeability by releasing



VEGF (also known as permeability factor, PF); in addition catecholamines such as dopamine and noradrenaline are delivered from dense granules in addition to histamine, all with synergistic effects in augmenting vessel permeability [12].

Polymorphonuclear cell (PMNs), including neutrophils (60-65% of the total leukocytes), eosinophils and basophils extravasate from the blood stream and perform a graded infiltration that reaches maximums in 12-24 h and is followed by decline, stop and apoptose. Excessive PMNs infiltration may be detrimental for the tissue because PMNs release a wide array of cytotoxic molecules. Granule components include several non-selective proteolytic enzymes, cytotoxins, antimicrobial peptides; in addition to the production of reactive oxygen species (ROS). The lifespan of neutrophils in the bloodstream is limited to hours but when they extravasate, the presence of DAMPs' agonists in the infiltrated tissues prolongs neutrophil survival.

PRP may influence both the amount of neutrophil infiltration and the survival of neutrophils in the injured tissues. In fact, PRP delivers both CCL and CXCL chemokines that attract different leukocyte subsets. In particular, CXCL7 (very abundant in platelets) in collaboration with NAP2 provides a strong chemotactic signal for neutrophil infiltration. In addition, PRP releases a known chemotactic cytokine for neutrophils, CXCL8/IL8. Moreover, we have recently shown that these chemotactic signals are reinforced and augmented by local cell synthesis in vitro [13]. PRP can also modify the lifespan of infiltrated leukocytes by modifying the molecular environment of the injury.

Thus, the administration of PRP would presumably modify the innate immune response, mainly by altering the molecular environment and the chemotactic driven pattern of neutrophil infiltration, the intensity and the timing. However, these effects may be dependent on the tissue conditions and anatomical location.

3.1.2.2. Monocyte/macrophage infiltration and polarization

During the initial days subsequent to injury (from 2 h to 72 h) monocyte/macrophages gradually infiltrate the tissue, ready to clean up apoptotic neutrophils. Indeed, macrophages are specialized in clearance of death cells.

The expression "macrophage polarization" refers to the ability of macrophages to change their functional phenotype in response to molecular signals they sense in their microenvironment.

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Macrophages have been categorized conventionally into pro-inflammatory M1 and tissue repairing M2 phenotypes. In the presence of LPS or IFN-gamma macrophages are "classically" polarized and denominated M1 macrophages. They have an inflammatory phenotype as they express IL-1b, IL-6, IL-8 and TNF-a.

Instead, in the presence of high levels of IL-4, M2 macrophages are "alternatively" polarized and they produce anti-inflammatory cytokines, including IL-10, IL-1Ra, CD-36, scavenger receptor A or mannose receptor. However, growing knowledge about macrophage plasticity indicates that M1/M2 polarization is an over-simplified view. As a matter of fact, a continuum range of polarization states exist between the two extremes M1 and M2.

Inflammatory mechanisms are protective mechanisms that should be ideally self-limited and lead to complete resolution returning to tissue homeostasis. Recent data indicate that M1/M2 activation states are extremely plastic to external signals and macrophages can be repolarized from M2 to M1 states although the mechanism is unknown [14]. Resolution of inflammation is an active process involving the biosynthesis of specialized pro-resolving mediators by M2 polarized macrophages.

Assuming that manipulation of macrophage polarization can be a tool for therapeutic exploitation, it is imperative to gain knowledge about how PRP influences macrophages. In fact, PRP modifies the environment and macrophages can gain distinct functions supporting their participation in inflammation or alternatively in the resolution of inflammation. Previous data showed that CXCL4/PF4 induces a polarization state distinct from M1 or M2 [15], and the term M4 polarization has been proposed. This is relevant because PF4 is one of the most abundant cytokines stored in platelets' alpha-granules (micromolar concentrations), and is released from platelets upon activation. However, M4 polarization has been studied in the context of atherosclerosis, but not in tissue repair.

Therefore, further research is indispensable to establish how PRP would influence the activation state of macrophages, and whether resolution of inflammation can be achieved by exposing macrophages to determined molecular environments.

3.1.3. Regulation of fibrotic pathways

Fibrotic tissue is characterized by excessive type 1 collagen accumulation that hinders tissue regeneration. The presence of myofibroblasts is central to fibrotic tissue production. They originate from a spectrum of cellular sources, and several molecular pathways can induce the transition of cells to myofibroblasts. In fact, myofibroblasts can describe a functional status rather than a fixed cell phenotype. Fibrosis is predominantly controlled by TGF-b1, which is secreted as an inactive protein associated to a latent protein. TGF-b1 enhances strongly the synthesis of type 1 collagen by creating an autocrine loop; additionally it is an antiapoptotic agent for myofibroblasts. TGF-b1 is abundant in PRP, stored in considerable amounts in a-granules and secreted upon platelet activation. Additionally leukocytes secrete TGF-b1. TGF-beta-stimulated M2-like macrophages have profibrotic activity [16]. Instead, serum amyloid protein present in plasma has been shown to inhibit fibrosis in different models by regulating macrophage function. Thus, PRP actions are theoretically paradoxical regarding the develop-

ment of fibrosis. However, clinical practitioners using PRP injections rarely report the presence of fibrosis.

Scarring is also a key problem for axon regeneration because fibrotic tissue may block axon growth and impair axon function.

3.2. Angiogenesis

The supply of oxygen is essential for cell metabolism and wound healing. Indeed, poor tissue perfusion creates a hypoxic environment that impairs the healing process. Angiogenesis involves multiple biological mechanisms including cell migration, proliferation, and differentiation.

Vascularization occurs through outgrowth of preexisting blood vessels (angiogenesis) and involves cell migration and proliferation. Upon injury, vessels consisting of naked endothelial cell channels have potential to sprout and branch providing nutrients and oxygen to regenerating tissues. Vessel sprouting and enlargement are driven by migratory endothelial cells (EC) called tip cells. Additional types of cells, i.e. smooth muscle cells and pericytes, are involved in vessel stabilization. Both mechanisms, angiogenesis and arteriogenesis, involve a wide array of cytokines and growth factors that can be supplied in physiological concentrations by PRP administration (Table 1). Essentially, PRP cause endothelial cell proliferation and capillary tube formation in vitro.

Alternatively to angiogenesis, new vessels can be formed through mobilization and domiciliation of progenitors of endothelial cells to sites of tissue injury (vasculogenesis) or ischemic tissues, a process mediated by VEGF and SDF-1a binding to CXCR4 receptors on EPCs.

PRP augments ischemic neovascularization presumably due to the stimulation of the three above described mechanisms: angiogenesis, arteriogenesis and vasculogenesis. Arteriogenesis is the main driver of restoration of blood perfusion in ischemia [17].

Indeed, the importance of coagulation factors and of platelet secretome (VEGF, TGF-b1, PDGF, bFGF, angiopoietin) is evident for angiogenesis, not only because of their individual actions but because of beneficial synergies between these GFs. For example, Ang-1, an EC survival factor, stimulates capillary tube formation synergistically with VEGF. Also synergy between both PDGF and VEGF results in the formation of a more mature vascular network than when each factor is given alone [18]. Also the angiopoietin system contributes to vessel maintenance growth and stabilization.

Paradoxically, platelets also provide several antiangiogenic factors necessary for vessel downregulation. Angiogenesis inhibitors (CXCR3 agonists) such as PF4 and TSP-1 are very abundantly stored in platelets. Additionally, angiostatin, a product of plasminogen proteolysis, inhibits angiogenesis. Both pro- and anti-angiogenic properties have been attributed to TGF-b1. At low doses it contributes to the angiogenic switch in part by upregulation of VEGF and uPA, whilst at high doses contributes to the resolution of angiogenesis by inhibiting EC proliferation and migration, promoting the reformation of the basement membrane.

PRP- associated angiogenesis stimulators	PRP-associated angiogenesis inhibitors
Vascular endothelial growth factor (VEGF)	Angiostatin
Basic fibroblast growth factor (bFGF)	Endostatin
Platelet-derived growth factor (PDGF)	Platelet factor 4 (PF4)
Epidermal growth factor (EGF)	Plasminogen activator inhibitor (PAI-1)
Hepatocyte growth factor (HGF)	Transforming growth factor (TGF-beta)
Insulin like growth factor 1, 2 (IGF-1, IGF-2)	Thrombospondin-1 (TSP-1)
Angiopoietin (ANGPT1)	Tissue inhibitor of metalloproteinases -1, -4
Matriz metalloproteinases 2, 9 (MMP-2 and -9)	(TIMP-1,-2,-3,-4)
Lipoprotein A (LPA)	Fibronectin
Sphingosine-1-phosphate (SIP)	α2-macroglobulin
Stromal cell derived factor (SDF-1, CXCL12)	
Heparanase	
Deoxyribose-1-phosphate	
CD40-L	
IL-8, CXCL8	
CXCL12	
Down-regulate vessel permeability	**Increase vessel permeability**
Angiopoietin-1	VEGF
Serotonin	Histamine
Sphingosin-1-P	Noradrenaline (NE)
	Dopamine (DA)

Table 1. PRP-associated positive and negative regulators of angiogenesis. Some of these molecules may have both pro- and anti-angiogenic potential depending on the situation at the time of their release and/or the expression of cryptic sites. Reproduced from International Journal of Clinical Rheumatology, August 2012, Vol.7, No4, Pages 397-412 with permission of Future Medicine Ltd.[21]

As shown above, PRP provides the opportunity to therapeutically manipulate angiogenesis by targeting multiple cell phenotypes. Crosstalk between cell types along with multiple signals constitutes the complex system that regulates angiogenesis. Not only the activities of endothelial cells, but also of endothelial progenitors, smooth muscle cells and pericytes are influenced by PRP signals. Pericytes, crucial for vessel stabilization, can arise from different cell sources since they can transdifferentiate from the endothelium, a common vascular progenitor or a mesenchymal progenitor. The association of pericytes with newly formed vessels regulates EC proliferation, survival, migration, differentiation and vascular branching, blood flow and vascular permeability. Pericytes have important roles in tissue repair. For example, in skeletal muscle perycites arise from blood vessels and express NG2 proteoglycan and alkaline phosphatase and they efficiently regenerate the muscle expressing myogenic markers only when fully differentiated [19-20]. However, no information is yet available about the interaction of the molecular pool released from PRP and pericytes.

Other cooperative mechanisms such as partial degradation of the ECM and basement membrane are necessary to facilitate cell migration during angiogenesis. Actually, several protease

families released from PRP including plasminogen activators (uPA and PAI-1), and MMPs have been characterized as having a role in the proteolytic degradation and remodeling of the subendothelial basement membrane and the surrounding ECM. By digesting ECM proteins, these enzymes create a path for tip cell migration. Platelets contain fibrinolytic factors and enzymes that may regulate precisely the pericellular proteolytic environment required for the control of cell migration and matrix remodeling. For example urokinase plasminogen (uPA) and plasminogen activator inhibitor type I (PAI-1) proceed as modifiers of the pathway that impact migratory events. Almost all cell types need to migrate under physiological or pathological conditions. Clearly the binding of PAI-1 with its several targets has the potential to influence the motile program at multiple levels. Further complexity is provided by the presence of endogenous proteases inhibitors (TIMPs) that control the activity of proteases.

3.3. Stem/precursor cell activation and differentiation

It has been demonstrated in last years that most organs have a resident pool of somatic, tissue specific cells. These stem cells are located in niches characterized by a typical spatial localization, the anchorage of stem cells to supporting cells, and the presence of typical extracellular matrix. These cells are docked in specific microenvironments that control their survival and self-renewal capabilities preventing them from exhaustion. In the niche, the integration of stimulatory and inhibitory signals determines cell quiescence.

In general, these cells are mitotically quiescent. PRP contains agents able to restore mitosis in quiescent precursor cells, consequently mitotically arrested cells are able to divide again. By definition, these precursor stem cells are capable of self-renewal and have various potentialities for differentiation. In general they are committed to differentiate in the local cell phenotype and are designed to substitute dying cells during turnover, trauma or pathology. PRP participates in mobilization of progenitor cells and proliferation but its effects in differentiation are controversial [22].

PRP influences the number of stem cells by virtue of its mitotic effect and maintains stemness in most settings. Actually, the effects of PRP on the differentiation of synovium derived MSCs are negative in all three lineages and PRP alone maintains MSCs stemness [23]. Besides PRP inhibits differentiation of adult rat tendon stem cells towards nontenocyte lineage [24]. Recent research in skeletal muscle repair has shown that PRP maintains stemness of muscle progenitor cells [25]. Though, PRP did not interfere with the osteogenic, chondrogenic and myogenic differentiation in the appropriate differentiation conditions.

In many cases of traumatic injury or disease the quantity and potency of this endogenous pool of precursor cells is insufficient to regenerate compromised tissues and migration and homing of mesenchymal stem cells circulating in the blood stream is required.

In fact, bone marrow contains several types of stem cells including hematopoietic cells that differentiate into mature blood cells, endothelial progenitor cells and MSCs which are proposed to give rise to the majority of marrow stromal cell lineages including chondrocytes, osteoblasts, fibroblasts, adipocytes, endothelial cells. Circulating MSCs in the blood stream

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can home injured tissue in response to chemoattractants released from platelets such as SDF-1a/CXCL12.

PRP was shown to be effective in promoting the migration of MSCs. In addition PRP can increase the number of MSC by stimulating proliferation. Actually the number of CMOs manufacturing cells for in-human trials are taking advantage of the mitogen properties and fetal calf serum [FCS) the typical cell culture supplement is being substituted by PRP. Several studies showed that population cell doublings is enhanced by PRP, that is to say PRP reduces the time needed to get a predefined cell number necessary for efficacious cell therapy [26].

3.4. Modulation of nerve repair

Peripheral nerve injury and regeneration

Peripheral nerves have the capacity to regenerate after an axonal injury. There are several kinds of peripheral nerve damages depending on the damage of the axon and surrounding tissue. After an injury or a breakdown, axon is able to regrow expressing repair-related molecules and aided by Schwann cell activation, proliferation, phagocytic activity and production of neurotrophic factors. These factors activate signaling cascades promoting synthesis of molecules related to axonal regeneration events. In addition to producing bioactive molecules, Schwann cells form structures called Bünger bands, which have as a function the physical guidance of growing axons [27]. They also recruit macrophages to the injury site to remove debris from the injury and help them supporting axon repair by secreting chemokines. As a result, axon healing is the result of the interaction between molecular signals and cellular events which allow proper growth of the axonal stump and consequent recovery of its functional activity at the end of healing process.

Peripheral nerve fibers are stimulated immediately after injury and release several neuropeptides into the microenvironment of the wound. Substance P, neuropeptide Y and calcitonin gene-related peptide (CGRP) influence endothelial cells, fibroblasts and are involved in vasoregulation and angiogenesis.

Growth factor and cytokine involvement in nerve healing

All these results have been attributed to growth factors released from platelets when activation occurs, but an accurate function and optimal concentration have not been identified [28]. Platelets release a high number of growth factors which may have precise effects on their own, or work synergistically depending on their concentration. Wound healing is influenced by diverse growth factors secreted by platelet such as PDGF, TGF-β, PF4, VEFG, EGF, PDEGF, IGF-I and others. Although these are not classically classified as neurotrophic, they have been demonstrated to have a role in Schwann cell migration, proliferation, neuron metabolism, synthesis of neurotrophic factors, matrix formation and myelinization and thus in axon regeneration [29-33]. Also, platelets release other molecules which are not growth factors such as catecholamines, histamine, serotonin, ADP, ATP and others which take part in blood vessel formation, immune reactions both innate and adaptive, and thus in tissue regeneration [30].

Nerve healing depends on equilibrium between Schwann cell proliferation and activation and neurotrophic molecules which create a regenerative milieu which helps axon repair and myelinization. Several growth factors present in PRP, such as PDGF, TGF-β1 and FGF-II, have shown to promote Schwann cell proliferation, activation and differentiation which may explain beneficial PRP effects shown in the previously commented studies [30, 33]. These growth factors, for which Schwann cells and neurons have membrane receptors, trigger the expression and subsequent synthesis of classic neurotrophic factors such as nerve growth factor [NGF), Glial derived growth factor (GDNF), brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CTNF)[30, 32]. Also, PDGF expression has been shown to be enhanced in neurons after a nerve injury, which may support the theory that this growth factor has an important role in axon healing. Another growth factor present in PRP, which has been signaled as neurotrophic is VEGF. It has been shown to be neuroprotective, to also augment Schwann cell proliferation and axon growth [30]. IGF-I has also been pointed out as a central promoter of nerve healing. *In vitro,* it has been observed that IGF enhanced neuron axonal growth and that myelinization does not occur when IGF is removed. Also, IGF has been shown to promote Schwann cell proliferation and migration. In vivo, IGF injections in the site of nerve injury have been proved to ameliorate nerve healing and myelinization [28, 29,30, 31].

In the clinical arena, perineural injections of PRP induced sensorial recovery in leprosy peripheral neuropathy [34].

4. PRP formulations and regulatory requirements

Innovative therapeutic tools appear in the horizon when basic knowledge and research surpasses a certain threshold and is ready for translation into the clinics. PRP technologies showed up in the late 80s, mainly based on increased knowledge about the functional role of platelets. Platelets are cytoplasmic fragments of the megakaryocyte in the bone marrow. A variety of molecules are stored in platelets' granules either synthesized by their parent cell the megakaryocyte or captured in the circulation. In PRP technologies platelets are used because of their capability to function as vehicles for growth factors and cytokine delivery.

Initially platelets were mainly studied because of their fundamental role in hemostasia. Allogeneic PRP, either derived from a single donor or pooled donors, has been used since the 60s as a transfusion product. In fact platelet transfusion is indicated in patients with platelet counts below 30,000 plt/ul or below 100,000 plt/ul if they are to follow a surgical procedure. Later in the 80s clinical researchers showed off that beyond their role in hemostasia, PRP derived product (PDWH) were effective in the management of chronic leg ulcers [35] and were an aid in cardiac surgery [36].

In the 90s, maxillofacial surgeons and oral implantologists introduced the clinical use of PRPs as autologous modifications of fibrin glue. They were confounded by the effects of PRP in bone regeneration in doing so accelerating the stability of dental implants. Of note, the anti-inflammatory properties in soft tissues, presumably attributed to the presence of platelets in the preparation, was another hallmark in PRP findings.

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In the new millennium, the use of PRP has been boosted not only by research in maxillofacial surgery and oral dentistry but also by new applications in orthopedics and sports medicine. In 2007, the term and definition of platelet rich plasma was introduce in Pubmed as a medical subject heading (MeSH) to be used for indexing scientific articles.

However, the definition of PRP in Pubmed is out of date by several reasons. First, PRP is not only used in surgical procedures but it is also used in the conservative management of non-healing ulcers and as an injectable in the management of chronic pathologies such as tendinopathies or osteoarthritis. Secondly, the current definition claims that GFs in platelets enhance tissue regeneration this is true but only up to a point. In fact, not only GFs from platelets but also plasmatic GFs have a crucial role in repair. Besides, this definition overlooks the hundreds of proteins released from platelets that also participate in healing. Despite all these limitations, PRP inclusion as MeSH term has served to harbor PRP research under a unique term.

PRPs differ from conventionally synthesized drugs in that they are products derived from living sources. Indeed platelets are lively cells and they may experiment several temporary transformations from preparation to local tissue delivery. The process is known as platelet activation and involves changes in platelet morphology, aggregation, centralization of granules, and secretion of their content to the extracellular milieu. Another peculiarity is that PRP products are complex multi-molecular mixtures that cannot be readily characterized and reproducibility in the composition is influenced by biological inter-individual variability.

4.1. Types of PRP products

PRP is prepared by taking a given volume of blood from a patient and processing it to separate blood components and concentrate the platelets and optionally the leukocytes. Importantly, the manipulation of blood in order to obtain PRP is minimal.

The nomenclature of PRP products reached a zenith of confusion at the beginning of the new millennium. In fact, more names than products appeared and the number of commercial terms was endless, including platelet concentrates (PC), autologous growth factors (AGF), plasma rich in growth factors (PRGF), platelet gel (PG), platelet rich fibrin matrix (PRFM) etc... It was evident that there were more names than PRPs to be named.

In 2009, Dohan [37] inspired the present nomenclature and classification of PRPs. Broadly speaking PRPs were categorized as pure PRP and leukocyte and platelet rich plasmas. Considering fibrin architecture and platelet counts we can differentiate further PRP subsets. Different PRP devices and harvest yield in terms of platelet and leukocyte count lead to the proposal of classification systems for PRP.

Alternatively to commercial automatic systems, PRP can be prepared in blood banks with highly standardized procedures. In this setting, PRP is prepared from a higher volume of blood, quality is assessed and aliquots of PRP are frozen for posterior applications. PRP obtained in blood banks are less expensive than PRPs obtained with automatic devices.

For blood withdrawal, many PRP protocols use anticoagulants to prevent blood from clotting. Most kits use ACDA or sodium citrate to chelate calcium ions in doing so preventing prothrombin conversion into thrombin. Other anticoagulants (i.e. heparin, EDTA) are avoided because they may compromise platelet stability and activation. Notwithstanding, ACDA and sodium citrate make the plasma acidic and some protocols recommend buffering the PRP back to a physiologic range prior to injection. Alternatively, PRP products such as leukocyte and platelet rich fibrin (L-PRF) do not use anticoagulants and fibrin is formed during the centrifugation step. Evidently, these products have a physiological pH but cannot be used as injectable.

Importantly, PRP activation is needed to induce the secretion of granule contents i.e. platelet secretome. This occurs spontaneously in blood but is inhibited if the blood is withdrawn in tubes containing anticoagulants. Reversion of anticoagulants inhibition of coagulation and platelet activation can be achieved by several procedures. One possibility is the addition of calcium or thrombin/Ca^{2+} to cleave fibrinogen with subsequent polymerization of fibrin monomers. Alternatively, physiological activation can be achieved by injecting the unactivated PRP that once in contact with collagen and other tissue factors will get activated. The mode of delivery of PRP has also to be taken into account, since it is a more involved process than the delivery of a drug or a single recombinant protein. The application protocol and post-application management involved will have a huge impact on determining whether the potential efficacy is seen.

Device Commercial name	Technology	FDA approved	Total process time	Disposable list Price	Increase above baseline	Platelet recovery	PRP formulations
GPS III™ (Biomet)	Floating Buoy	510(k)	15 min	$700	3.2x	90%	L-PRP
Angel (Arthrex)	Computer Aided system	510(k)	25 min	$495	4.3x	76%	L-PRP
ACP (Arthrex)	Standard Centrifugation Thixotropic gel	510(k)	5 min	$295	2.1±2x	60%	Pure PRP
AutoloGel System (Cytomedix)	Standard Centrifugation	510(k)	1-2 min	$325	1x	78%	L-PRP
GenesisCS (Emcyte)	Direct Siphoning	510(k)	16 min	$1550	10±3x (4ml)	68±17.1%	L-PRP
Pure PRP 2 (Emcyte)	Standard Centrifugation	Pending	5.5 min	N/A	8-16x	76%	Pure PRP
Harvest® SmartPrep2	Floating Shelf	510(k)	16 min	$395	4x	72.0±10%	L-PRP

Device Commercial name	Technology	FDA approved	Total process time	Disposable list Price	Increase above baseline	Platelet recovery	PRP formulations
BMAC™							
Symphony II (Depuy)	Floating shelf	510(k)	16 min	$395	4x	72.0±10%	L-PRP
Magellan™ (Arteriocyte)	Computer Aided System	510(k)	17 min	$350-495	5.1x	70%	L-PRP
Dr PRP USA (Rmedica-Korea)	Standard Centrifugation	Approved	12 min	N/A	N/A	97%	L-PRP
Prolo 30-50 (RM Bio Co, Ltd.)	Standard Centrifugation	None	N/A	N/A	4-6x	N/A	L-PRP
Prolo-High (RM Bio Co Ltd.)	Standard Centrifugation	None	N/A	N/A	5-8x	N/A	L-PRP
PRGF®-Endoret® (BTI)	Standard Centrifugation	510(k)	28 min	N/A	1-3x	70%	Pure PRP
CASCADE® Autologous Platelet System (MTF)	Standard Centrifugation Thixotropic gel	510(k)	20 min	N/A	1-2x	N/A	Pure PRP
YCellBio PRP YCELLBIO MEDICAL	Standard Centrifugation	510(k)	15 min	$80	7-9X	N/A	L-PRP

Table 2. Main characteristics of the devices and/or kits used to prepare PRP. Modified from: http://www.perfusion.com/perfusion/prpdevicesummary.asp

4.2. Regulatory

Regulatory requirements for PRPs are not uniform across the world. For example, in the US, PRP is not a product instead administration of PRP is a procedure and is, therefore, not subject to regulation by the FDA. However, the devices used to prepare PRP are regulated by the FDA premarket approval process and have to get a 510(k) clearance.

Devices and procedures destined to prepare PRP are classified for their intended use as class III medical devices and reach the market via premarket approval application. The product is evaluated to ensure that the product is safe and effective and displays consistent performance characteristics. 510(K) premarket notification exists for products that are similar to those

already marketed usually called predicate device. In these cases 510(k) clearance is evaluated only for substantial equivalency.

Table 1 shows devices and/or kits for PRP preparation

At the European level there is no harmonized regulatory framework for PRP therapies, and each country has its own approach to PRP regulation within the jurisdiction of national authorities. Devices must comply with Class II-a medical device directive 93/42/EEC. Device approval in EU is overseen in each EU country by a governmental body called a Competent Authority. Instead, the surgical use of PRP can be considered as an autologous graft within the surgical procedure as regulated by Directive 2004/23/EC.

Of note, if regulatory requirements for PRP therapies were over-interpreted, unnecessary work derived therein will increase costs and hamper the clinical use of PRP therapies. This hypothetical situation would be prejudicial for many patients since advancements in PRP science can provide effective treatments for pathologies with substantial social and economic burden.

4.2.1. Reimbursement

Currently most insurance plans do not reimburse for PRP treatment due to the lack of data about their efficacy. Interestingly, in the US Category III, code 0232T is used for emerging technologies and applies for nonsurgical uses of PRP. This code allows data collection to be used to document widespread use for FDA approval and potential reimbursement. Besides, this code will allow the AMA (American Medical Association) to track the use of PRP, since codes T are considered experimental they will require pre-authorization for payment. If a physician feels that the patient would benefit from PRP injections, typically as a step to avoid a more costly and invasive procedure preauthorization for PRP reimbursement should be requested. Presentation of cost savings rationale can be the key to successful preauthorization. In general managers are concerned about physician's plans to get injured patients back to productivity or work.

5. Translational uses of PRP biotechnology clinical relevance of PRPs

The goal of this section is not to provide an exhaustive overview of current clinical studies but to identify and briefly describe the miscellaneous clinical applications. PRP is considered investigational because currently, there is insufficient evidence to support the use of PRP for all the indications included below.

The use of PRPs has extended to multiple clinical fields and novel applications are emerging to meet varied clinical needs. The increasing use of PRPs in sports medicine, (after withdrawal of restrictions imposed by the IOC International Olympic Committee) and PRP applications in areas such as dentistry and plastic surgery is expected to drive the financial growth of PRPs, that was estimated in the US market as CAGR annual growth rate of 14% from 2009 to 2016 ($45m in 2009 will reach $126m in 2016) (http://www.researchviews.com/healthcare/medical/

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orthopedicdevices/Viewpoints.asp x?sector=Orthopedic%20Devices&DocID=10728). The diffusion of PRP is attributed to the biosafety of the product due to its autologous origin.

In general, clinical uses of PRP can be categorized in two. First surgical applications, when PRP is used in surgery as an aid to enhance repair, not only of target tissues but PRP also aims to enhance the healing of all the adjacent tissues damaged during the procedure, and secondly conservative uses of PRP, most often involving more than one application and used in outpatient settings. Besides, the use of PRP associated to other medical procedures (laser therapies, etc) offers potential to enhance such procedures. Table 3 and Table 4.

5.1. Treatment of non-healing wounds

PRP has been used to treat non-healing wounds for more than 2 decades. In fact, the topical management of chronic leg ulcers was the first clinical application of platelets outside the blood stream with healing purposes. Theoretically PRP or its derivatives are stimulants for non-healing wounds; the goal is to re-activate healing. The rapid formation of granulation tissue can prevent further deep tissue involvement and associated co-morbidities.

Complex non-healing wounds can have different etiologies including pressure ulcers, diabetes, venous, arterial, or surgical trauma. Impaired wound healing is the major complication that results in the development of chronic wounds often leading to amputations as often occurs in the diabetic foot. Components of lower extremity amputations in the diabetic patient include ulceration (85%), faulty wound healing (81%) initial minor trauma (81%), neuropathy (61%), infection (59%) gangrene (55%) and ischemia (46%) (38]. Actually, the wound healing society treatment guidelines for diabetic ulcers advises re-evaluation of the wound and treatment based on failure to reach a 40% reduction of initial wound size by week 4 (39]. Cost for amputations has been estimated to be between $ 20.000 and $ 60.000 per case. PRP is deemed useful in this context because it may provide a way to reduce this cost burden to society.

Several RCT have provided data about the efficacy of PRP or PRP derivatives in non-healing wounds. The initial platelet product known as PDWHF (platelet derived wound healing factors) stimulated the formation of granulation tissue in chronic leg ulcers [40]. Later, PDWHF of pooled donors was also examined in the treatment of diabetic ulcers (41]. In general, concomitant pathologies such as diabetes do not hinder the therapeutic effects of PRP, and autologous PRP is effective in the diabetic foot [42-43] or systemic sclerosis [44].

Despite several studies reporting the benefits of PRP in this setting, a recent meta-analysis of ulcer care studies and PRP failed to show any statistically relevant difference favoring PRP for the treatment of chronic wounds. Nevertheless, conclusions are not sound because they are based on 9 RCTs with high or unclear risk of bias [45]. Additional evidences complementary to RCT data can be obtained from practice based medicine and observational studies. These data obtained in a real-world setting provide pragmatic evidences of PRP benefits.

Actually, some PRP manufacturers such as Cytomedix, AutoloGel™ have created wound registries to evaluate the use of PRP and calculate cost savings based on mean treatment times; this is realistic using patients in pretreatment run-in periods as their own controls during standard wound care treatment [46].

The reduction in treatment time should impact clinical and financial decisions. Significant clinical outcomes indicated many previously nonresponsive wounds began actively healing in response to PRP therapy. Cost effectiveness analysis comparing the potential economic benefit of PRP to alternative therapies in treating non-healing diabetic foot ulcers, using an economic model based on peer-reviewed data showed that PRP resulted in improved quality of life and lower cost of care over 5-year period than other treatment modalities for non-healing diabetic ulcers [47].

5.2. Maxillofacial and oral surgery

The field of PRP gained new impetus at the end of the nineties when maxillofacial surgeons and dentists introduced PRP to augment oral reconstruction procedures. Since then a number of protocols have been developed for different applications. These include socket filling after molar extractions, implant surgery, PRP mixed with bone grafts during osteodistraction, and in the treatment of mandibular tumor resection. Numerous articles describe earlier stabilization of dental implants when PRP is used to enhance the properties of bone grafts, and to modulate the inflammatory status in the surrounding soft tissues.

The utility in several of these procedures seems evident. However, after meta-analyzing 24 studies addressing the use of PRP in the surgical treatment of periodontal diseases, it was concluded that PRP exerts a positive effect only when used with graft materials for the treatment of intrabony defects, but not in guided tissue regeneration. No significant benefit of PRP was found for the treatment of gingival recessions [48].

In sinus augmentation surgery the combination of PRP with autologous bone graft led to increased bone density at 6 months but not at three months [49].

The use of PRP in children is less known because they need little help for healing since young cells and young blood has stronger healing potential and plasticity when compared to adults. However, in the most frequent congenital facial malformation, i.e. cleft lip and palate, the use of PRP is being investigated [50- 53]. The goal here is to reestablish the maxillofacial arch and to close any oro-nasal communication. Besides, PRP is used in the closure of recurrent cleft palate fistulas.

5.3. Plastic surgery and dermatology

A recent review in plastic surgery including 15 randomized controlled studies and 25 case-controls showed that the outcomes were favorable in three main PRP indications: wound healing, fat grafting and bone grafting [54].

PRP is used in breast reconstruction associated to fat grafts because it enhances the survival of fat grafts that otherwise had a tendency to be resorbed by the organism. Similarly, the outcome of plastic reconstruction using skin grafts can be improved taking advantage of the pro-survival effects of PRP.

The use of PRP reduced recovery time in facial rejuvenation. In fact the addition of PRP to lipofilling procedures resulted in a significant reduction in the number of days needed to

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recover before returning to work or restart social activities. Also the aesthetic outcome was significantly better with PRP [55].

Combined treatments as fat graft, laser CO2 and PRP showed clinical benefits in the treatment of atrophic and contractile scars [56].

PRP combined with erbium fractional laser therapy is effective for treating acne scars or acne, at the same time PRP enhances the recovery of laser damaged skin [57].

PRP injected into the scalp is also used to manage androgenetic alopecia [58]. When the efficacy of the interventions was examined in 64 patients, half of the patients showed a clinically meaningful improvement [59]. PRP is also used in hair transplantation [60]. In this situation, PRP shortens the time for hair formation.

5.4. Treatment of orthopedic problems

The use of PRP to solve clinical problems in orthopedics has increased with impetus in the past five years the main reason can be the biosafety of the treatment and the fact that actual strategies for management are insufficient. PRP is used in open and arthroscopic surgery and as a conservative treatment for the management of chronic pathologies, most importantly tendinopathies, chondropathies and osteoarthritis. Main properties are attributed to modulatory effects on inflammation and angiogenesis along with reduction in pain [61-63].

Research in orthopedics encompasses a wide range of applications. In sports medicine, physicians are more deeply engaged in conservative management of tendinopathies and muscle injuries

Proposed clinical and surgical applications include spinal-fusion, osteoarthritis [hip and knee), tendinopathy enhancement of healing after ACL reconstruction and muscle strains. The challenge is to show that PRP is superior to the optimal available treatment.

5.4.1. Bone regeneration

In some applications PRP is used as a coadjuvant associated to autologous or homologous graft and also to bone marrow graft. There is no compelling evidence to demonstrate the efficacy of PRP alone in facilitating the union of long bones for union of tibial osteotomies or pseudoarthrosis or fractures [64). An randomized study involving 21 participants compared PRP+allogeneic graft versus allogeneic bone alone in patients undergoing tibial osteotomy in the medial compartment in patients with OA [65]. There was a significant difference in the proportion of bones that were united after one year in favor of PRP but evidence from a single trial is insufficient to support routine interventions with PRP.

5.4.2. Lumbar fusion and intervertebral disc degeneration

Posterolateral arthrodesis in lumbar spine surgery was enhanced when PRP was combined with cancellous bone graft as shown by densitometry in a prospective study [66]. However, a randomized clinical study did not show any benefit when PRP is used with autologous bone in mono-segmental posterior lumbar interbody fusion [67].

Intervertebral disc degeneration is also common in orthopedics and current treatments are of limited value to enhance the regenerative process. In fact some studies point out the efficacy of PRP in reversing the degenerative trend of the intervertebral discs based on basic science research [68]. However this application lacks translation in published reports.

5.4.3. Osteoarthritis

The efficacy of PRP has been most studied in knee osteoarthritis while few clinical data are available about the therapeutic effects in hip OA.

There are several randomized clinical trials comparing the efficacy of PRP treatment with HA administration. Also PRP has been compared with placebo administration. Two recent metaanalysis [69,70] concluded that multiple PRP injections ameliorate pain and improves function and tends to be more effective that HA administration. Patients with lower levels of knee degeneration achieve better results than more advanced knee deterioration. Although less investigated, PRP injections in the hip ameliorate symptomatology and function, however the clinical level of evidence is low and more clinical studies are needed before claiming therapeutic effects in this joint [71].

5.4.4. Tendon pathology

Most studies in tendon pathology involve guided injections of PRP. Indeed, the development of real-time imaging techniques such ultrasonography enhances the safety and accuracy of PRP delivery during percutaneous management. Consequently, investigation on the efficacy of PRPs for managing chronic tendinopathies has grown in the last years and the quality of the studies has improved considerably. Limitations of observational studies have been overcome by level I and level 2 clinical trials. However due to tendon diversity and function, the various PRP products and the diversity of application protocols, quantitative synthesis and meta-analyses are difficult to perform.

Most frequent upper limb tendinopathies involve the supraspinatus and the medial and lateral epicondyle. In these pathologies, the quality of clinical studies is high/moderate. A quantitative synthesis evaluating the efficacy of PRP as an adjuvant in rotator cuff arthroscopy failed to show any benefit associated to PRP. The only uncertainty in favor of PRP is that it may decrease the proportion of retears, but this needs further confirmation [72].

Ultrasound guided injections and real-time follow-up has fueled the use of PRP in tendinopathies

A recent metaanalysis examining the conservative management of tendinopathy has shown that PRP provide some benefits in pain [61]. Moreover, subgroup analysis showed a modest reduction of pain in epicondylitis [73]. However, major limitations for pooling data and drawing firm conclusions comprise different outcome measurements and follow-up periods.

The most commonly treated tendons in the lower limb were Achilles, the patellar tendon and the plantar fascia. PRP shows potential benefits in these anatomical locations, current eviden-

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ces are encouraging but limited. The need is clear to compare PRP treatment with the most adequate control for each condition.

5.4.5. Muscle injuries

PRP injections are used in professional athletes; the goal is to accelerate muscle healing, and avoid relapses through true muscle regeneration, i.e. absence of scar tissue. Especially in elite athletes the goal is to achieve rapid healing and resume competition faster than with conventional care. Although case series provide promising results, two recent randomized controlled trials showed divergent results [74,75]. Protocols and PRP formulations were different in both studies, thus further research is warranted.

5.5. Treatment of eye problems

5.5.1. Macular hole

Platelet concentrates were used in the 90s as an adjunct to macular hole healing. PRP is an autologous alternative to fibrin glue with much more biological activity conferred by hundreds of GFs and cytokines stored in alpha-granules. The efficacy of platelet concentrates was examined in a multicenter double blind study involving 53 eyes in the experimental group and 57 eyes in the control group. Injection of autologous platelet concentrates during macular hole surgery improved significantly the anatomic success of the intervention but did not influence visual acuity [76].

5.5.2. Dry eye

Autologous serum administered topically has been used to treat dry eye symptoms because it can improve not only lubrication but also enhance lacrimal production since its composition includes GFs important in this context such as EGF. However, after metaanalysing current studies there were inconsistency in relation to the benefits provided by autologous serum [77].

As an alternative, PRP derivatives mainly the PRP released supernatant or the PRP lysate are being investigated to improve dry eye conditions, administered topically. Alternatively, in severe cases PRP is injected adjacent to the lacrimal gland [78]. Results indicate a significant increase in lacrimal volume and patient's self-reported improvements. PRP also enhanced epithelial status after LASIK but did not affect the recovery of corneal sensitivity.as evaluated in a controlled study in which the contralateral eye was used as control [79].

5.5.3. Corneal ulcers

Perforated corneal ulcers have been treated with PRP fibrin in a reduced number of patients (N=11=. In all cases the corneal perforation was sealed after 3-5 days of stability of the membrane in the ulcer [80]. When PRP eyedrops were compared to autologous serum eyedrops in the treatment of persistent epithelial defects PRP was more efficient than serum [81]. After photorefractive keratectomy, the use of PRP enhanced wound healing and reduced haze formation [82]

In a prospective controlled study in acute ocular chemical injury PRP eyedrops as adjunct to standard care was superior to artificial tears, autologous platelet lysate was also effective for the treatment of refractory ocular GVDH in unresponsive patients [83].

5.5.4. Other therapeutic applications

Some anecdotic uses of PRP in urology for fistula repair [84], in the management of infantile extravasation injury [85], and in gynecology for vaginal prolapse [86-87] have been published.

6. Challenges ahead

As PRP is eminently a translational technology, challenges must be addressed through three interlinked angles: scientific, clinical and socioeconomic.

From the scientific aspect, evidence continues to build for the mechanisms of PRP in tissue repair regulating the behavior of different cell types. At present we have a more complete picture on how PRP influences healing mechanisms however, we do not know enough to design formulations for specific medical problems. Difficulties to advance are attributed to the lack of characterization of the host tissue. In fact, the host response to PRP administration will drive the healing mechanisms. The host response invariably starts with activation of the immune innate system as a result of PRP administration. Moreover, considering the local pH, O_2 levels, and cellular conditions of the host (i.e. number of dying cells apoptotic or necrotic) is important not only in terms of GFs and cytokine stability but also to have information about the main mechanism affected.

Clinical challenges: PRP research may eventually lead to superior therapies. The translational imperative is making PRP formulation effective in defined indications. The challenge is to customize PRP formulations. Essentially a single formulation cannot fulfill every need, from treating a degenerative knee to repairing a pseudo-arthritic condition.

The modest benefits achieved with clinical trials addressing PRP efficacy should not reduce our motivation, instead we need to understand better the underlying mechanisms so that the most appropriate indications can be found and appropriate trials conducted to test the specific indications. Novel approaches to clinical trial design shall recognize that the efficacy of these therapies will be dependent upon delivery protocol as well as the PRP product itself.

Socioeconomic challenges: we should consider carefully the potential of PRPs to reduce costs in chronic diseases. This could be achieved by the creation of proper registries and economic models considering the costs generated in pre-treatment run-in periods. In patients with recalcitrant pathologies, candidates to surgical interventions, economical algorithms will help to estimate savings attributable to surgical and post-surgical costs relative to the conservative management.

Cardiac surgery	Coronary artery bypass graft surgery*
	Aorta surgery*
Plastic surgery	Breast reconstruction**
	Infected high-energy soft tissue injuries*
	Skin grafts**
	Fat grafts***
	Pilodinal sinus abscesses*
Pediatric surgery	Alveoloplasty of cleft palate*
	Extravasation injury (premature infants)*
Maxillofacial and oral surgery	Molar extractions***
	Implant surgery***
	Osteodistraction**
	Periodontal disease**
	Mandibular tumor resection**
	Sinus lift**
Urology	Uretracutaneous fistula repair *
	Vesicovaginal fistula*
Gynecology	Vaginal prolapse*
Orthopedics and Arthroscopic surgery	Non-union (aseptic pseudo-arthrosis)*
	Tendon surgery*
	Morton neuroma*
	Peripheral nerve regeneration*
	ACL reconstruction***
	Rotator cuff surgery***
	Lumbar interbody fusion*
	Tibial osteotomy*
	Hip conditions (sacroiliac joint dysfunction)*

*** Broadly used, that is RCTs, several clinical evidences and different levels of evidence

** Moderate use, case series and at least one controlled study

* Anecdotic use, case series or an isolated prospective cohort

Table 3. Miscellaneous applications of PRPs in open surgery

Eye disease	Macular hole**
	Restoration of human lacrimal function (dry eye)**
	Corneal ulcers and lesions **
	Chemical acute wounds
	Neurotrophic wounds
	epithelial
	Nerve regeneration after LASIK*

	Refractory ocular GVDH*	
Cardiology	Complications of sternotomy**	
	Severe deep sternal wound infection**	
Dermatology	Non-healing ulcers***	
		Diabetic ulcer
		Neurogenic
		Arterial
		traumatic
		Pressure ulcers
		Vascular ulcers
	Treatment of striae distensae*	
	Androgenetic alopecia*	
	Peripheral neuropathy*	
Sports Medicine and Orthopedics	Chondropathy and Osteoarthritis***	
	Tendinopathies***	
	Muscle injuries**	
	Ligament injuries*	
	Fasciitis plantar**	
	Recalcitrant hindfoot and ankle disease**	
	Per-cutaneous non-unions*	

Table 4. Conservative clinical applications of PRP therapies

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Chapter 2

Molecular Fingerprinting and Selection of Appropriate Media for Rapid *In Vitro* Multiplication of Three Yam Varieties

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Additional information is available at the end of the chapter

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1. Introduction

Yam is the colloquial name for some species in the genus *Dioscorea*, members of the family *Dioscoreaceae*. Yams are perennial monocotyledonous plants with a vine and underground tubers. There are about 700 species within the family, nine of which are medicinal plants that accumulate steroid saponins in their rhizomes and six species namely *D. bulbifera*, *D. cayenensis*, *D. dumentorum*, *D. prahensilis*, *D. alata* and *D. rotundata* are edible. The cultivation of the edible tuber is mostly in Africa, Asia, Latin America and the Oceania regions. In West and Central Africa, especially Ghana the underground yam edible tuber is very important as a staple providing food security and income for some 26.2% of the population [6]. The daily average yam consumption is approximately 300 kcal per capita [4]. Being the third most important energy source in the Ghanaian diet, yam accounts for 20% of total caloric intake [4]. It is a versatile root crop which has various derivative products after process as it can be barbecued, roasted, fried, grilled, boiled, smoked and when grated it is processed into a dessert recipe. At present farmers are only getting about 20% of the potential yield of yams. In Ghana, the consumer has developed preference for a particular *Dioscorea rotundata* variety locally called "Pona" because of its peculiar taste and texture. It is with this in mind that the CSIR-CRI Yam breeding program evaluated and selected for release three new yam varieties (CRI-Pona, CRI-Kukrupa, and Mankrong Pona). These varieties were officially released in May 2005 and are all high and stable yielding, pest and

disease tolerant and have good culinary characteristics [14]. These released yam varieties are in high demand by farmers and all possibilities are being explored to enhance the production of clean planting materials which could be achieved using tissue culture techniques.

1.1. Yam production and associated challenges

Traditionally, yams are grown from the edible tuber in the form of whole tuber or sections, or setts on mounds and as they produce vines, they are staked with sticks to permit the vines to climb up the stake (Figure 1). The growth period is between 6 and 10 months, depending on the variety, after which they produce the edible underground tubers (Figure 2). Annually production of yam stands at about 48 million tonnes in the sub-saharan West African region and this represents 93% of the global production indicating that the region is the hub for production of the tuber. The countries involved in the production are Benin, Cote d'Ivoire, Ghana, Nigeria and Togo. Globally, Ghana is the leading yam exporter, having exported 20,841 metric tons of yams in 2008, but with the increasing global demand for yam from Europe, the U.S and neighbouring African countries, there is a potential for higher production and export volumes [10]. Research conducted in Council for Scientific and Industrial Research-Crops Research Institute (CSIR-CRI) showed there is a potential to increase the yields of yam from the average of 12 tons/ha to between 65-70 tons/ha. The major challenge in meeting the production targets are inadequate access and high cost of seed yam, hence despite the availability of fertile land and demand for yam domestically and abroad cultivation is low. Typically, cost of seed covers about 58% of production cost. This challenge is due to the fact that yams are vegetatively propagated from the edible tuber, and there is the general lack of high quality disease-free seed yam as the planting materials are usually infested with Fungi, Bacteria and Viruses. In order for farmers to maintain the clonal materials yams have been propagated vegetatively. This means of propagation has the potential of contributing immensely to the spread of diseases and pathogens endemic in the planting materials since same planting materials are used for propagation year after year which leads to accumulation when clean materials are not used. Currently the use of tissue culture techniques along with cryotherapy [7], chemotherapy and thermotherapy are the sure means of producing clean virus-free planting materials of vegetatively propagated crops. The presence of several viruses in West African yam was reported in 1992 [20] and these are supposed to have led to significant losses in crop production [9]. *Yam mosaic virus, a Potyvirus* is the most important infection in West African yams. It was first detected in *D. cayenensis* in Côte d'Ivoire [21]. The symptoms include mosaic, shoestring, green vein banding and stunting of the yam plant [17; 5]. Other viruses reported in yams include Badnavirus and Cucumber mosaic virus. It has been reported that sequences of badnaviruses have been integrated into the yam genome [18]. This has culminated from years of cropping yam with infected planting material. It is therefore important that tissue culture techniques be developed to facilitate the production and mass propagation of clean planting material. Other pathogens commonly associated with yams are nematodes and anthracnose.

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Figure 1. Yam plant vines growing on a stake in the field

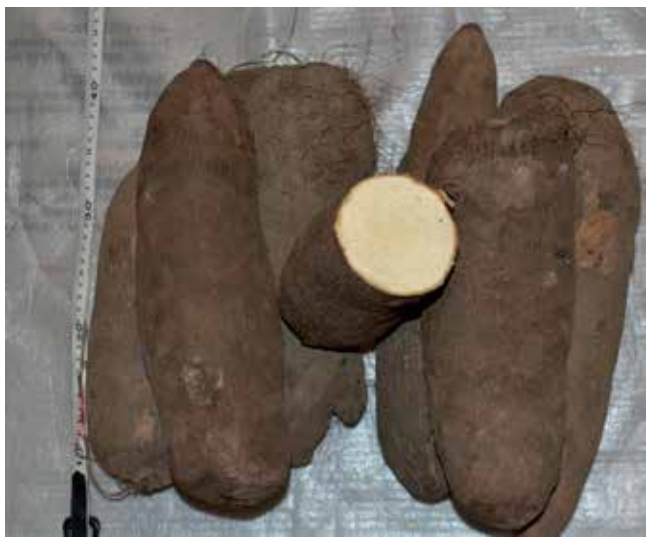

Figure 2. Edible yam tubers harvested from underground

1.2. Tissue culture production

Crop propagation through *in vitro* approaches offers a scope for improving root and tuber crops with desirable traits in larger quantities. The technique allows for rapid mass propagation of clean planting materials all year round in a limited space and is ideal for the conservation of germplasm. This notwithstanding, the widespread application of tissue culture has few

limitations such as high initial cost of production, choice of crops restricted to species with acceptable propagation protocols and reproducibility of protocols.

Propagation of plants through tissue culture has become an important and popular technique to reproduce crops that are otherwise difficult to propagate conventionally by seed and/ or vegetative means. Specialised and matured cells are manipulated to give rise to multiple copies of the parent plant under optimum aseptic environmental conditions and appropriate stimuli. It offers many unique advantages over conventional propagation methods such as rapid clonal multiplication of valuable genotypes, expedition release of improved varieties, production of disease free plantlets, non-seasonal production, germplasm conservation and facilitating their easy international exchange. The application of tissue culture techniques towards the production of clean planting material is critical for vegetatively propagated crops. On the field, tissue culture produced plants were found to establish more quickly, grow more vigorously and produce higher yields than conventional propagules with approximately 30% higher yield [15].

A number of factors come into play when establishing crops *in vitro*, and these include the plant part used (explant), its developmental stage, conditions under which it was grown, and the choice of growth conditions. Success of most tissue culture works depend much on the levels and kinds of plant growth regulators included in the medium. Root and shoot initiation, callus formation and differentiation are closely regulated by the relative concentration of auxin and cytokinin in the medium [3]. Plant growth regulators are critical media component in determining the developmental pathway of the plant cell. When establishing yam cultures *in vitro*, it has been shown that the age of the explant is critical [2] and also there seem to be a good reserve of endogenous auxins hence cultures do not require the supplementation of auxins [2]. Cytokinins such as benzylaminopurine (BAP) and kinetin are generally known to reduce apical meristem dominance as well as enhance both axillary and adventitious shoots formation from meristematic explants [8]. BAP has a marked effect in stimulating the growth of axillary and adventitious buds, and foliar development of shoot tip cultures [1]. In *Dioscorea* it has been shown to enhance the development of multiple buds and shoots under high concentrations [2]. Efficient growth and development is achieved when *in vitro* growth media and conditions are determined for the various growth stages namely culture establishment, mass propagation and plantlet development prior to transferring crops to the field. In yams, nodal culture establishment requires the addition of NAA and BAP to the medium, whereas meristem establishment requires the inclusion of GA_3 and adenine sulphate to the medium. The mass propagation state requires only cytokinin be it Kinetin or BAP, whereas the rooting and plantlet establishment stage does not require any growth regulator in the medium [2]. *In vitro* manipulations when established are very important for germplasm maintenance.

1.3. DNA fingerprinting

In Ghana, crops developed, evaluated and selected for utilisation are given to the farmers through the agriculture extension agents. Most of the time as much as each crop variety has a

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name, they are all referred to as agriculture varieties. There are even incidents where different settlements give different names to the same crop variety, although the selection of the name at the variety developmental stages is done with the farmer. This makes it very difficult for the researcher after releasing a variety to track the extent of spread and adoption. Fingerprinting and documentation of genetic make up at the DNA level is thus vital to facilitate the researchers efforts. Fingerprinting can also be referred to as genotyping; which is the process of determining the genetic constitution – the genotype – of an individual by examining their DNA sequence. This provides information necessary to characterise germplasm and is a vital tool for identification of germplasm, as well as ensuring the genetic integrity with time. Genotyping can be applied to a broad range of organisms, including microorganisms. The genotype of an individual provides the fingerprint and comparing fingerprints allows you to determine the similarity between two individual, to find matches. This kind of information is vital to document the identity of crop varieties released to facilitate the ability to trace individual at any point in time.

Currently, methods of characterization used by breeders include morphological, agronomic, and biochemical systems. Characterization based on morphologic characteristics alone may be limited since the expression of quantitative traits is subjective to strong environmental influence. Alternatively, molecular characterization techniques are capable of identifying polymorphism represented by differences in DNA sequences. This has the ability of analyzing variation at the DNA level during any stage of the development of the plant, where environmental influences are excluded. The PCR-based methods constituted a new milestone in the field of DNA fingerprinting that has to be included in the requirements for varietal release in Ghana.

1.4. Study objective

The need for clean planting materials on mass production scale is crucial to complement limitations of seed production in yam industry. *In vitro* rapid multiplication offers the best system to be used in efforts to meet seed yam targets in the dissemination of clean planting material of released root and tuber varieties. This system is not in existence in Ghana, therefore first objective of this paper is to establish appropriate medium for each of the three released yam varieties to enhance *in vitro* rapid multiplication, and document *in vitro* production scheme for the released yam varieties.

Following the evaluation and selection of a crop variety for dissemination, it is vital to have a system that will permit the ability to trace the product. Genomic fingerprinting is one such tools that when used can facilitate variety identification. This study uses SSR Microsatellites to establish the molecular identity of three released yam varieties alongside 21 other yam accessions comprising species *Dioscorea rotundata, D. cayenensis, D. bulbifera, D. alata, D. dumentorum,* and *D. esculenta.* The documentation of this information will enable the researcher to identify their samples at any point in time and also provide genetic relatedness information that is vital for breeding.

2. Materials and methods

2.1. *In vitro* manipulations

2.1.1. Establishment of Mother Plant under screenhouse conditions

Three yam varieties released by CSIR-CRI namely CRI-Pona, Mankrong Pona and Kukrupa were used in the study. Samples of yam tubers were obtained from the CSIR-CRI yam breeding program, sectioned into minisetts and treated with ash and benlate prior to planting in pots at the Screenhouse. Mini setts sprouted after approximately six weeks and vines older than three weeks were harvested for *in vitro* manipulations.

2.1.2. Preparation of explants

Yam vines were harvested from plants growing in the screenhouse, labelled appropriately and the cut end was dipped in deionised water and sent to the Tissue Culture laboratory. Vine was thoroughly washed under running tap water and the nodal cuttings and shoot tips were excised (Figures 3 and 4) into autoclaved deionised water in labelled beakers for surface sterilisation.

Figure 3. Yam Nodal cutting explant freshly harvested from the field for initiation *in vitro*

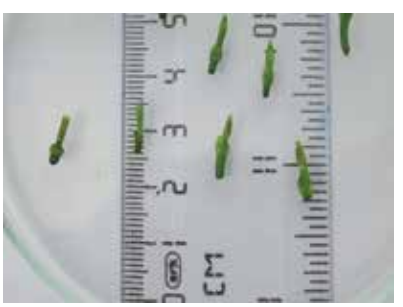

Figure 4. Yam Shoot tips explant freshly harvested from the field for meristem excision and initiation *in vitro*

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Surface sterilization was carried out under sterile conditions in the laminar flow cabinet as follows: Explants were transferred into beakers containing 70% ethanol for five minutes and then surface sterilized with 20% sodium hypochlorite solution (with 6% active chlorine) containing 2-3 drops of tween 20 for 15 minutes. They were washed with sterile distilled water three times after which edges of explants were trimmed. Explants were then further surface sterilized with 10% sodium hypochlorite solution containing 2-3 drops of tween 20 for 10 minutes and the edges of the explant trimmed, rinsed three times in autoclaved distilled water and kept in autoclaved water prior to culturing. After sterilization, meristems (approximately 1x1 mm) were excised from the shoot tips using a dissecting microscope. The meristems and nodal cuttings were then labelled appropriately and cultured on appropriate media and labelled accordingly. The individual cultured explants were code labelled for ease of tracing the material used for initiation. This was vital because once a culture is screened for the presence of a virus and it test negative or positive, the implication is that all clonal materials generated from that particular explant are either cleaned or infected respectively. Infected explants can therefore removed from the mass propagation system.

2.1.3. Nutrient media preparation

2.1.3.1. Initiation medium for Yam

Murashige and Skoog [11] basal salts complimented with growth hormones and vitamins were used. The growth regulators used for meristem cultures were BAP, NAA and GA3. Where nodal cuttings were used, GA3 was not included in the medium. The medium was further supplemented with Adenine Sulphate ($AdSO_4$) (80 mg/l) as a cytokinin additive and L-cysteine (20 mg/l) as an antioxidant, 30 g sucrose and 7 g agar were used as carbon source and gelling agent respectively. The details of the media composition are as in the table 1 below. The following vitamins Myo- inositol, Nicotinic acid amide, Pyridoxine, Thiamine- HCl and Glycine were used as documented by Murashige and Skoog [11]. The pH of the medium was set at 5.7 ± 1 and sterilised in an autoclave at a temperature of 121°C at 15 psi for 15 minutes. Culture vessels used were pyrex test tubes with dimension 16 X 125mm. Medium to be used for meristem cultures were slated after removal from the autoclave prior to allowing them to cool down. This provided a broad surface for the excised meristem to be placed on the upper part as represented in the Figure 5. Meristem cultures were transferred every eight weeks onto fresh medium till shoots differentiated from the explants after ten months. Shoots differentiating from nodal buds were excised onto the same medium till actively growing shoots were obtained.

2.1.3.2. Rapid multiplication medium for Yams

Actively growing shoots (Figure 6) from both meristem and nodal bud cultures were subcultured onto complete MS medium with vitamins supplemented with 2.5 μM kinetin referred to as yam multiplication medium. During subculture, the shoot tips (Figure 7) and nodal cuttings (Figure 8) were excised from a growing shoot and grown on freshly prepared medium. Kinetin concentration in the rapid multiplication medium was manipulated to optimize yam

Figure 5. Growing Yam meristem placed on slanted medium

multiplication medium. The complete MS medium with vitamins was supplemented with kinetin at 0, 2.5, 5, and 10 μM concentration. Another medium that was used was the complete MS medium, supplemented with the following vitamins Myo-inositol, Nicotinic acid amide, Pyridoxine, Thiamine-HCl and Glycine and labelled as "mm". The pH of all the media was set at 5.7±1 using 0.1 M NaOH for adjusting it from a lower pH. The media were sterilised at a temperature of 121°C at 15 psi for 15 minutes in an autoclave. The various media used in these experiments are presented in the Table 1.

Figure 6. Actively growing yam cultures

2.1.4. Incubation

All cultures were incubated at a temperature of 26 ± 2°C with a photoperiod of 16 hours of light and 8 hours of darkness.

http://dx.doi.org/10.5772/60411

Initiation/Merisem Medium	**Quantity / Final Concentration in Media**		
Component	**Meristem Initiation medium**	**Nodal Cutting Initiation medium**	**Rapid Multiplication medium**
MS Basal Salts*	4.33 g/l	4.33 g/l	4.33 g/l
MS Vitamins*	Manufacture's instruction	Manufacture's instruction	Manufacture's instruction
$AdSO_4$	80 mg/l	80 mg/l	80 mg/l
BAP	5 X 10^{-6} M	5X10^{-6} M	-
NAA	1 X 10^{-7} M	5X10^{-7} M	-
GA_3	2.25 X 10^{-7} M	-	-
Kinetin	-	-	0 – 10 X 10^{-6} M
Sucrose	30 g/l	30 g/l	30 g/l
L-Cysteine	20 mg/l	20 mg/l	20 mg/l
Agar	7-7.5 g/l	7-7.5 g/l	7-7.5 g/l
pH	5.7 ± 0.1	pH	5.7 ± 0.1

*Murashige and Skoog basal salts and vitamins [11]

Table 1. Medium composition for yam (1 litre)

Figure 7. Nodal cutting excised from actively Growing cultures for rapid multiplication

2.1.5. Biomass determination

Dry matter was also estimated as a measure of growth using 5 plantlets per treatment. Fresh weight was estimated by weighing the plantlets while dry weight was estimated by drying the plantlets in an oven at 80°C for 48 hours. Each treatment was replicated thrice.

Figure 8. Yam Shoot tips excised from actively Growing cultures for rapid multiplication

2.1.6. Data collection and analysis

Data was taken after 8 weeks of initiation and subsequent data were taken every 8 weeks by counting the number of leaves, shoots, multiple buds as well as root development and the general performance of the cultures was also noted. Statistical package used to analyse data was SAS 9.1.

2.2. Fingerprinting

2.2.1. Plant materials

Sampling of leaves for DNA isolation towards genomic studies was made from the field germplasm holding of the CSIR – Plant Genetic Resources Research Institute, located at Bunso in the Eastern Region of Ghana. For this study, a total of 21 samples were selected at random from six different species of yam grown in Ghana namely *Dioscorea alata, D. dumenterom, D. rotundata, D. cayenensis, D. bulbifera* and *D. esculenta* (Table 2). There were at least three different samples of each species and these were screened alongside the three released yam varieties.

2.2.2. Extraction of genomic DNA

Genomic DNA was isolated from 100 mg of young tender leaves. They were weighed into 2 ml eppendorf tubes and grounded with liquid nitrogen into fine powder. The genomic DNA was extracted using the following manufacture's instructions of the Qiagen protocol from the DNeasy plant mini kit.

2.2.3. DNA quantification and gel electrophoresis

The quality of DNA was checked on 0.8% agarose in 1x TAE (Tris-acetic EDTA) buffer by gel electrophoresis with Ethidium bromide (0.5 ug/ml). Electrophoresis of the DNA was carried out at 120 V for 40mins and then visualized with a UV transilluminator. The quality of DNA was ascertained and the concentration was projected by the intensity and comparison to 1 kb lambda DNA mass ladder (1 kb invitrogen). Quantification of DNA was

Accession	Lab Code	Specie	Population Identity
KT/01/015	Dr 013	*D. rotundata*	Pop1
UWR/97/101	Dr 026	*D. rotundata*	
UWR/97/059	Dr 039	*D. rotundata*	
UWR/97/085	Dr 052	*D. rotundata*	
FA/89/039	Da 110	*D. alata*	Pop 2
82/326	Da 130	*D. alata*	
FA/89/026	Da 137	*D. alata*	
AGA/97/173	Da 143	*D. alata*	
82/430	Dd 178	*D. dumeterom*	Pop 3
AGA/97/202	Dd 183	*D. dumeterom*	
BD/96/023	Dd 186	*D. dumeterom*	
TA/97/013	Db 187	*D. bulbifera*	Pop 4
TA/97/093	Db 193	*D. bulbifera*	
TA/97/141	Db 201	*D. bulbifera*	
SO/89/093	De 202	*D. esculenta*	Pop 5
TA/97/071	De 208	*D. esculenta*	
82/407	De 215	*D. esculenta*	
TA/97/057	Dc 109	*D. cayenesis*	Pop 6
SCJ/89/001	Dc 221	*D. cayenesis*	
BD/96/026	Dc 249	*D. cayenesis*	
82/129	Dc 251	*D. cayenesis*	
CRI Pona	CRI-Pona	*D. rotundata*	Pop 7
Mankrong Pona	Mankrong Pona	*D. rotundata*	
Cri-Kukrupa	CRI-Kukrupa	*D. rotundata*	

Table 2. List of accessions used for the study

evaluated by reading absorbance at 260 nm and 280 nm with the spectrophotometer. The DNA was diluted to 10 ng/μl for PCR amplifications.

2.2.4. PCR amplification

A set of 16 set of primer pairs [19] were used in the experiment (Table 3). DNA amplification was carried out with a 96 well plate Bio-Rad™ Thermocycler from BIO-RAD. The PCR conditions were optimized for cycling number, concentrations of the primer, $MgCl_2$ and DNA template. The reaction mixture (10 ul) contained 6.075 ul of Nuclease free sterile water (DNA

grade water), 1 ul of 10x PCR Buffer, 0.9 ul of $MgCl_2$ (25 mM), 0.4 ul dNTPs (10 mM), 0.25 ul primer (50 ug/ml) of each forward and reverse, 0.125 ul of SuperthermTaq Polymerase (1unit) and 1 ul of 10 ng DNA template. The cycling conditions were as follows: an initial denaturing step of 94°C for 5 mins, 35 cycles of 94°C for 30 secs, 51°C for 1min, 72°C for 1 min and a final elongation step of 72°C for 1 min. In every experiment, a negative control was included where the template DNA was replaced with PCR grade water. Amplification products were examined on a 6% polyacrylamide gel (Water, 10x TBE, 4% acryl amide (19:1), 10% APS and TEMED) and stained with silver nitrate. A 100 bp ladder (Gene Ruler ™, Fermentas) was used as a size marker.

Primers	Sequence	Tm/°C
Da1F08R	5'CTATAAGGAATTGGTGCC	54.4
Da1F08F	5'AATGCTTCGTAATCCAAC	54.9
Da1D08F	5'GATGCTATGAACACAACTAA	52.5
Da1D08R	5'TTTGACAGTGAGAATGGA	54.6
Da1C12R	5'AATCGGCTACACTCATCT	54.4
Da1c12F	5'GCCTTTGTGCGTATCT	54.2
Da1A01F	5'TATAATCGGCCAGAGG	54.1
Da1A01R	5'TGTTGGAAGCATAGAGAA	53.9
Dpr3F10R	5'ACGCACATAGGGATTG	54.9
Dpr3F10F	5'TCAAAGGAATGTTGGG	54.8
Dpr3F12R	5'TCAAGCAAGAGAAGGTG	54.4
Dpr3F12F	5'TCCCCATAGAAACAAAGT	54.2
Dab2E07F	5'TTGAACCTTGACTTTGGT	55.3
Dab2E07R	5'GAGTTCCTGTCCTTGGT	54.5
Dpr3F04R	5'GCCTTGTTACTTTATTC	46.2
Dpr3F04F	5'AGACTCTTGCTCATGT	46.7
Dpr3D06R	5'ACCCATCGTCTTACCC	55.3
Dpr3D06F	5'ATAGGAAGGCAATCAGG	54.8
Dpr3B12R	5'CCATCACACAATCCATC	54.9
Dpr3B12F	5'CATCAATCTTTCTGCTT	54.3
Dab2D08R	5'GATTTGCTTTGAGTCCTT	54.1
Dab2D08F	5'ACAAGAGAACCGACATAGT	53.4
Dab2E09F	5'AACATATAAAGAGAGATCA	45.3
Dab2E09R	5'ATAACCCTTAACTCCA	46.3

Primers	Sequence	Tm/°C
Dab2D06F	5'TGTAAGATGCCCACATT	54.4
Dab2D06R	5'TCTCAGGCTTCAGGG	55.1
Dab2C12R	5'CGAACGATCCAATAAAA	54.2
Dab2C12F	5'AGGCATCTTGGGAAA	54.3
Da3G04F	5'CACGGCTTGACCTATC	54.5
Da3G04R	5'TTATTCAGGGCTGGTG	55
Dab2C05F	5'CCCATGCTTGTAGTTGT	53.9
Dab2C05R	5'TGCTCACCTCTTTACTTG	53.5

Source: Tostain *et al*., [19]

Table 3. List of primers, their sequences and melting temperatures

2.2.5. Silver staining

The gels were placed on a shaker with a minimal shaking to allow solution flow over gel swiftly. Fixation was done with 10% Glacial Acetic Acid (100 ml acetic acid, 900 ml water) for 15 mins. This was washed off with distilled water and 1.5% Nitric acid (15 ml Nitric acid, 985ml water) solution was added for 5 mins. The silver stain solution (1.0 g Silver nitrate, 1.5 ml 37% formaldehyde, topped it up with water up to 1000 ml) was preceded after washing off the nitric acid solution for 20mins. Finally, the developer (30 g Sodium carbonate, 1.5 ml 37% formaldehyde and 0.25 ml Sodium thiosulphate 10 mg/ml, water up to 1000 ml) was added and allowed to develop the photographic stains/ bands for visualization. This was stopped with 10% acetic acid and then stored in distilled water for photographic capturing and scoring.

2.2.6. Gel scoring and data analysis

Bands were scored manually as present (1) or absent (0) from the gels. Similarity matrix was calculated using NYSTS software while cluster analyses were also carried out using Genstat and dendrograms were constructed. Similarity matrices from each primer were compared pair wise using a randomization test. POPGENE32 [22] was used for genetic population analysis as well as to test the effectiveness of loci used.

3. Results and discussion

3.1. *In vitro* manipulations

3.1.1. Explant response on initiation medium

The growth and development of different crop species vary considerably. The data obtained following *In vitro* initiation of nodal cuttings and meristems are presented in Tables 4 and

5 respectively. The nodal cuttings for Kukrupa and Mankrong-Pona when grown in yam initiation medium, had higher success rate than CRI-Pona (Table 4). The success rate was higher in the nodal cuttings (52.9 – 86.6%) than in the apical meristems (46.4 – 53.33%). In both situations, Mankrong-Pona had a higher success rate than CRI-Pona. The variety Kukrupa was however not included in the apical meristem experiment. The measure of percentage success was based on explants that developed to the extent of producing shoots. Well-developed shoots were obtained in all the successful nodal cutting explants after eight weeks in culture although by two weeks, some explants had already started producing shoots (Figure 9). Meristem development was however very slow since it took six months for shoot differentiation to occur and following that, by ten months, multiple shoots had started developing (Figure 10 a&b). Following culture initiation, successful meristems initially expanded due to cell division, turned dark with green clusters of cells, which later differentiated into buds and then further into shoots. Shoot differentiating from most of the Makrong-Pona meristem cultures had more than ten leaves and up to eight shoots per culture however, on the average, there were 4.43 shoots per culture, whereas CRI-Pona had 1.25 shoots per culture (Table 5). Shoots differentiated from nodal bud explants 12 days after culture (Table 4). The extent of success on meristem cultures was expected to be low, mainly due to the minute size of the explant used in initiation. Culture development is slow as it takes up to ten months for shoot to develop. However multiple shoots develop from the meristems and this facilitates rapid *in vitro* development. Hence if only a few meristems are successful *in vitro*, mass propagation is achieved. Development of shoots from nodal cutting explants is notably high and reliable. However, this method cannot be reliable if pathogens especially viruses have to be eliminated from the crop variety.

Variety	Total number of explants initiated	Number successful	% Success	Average Shoot formation
Cri-Pona	51	27	52.9	1.3
Kukrupa	69	54	78.3	2.1
Mankrong-Pona	45	39	86.7	1.5

Table 4. Yam Nodal Cutting initiation Success Rate of the three released varieties

Variety	Introduction number	Number successful	% Success	Mean No. of leaves	Mean No. of shoots
Mankrong Pona	90	48	53.3	7.4	4.4
Cri-Pona	84	39	46.4	3.3	1.3

Table 5. Yam Meristem initiation success Rate of the three released varieties

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Figure 9. Yam nodal cultures sprouting *in vitro* after two weeks in culture

A **B**

Figure 10. Four months old yam meristem (A) differentiating into multiple shoots after 10 months in culture Yam meristem explant forming multiple shoots after ten months (B) in culture.

3.1.2. Selection of appropriate rapid multiplication medium

Media supplemented with kinetin at different concentrations as well as MS medium enriched with vitamins and growth additives were used in this study. The responses of the three

released varieties on different media are presented in Figures 11 and 12 below. The performance of the three released varieties varied on the four different media. Considering shoot development, the mean was highest on medium containing 10 μM kinetin for CRI-Pona at 4±1.39, 8.6±1.08 for CRI-Kukrupa on 5 μM kinetin and 8.29±0.7 for Mankrong-Pona on 2.5 μM kinetin. With the exception of CRI-Kukrupa where highest number of leaves (9.33±1.8) occurred on the control medium (no kinetin), CRI-Pona had 4.67±1.8 leaves on the same medium as medium with highest number of shoots (10 μM kinetin), and Mankrong-Pona also had 13±1.18 leaves on medium containing 2.5 μM kinetin. Notably this attempt is to maximise *in vitro* rapid multiplication. In tissue culture, nodal cutting are used to generate shoots, and the number of leaves generating is the determining factor for the multiplication rates that can be attained, since within each leaf axil is a bud that can develop into a whole shoot. Hence the higher the number of leaves the higher the multiplication rate. Shoots differentiate from buds and within each bud are clusters of meristematic cells which are capable of differentiating in shoots when the growth conditions are appropriate. Reports in previous research where BAP was the cytokinin used had a maximum of four shoots developing on the average [2]. Later efforts on generating somatic embryos reported 7-9 shoots developing per culture where kinetin was used [16]. This present study reported 3 to 8 shoots per culture, it is therefore indicative that Kinetin is an appropriate growth regulator to be used for rapid multiplication on yam.

Comparing the effect of different media on rapid multiplication of yam nodal culture in terms of shoots and leave (Table 6), medium supplemented with 2.5 uM Kinetin was the best although the difference was not significant. In tissue culture since a lot of clonal materials can be generated from one culture differences in terms of number are significant although statistically it may not be significant. The performance of cultures on the medium labelled "mm" which was not supplemented with Kinetin was very poor and significantly low numbers of leaves and shoots were recorded. This confirms that to achieve rapid multiplication of yam *in vitro*, the inclusion of kinetin is critical. Although the data obtained indicated that to maximize rapid multiplication *in vitro*, different media have to be used for the different varieties released, medium supplemented with 2.5 μM kinetin may be appropriate for all the varieties.

Comparing effect of the yam different varieties, nodal cultures during rapid multiplication (Table 7) in terms of shoots response of Mankrong Pona and CRI-Kukrupa were similar. The variety CRI-Pona had significantly low number of shoots. In terms of leaf development, CRI-Kukrupa had a significantly higher (8.3) number of leaves whereas CRI-Pona had the lowest (3.7). This response was due to varietal differences and Mankrong Pona will be recommended over CRI-Pona where high numbers are needed to be produced within a limited time. There was significantly very high positive correlation between leave and shoot development during rapid multiplication (Table 9). This indicates that the concentration of Kinetin used promote both leaf and shoot development as high concentrations of some cytokinin (BAP) can inhibit leaf development will occur [2] and this will not favour mass propagation.

http://dx.doi.org/10.5772/60411

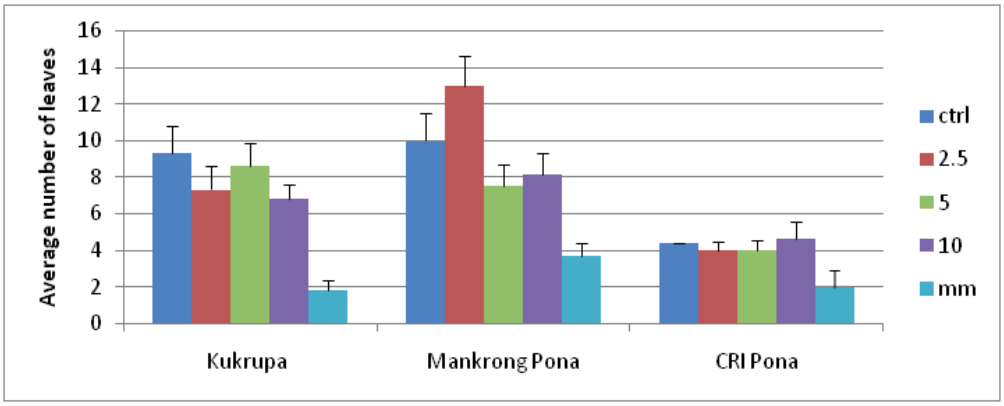

Figure 11. Development of Leaves in cultures of the three released varieties during rapid multiplication

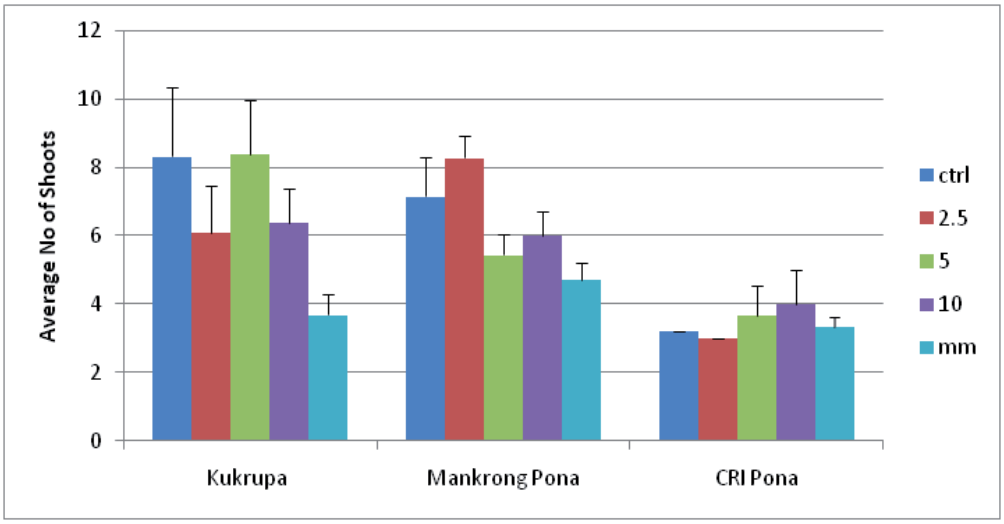

Figure 12. Development of Shoots in cultures of the three released varieties during rapid multiplication

The study considered biomass as an additional measure of growth. The data, as shown in Figure 13 below revealed that plant biomass was consistently low and on the medium labelled "mm". The variety Kukrupa had the highest biomass on the control medium. Considering Mankrong Pona high biomass was on the control medium as well as 2.5 and 5 μM Kinetin supplemented medium. Significantly high biomass was recorded for CRI-Pona cultures growing on 10 μM Kinetin supplemented medium, it is the same medium on which highest number of leaves and shoots were observed for that variety. It is therefore possible that CRI-Pona accumulated biomass at the expense of sacrificing organ differentiation.

Media	Mean(±Stder)	Shoots	Leaves
0	Mean	6.000ab	7.857a
	Stder	0.839	0.988
2.5	Mean	6.421a	8.895a
	Stder	0.788	0.1.149
5	Mean	6.000ab	7.211a
	Stder	0.658	0.801
10	Mean	5.809ab	7.333a
	Stder	0.519	0.773
mm	Mean	3.952b	2.476b
	Stder	0.355	0.423

Similar letters are not significant according to Tukey test ($p<0.05$); Stder – Standard error

Table 6. Comparison of effect of different media on nodal culture of Yam (Number of Shoots and Number of Leaves)

Media	Mean(±Stder)	Shoots	Leaves
CRI Pona	Mean	3.389b	3.778c
	Stder	0.617	0.919
Kukrupa	Mean	6.000a	5.875b
	Stder	0.462	0.689
Mankrong	Mean	6.182a	8.318a
	Stder	0.394	0.588

Similar letters are not significant according to Tukey test ($p<0.05$); Stder – Standard error

Table 7. Comparison of effect of different varieties on nodal culture of Yam (Number of Shoots and Number of Leaves)

		Leaves	Shoot
Leaves	Pearson Correlation	1	.816**
	Sig. (2-tailed)		.000
	N	94	94
Shoot	Pearson Correlation	.816**	1
	Sig. (2-tailed)	.000	
	N	94	94

**. Correlation is significant at the 0.01 level (2-tailed).

Table 8. Correlations

http://dx.doi.org/10.5772/60411

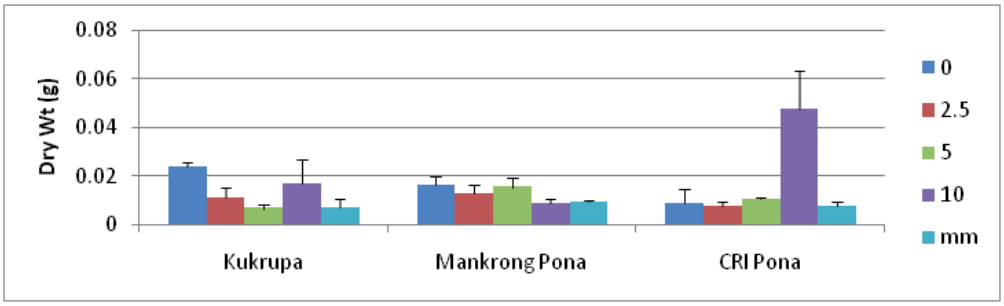

Figure 13. Dry weight and moisture content among the different treatments of the three released varieties

3.2. Fingerprinting results and discussion

Microsatellite in DNA represents repetitive DNA based on very short repeats such as dinucleotides, trinucleotides or tetranucleotides, consisting of repeats of a motif, and is otherwise referred to as Simple Sequence Repeats (SSR). These repeats serve as molecular markers by which genetic identity can be documented. Although there are several molecular marker systems namely: Amplified fragment length polymorphism (AFLP), DNA amplification fingerprinting (DAF), Inter-simple sequence repeat (ISSR), and Random amplified polymorphic DNA (RAPD) just to name a few, microsatellites or SSRs (Simple Sequence Repeats) has been reported as being useful for genotyping due to its high polymorphic information content (PIC). It is a codominantly inherited marker with locus specificity and genomic coverage is extensive. Also, systems provide simple PCR amplification detection methods. This present study used a set of yam simple sequence repeat (SSR) markers developed in different species of yam (*Dioscorea* sp.), where microsatellite-enriched bank was created from *Dioscorea alata*, *Dioscorea abyssinica* and *Dioscorea praehensilis*. That study identified and characterized 16 polymorphic loci, which were found to be transferable to species of other *Dioscorea* sections [19].

In this present study, when the 16 SSR primers were screened, 15 produced scorable bands. These 15 primers were used to screen a total of 25 yam accessions (including the three released varieties). Following amplification of PCR products, there were a total of 94 alleles and an average of 6.26 alleles per loci. Similarly when used to screen 22 *D. rotundata* accessions from Benin, 117 alleles were observed with and average of 7.3 alleles per loci [19]. The data obtained in this study was subjected to NTSYS analysis and this revealed the three released varieties clustering into one group (Figure 14). One *D. rotundata* accession from the CSIR-PGRRI collections was grouped with the released varieties. A similarity matrix (Table 9) established the percentage similarity between Mankrong-Pona and CRI-Pona to be 97%. The data obtained indicated that the released yam varieties are distinct from other *D. rotundata* accessions being conserved at the CSIR-PGRRI. It is argued that similarity above 95% is indicative that the two samples are duplicates. This has to be investigated further since *in vitro* growth rates of the two released varieties (Mankrong Pona and CRI Pona) are distinctively different in this current study. It is possible that the set of loci used to conduct molecular diversity assessment are unable to detect much differences in the released *Dioscorea* varieties as the SSR libraries used

to design the primers were generated from *Dioscorea alata, Dioscorea abyssinica* and *Dioscorea praehensilis* and not *D. rotundata*. The investigator who developed the primers however demonstrated that they are transferable to *D. rotundata*, although only 3 accessions of rotundata were used in that particular study [19].

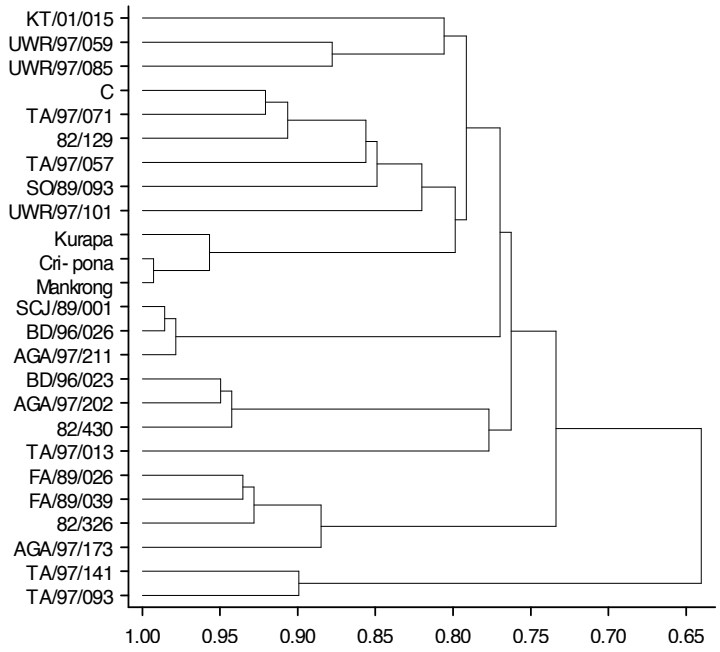

Figure 14. Dendrogram of 25 accessions of yam based on unweighted neighbour joining cluster analysis

	Dr 013	Dr 026	Dr 089	Dr 052	Da 110	Da 130	Da 137	Da 143	Dd 187	Dd 183	Dd 186	Db 187	Db 193	Db 201	De 202	De 208	De 215	Dc 109	Dc 221	Dc 249	Dc 251	Cri- pona	Mankrong	Kukrapa
Dr 013	1																							
Dr 026	0.10	1.00																						
Dr 089	0.66	0.21	1.00																					
Dr 052	0.40	0.42	0.69	1.00																				
Da 110	0.24	0.16	0.27	0.46	1.00																			
Da 130	0.16	0.14	0.23	0.47	0.85	1.00																		
Da 137	0.16	0.20	0.28	0.47	0.85	0.85	1.00																	
Da 143	0.23	0.27	0.25	0.44	0.72	0.58	0.68	1.00																
Dd 187	0.02	0.21	-0.13	0.04	-0.01	-0.03	0.07	0.24	1.00															
Dd 183	-0.01	0.24	-0.10	0.08	0.02	0.00	0.11	0.23	0.89	1.00														
Dd 186	0.02	0.27	-0.07	0.12	0.00	-0.02	0.09	0.16	0.89	0.94	1.00													
Db 187	-0.02	-0.02	-0.02	0.04	0.15	0.12	0.22	0.14	0.28	0.37	0.36	1.00												
Db 193	-0.11	-0.01	-0.09	-0.10	0.02	-0.02	0.03	-0.09	-0.05	0.04	0.04	0.37	1.00											
Db 201	-0.11	-0.01	0.00	-0.06	-0.03	-0.07	-0.07	-0.14	-0.15	-0.06	-0.07	0.23	0.78	1.00										
De 202	0.08	-0.08	0.10	0.12	0.17	0.20	0.20	0.05	-0.07	0.02	-0.02	0.24	0.20	0.20	1.00									
De 208	0.05	-0.15	0.14	0.04	-0.07	-0.09	-0.09	-0.16	-0.04	-0.03	-0.01	0.09	-0.01	0.06	0.33	1.00								
De 215	0.23	0.03	0.23	0.13	-0.03	0.00	0.00	-0.10	-0.09	-0.07	-0.04	0.04	0.04	-0.06	0.20	0.38	1.00							
Dc 109	-0.01	0.33	0.20	0.24	0.17	0.18	0.23	0.06	-0.05	-0.01	0.03	0.05	0.07	-0.02	0.08	0.16	0.56	1.00						
Dc 221	0.02	0.36	0.22	0.25	0.14	0.15	0.20	0.03	-0.07	-0.03	0.01	0.02	0.07	-0.02	0.06	0.15	0.63	0.96	1.00					
Dc 249	0.04	0.33	0.20	0.19	0.12	0.13	0.18	0.01	-0.10	-0.06	-0.02	0.00	0.07	-0.07	0.03	0.09	0.56	0.91	0.96	1.00				
Dc 251	0.03	0.10	0.16	0.31	0.08	0.07	0.07	-0.01	0.11	0.13	0.15	0.27	0.17	0.09	0.15	0.43	0.32	0.28	0.27	0.12	1.00			
Cri- pona	0.02	0.34	0.19	0.20	-0.06	-0.03	-0.03	-0.02	-0.01	-0.04	-0.01	-0.05	0.00	0.05	-0.01	-0.04	0.25	0.31	0.38	0.31	0.30	1.00		
Mankrong	0.04	0.36	0.21	0.22	-0.05	-0.02	-0.02	-0.01	0.01	-0.02	0.01	-0.03	0.02	0.07	0.01	-0.04	0.27	0.33	0.40	0.33	0.31	0.97	1.00	
Kukrapa	-0.01	0.31	0.07	0.08	-0.09	-0.06	-0.06	-0.04	-0.04	-0.07	-0.04	-0.02	0.10	0.10	0.08	-0.11	0.17	0.25	0.33	0.30	0.13	0.89	0.85	1.00

Table 9. Jaccard's coefficient similarity matrix of 25 yam genotypes using 15 SSR primers

Data analysis using population genetic analysis software PopGen32 [22], revealed 100% overall polymorphic loci overall the populations. According to the genetic variation statistics at all

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loci [12] presented in Table 10, the mean number of effective alleles (ne) was 4.05±1.43, loci DalA01 and Dab2C05 had the highest value at 6.82±1.2, however, locus Dab2E07 had the lowest number at 1.73±0.7. The mean observed number of alleles was 5.33±1.45. Again, loci DalA01 and Dab2C05 recorded the highest number of observed alleles (8), however, locus Dab2E07 had the lowest number of alleles (3). With an average allele sample size (n) of 38, locus Dpr3F04 had a high sample size of 42 whereas a value of 26 was recorded in loci Dpr3F10 and Dab2E07. The mean, highest (loci-DalA01) and lowest (locus Dab2E07) Shannon index (I) were 1.46±0.37, 1.99, and 0.74 overall respectively, indicating that locus DalA01 estimated the highest level of genetic diversity among the samples used in this study. The overall allele frequency (Table 11) revealed that Allele A of locus Dpr3F04 was the least frequent allele (0.022) among the samples as this allele was unique to only one sample in the *D. alata* population. However allele A in locus Dab2E07 was the most frequent allele (0.73) as this allele was present in *D. rotundata, alata, cayenensis* and the released varieties, however it was absent in *D. alata* sample, *D. bulbifera*, *D. dumentorum* and *D. esculenta*. The Nei's original measures of genetic identity and genetic distance [13] as shown in Table 12 revealed that the released varieties population are closer to *D. cayenensis* accessions (0.57) than *D. rotundata* population (0.89) used in this study. However they were very distantly related to *D. bulbifera* (2.39).

Locus	Sample Size	na*	ne*	I*
DaIAO1	44	8.0000	6.8169	1.9937
DaIC12	40	6.0000	4.0609	1.5552
DaID08	42	5.0000	4.3881	1.5384
DaIF08	44	4.0000	3.6391	1.3278
Da3G04	42	5.0000	4.0091	1.4837
Dab2CO5	44	8.0000	6.8169	1.9904
Dab2C12	44	5.0000	3.3379	1.3307
Dpr3D06	30	4.0000	3.1915	1.2609
Dab2D06	32	6.0000	5.1717	1.7020
Dab2D08	34	5.0000	2.8614	1.2822
Dpr3F04	46	5.0000	3.5986	1.3764
Dpr3F10	26	7.0000	5.2000	1.7756
Dpr3F12	42	5.0000	3.0625	1.3108
Dab2E07	26	3.0000	1.7333	0.7436
Dab2E09	32	4.0000	2.9767	1.2342
Mean	38	5.3333	4.0576	1.4604
St. Dev		1.4475	1.4276	0.3206

* na = Observed number of alleles

* ne = Effective number of alleles

* I = Shannon's Information index

Table 10. Summary of Genetic Variation Statistics for All Loci

Allele \ Locus	DaIAO1	DaIC12	DaID08	DaIF08	Da3G04	Dab2CO5	Dab2C12	Dpr3D0
Allele A	0.1818	0.0500	0.2857	0.2955	0.1429	0.0455	0.3409	0.4333
Allele B	0.2273	0.3750	0.1190		0.1667	0.1136	0.3864	0.3000
Allele C	0.1136	0.2000	0.1905		0.2619	0.1591	0.0909	0.1333
Allele D	0.0909	0.1000	0.2857	0.2955	0.3571	0.1364	0.1591	0.1333
Allele E	0.1136		0.1190	0.2955	0.0714	0.1136		
Allele F	0.1136	0.0500		0.1136		0.2273	0.0227	
Allele G	0.0455	0.2250						
Allele H	0.1136					0.1364		
Allele I						0.0682		

Allele \ Locus	Dab2D06	Dab2D08	Dpr3F04	Dpr3F10	Dpr3F12	Dab2E07	Dab2E09
Allele A	0.0625	0.0588	0.0217	0.0385	0.1667	0.7308	0.1875
Allele B	0.1875	0.0588	0.1957	0.0769	0.1429	0.1923	0.5000
Allele C	0.2188	0.5294	0.3478	0.3077	0.5000	0.0769	0.1250
Allele D	0.2500	0.1765	0.3261	0.0769	0.1667		
Allele E	0.0938	0.1765		0.1538	0.0238		0.1875
Allele F	0.1875			0.1923			
Allele G				0.1087	0.1538		
Allele H							
Allele I							

Table 11. Overall Allele Frequency :

pop ID	1	2	3	4	5	6	7
1	****						
2	0.4794	****					
3	1.4823	1.4745	****				
4	0.9579	0.9490	1.6502	****			
5	0.7289	1.1958	1.6637	0.7025	****		
6	0.5685	0.9745	2.1447	0.9580	0.3903	****	
7	0.8903	1.4844	2.3915	1.3699	0.9354	0.5665	****

Table 12. Nei's original measures of genetic identity and genetic distance

4. Conclusion

In this study, *in vitro* performance of the three released yam varieties revealed that they respond differently to the same medium. Maximising the used of tissue culture for mass

propagation of clean healthy planting materials is crucial and to achieve this individual varieties have to be micropropagated in appropriate medium. Other growth regulators may have to be screened to further optimise the performance of CRI-Pona. The study has made fingerprint information available to monitor the integrity of the released varieties.

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Chapter 3

Utilization of *Beta vulgaris* Agrowaste in Biodegradation of Cyanide Contaminated Wastewater

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Additional information is available at the end of the chapter

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1. Introduction

Recent developments in biotechnology for agro- /agro-industrial waste utilization have identified a plethora of agricultural waste (agrowaste) that is suitable for microbial proliferation and production of a variety of high value biological products, which are useful in industrial as well as environmental applications. About 1.6 billion tons of agrowaste is reportedly generated globally per annum [25]. Considering the environmental degeneration caused by such waste, and the fact that they are readily available, research studies have been geared toward assessing the feasibility of converting such waste into value added products. Studies into the chemical and nutritional composition of agrowaste have equally identified some of them as suitable substrates for microbial cultivation [54, 40, 63, 69, 5].

In environmental bioremediation applications, microorganisms can be supported on solid agrowaste to provide the required macro- and micro-nutrients required for biofilm formation, which usually enhances the metabolic activities of the microorganisms for solubilization and biodegradation of contaminants, some of which are known to be potential human carcinogens [18, 22]. The paradigm shift from conventional substrates such as refined glucose, to unconventional substrates such as solid agrowaste or agro-industrial waste could be due to the fact that the latter mitigates operational costs, particularly for large-scale processes. Nutrients are considered the largest expense in industrial bioprocesses whereby the fermentation medium can account for a large proportion of fermentation costs [10, 39, 60]. Suitable agrowaste such as orange peel, apple pomace, wheat bran, sugar cane bagasse, wheat bran, soybean oil cake, jatropha curcas, whey waste, and *Beta vulgaris*, have been identified to support microbial growth and the synthesis of metabolites which can catalyze a number of reactions under suitable conditions[46, 42, 62, 5].

One of the most common wastewater pollutants is cyanide. It is usually released through various anthropogenic activities in the form of industrial effluent discharged from numerous industries. Another incessant anthropogenic source of cyanide deposition into the environment is through petroleum oil processing and its derivatives. Naturally, hydrocarbon oils such as petroleum contain cyano group compounds, which react with metals during thermal cracking operations to form metal cyanide complexes that culminate in wastewater [14]. Many of these cyanide complexes are known to be highly unstable, mainly due to thermal instability, thus releasing free cyanide into the environment under high temperature. It has been reported by Acheampong *et al.* [1] that, cyanide concentrations from facilities that serve industrialized areas could have cyanide concentration higher than 21.6 mg F-CN/L. Cyanide exposure is known to result in neurological disorders and thyroid abnormalities in humans [69, 55]; hence, a robust and economically feasible bioremediation process using renewable resources (agrowaste), i.e. an environmentally benign approach, is necessary to ensure a sustainable and an effective bioremediation process for cyanide deposited into the environment.

It is common to use oxidation methods for cyanide degradation and its complexes, such as the use of metal catalyzed hydrogen peroxide, and alkaline chlorination processes, including removal by ion-exchange resin [17]. This approach, though effective, has some drawbacks that are of major concern. The excess reagents used in the treatment tend to further pollute the environment, as well as increase operational costs. In addition, due to municipal regulations in some countries, the application of chemical methods on a large scale is not permissible. Considering that cyanide in wastewater is undesirable, if present, it must not exceed the discharge limit of 0.01 mg F-CN/L [23]. Thus, cyanide degradation using biotechnological processes is desirable.

It has been shown that several microorganisms such as algae, bacteria, and fungi, can produce enzymes that are capable of degrading free cyanide, cyanide complexes and by-products produced [3, 24, 33, 59]. Recently, studies have established sustainable cyanide biodegradation processes using various microorganisms such as *Klebsiella sp.*, *Pseudomonas sp.*, *Acinetobacter sp.*, *Bacillus sp.*, and many others [41, 58]. A fungal specie - *Fusarium oxysporum*, has equally been reported for its ability to produce enzymes such as nitrilase which readily hydrolyses cyano-compounds into a corresponding weak acid and ammonium-nitrogen, thus bioremediating the contaminated wastewater, with both the acid and ammonium-nitrogen produced being consumed for metabolic functions [37, 32]. Several agrowaste have also shown to be effective substrates for the cultivation of microorganisms and for the biodegradation of cyanic compounds [15, 30, 49].

The application of agrowaste as a substrate in cyanide biodegradation systems is particularly promising, as reported by Santos *et al.* [62]. Having a readily accessible waste material, microorganisms will be able to produce enzymes suitable for bioremediating contaminants in wastewater [59]. Besides their application as effective biosorbents, agrowaste can serve as a sole substrate for bioremediation purposes, on condition that it is compatible to the microbial community to be used [67, 45, 17, 16]. In South Africa, approximately 10 million tons of agrowaste is generated per annum [53], of which 96% is classified as pre-consumer waste

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(Figure 1). This is a large quantity of waste for a developing economy and should be put into profitable use to safe our environment.

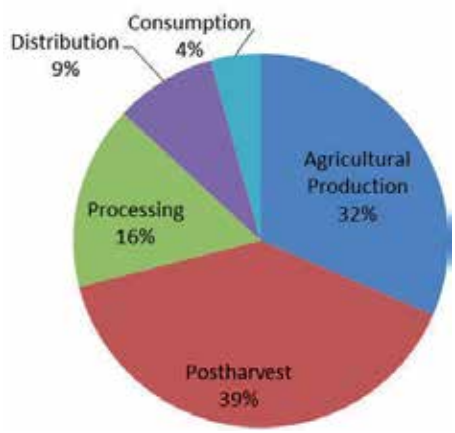

Figure 1. Classification of agrowaste production in South Africa [53]

2. Application of *Fusarium sp.* and *Beta vulgaris* in cyanide biodegradation

Fusarium sp. are widely distributed in environmental samples, particularly in soil. They can cause spoilage of agricultural produce and produce mycotoxins which contaminate cereal crops, affecting human and animal health, if the mycotoxins enter the food chain. *Fusarium sp*. has also been found useful in the hydrolysis of starch. The hydrolysed agricultural produce can be used to sustain the production of extracellular enzymes such as pectinase, cellulase, xylanase, amylase, and organic acids [43]. The fungus is also known for the production of cyanide hydratase and nitrilase including cyanidase. *Fusarium sp.* has been identified as having the ability to degrade cyanides through hydrolysis at varying temperature and pH, then metabolise the by-products as either nitrogen and carbon sources, respectively [52, 31]. The cyanide hydratase, converts the cyanide to amide products and ammonium-nitrogen while the nitrilase hydrolyse cyanide to produce a carboxylic acid [50]. Compared with other enzymes derived from bacteria, nitrilase and cyanide hydratase are of higher activity and can degrade various cyanides [59].

Beta vulgaris waste consists of water, carbohydrates, minerals and proteins which makes it a suitable substrate for microbial growth in the production of high value compounds [5, 45]. However, limited studies have shown its potential as a feed stock and solid support in a bioreactor for the biodegradation of cyanide in the presence of heavy metals [46]. Additionally, hydroxyl functional groups found in *B. vulgaris* waste can act as pseudo-catalysts for the conversion of cyanide to ammonium-nitrogen. Alhough the free hydroxyl functional group is a weak acid, they are able to deprotonate to produce alkoxides in the presence of a strong base like cyanides especially at high alkaline pH (Figure 2) [31, 62].

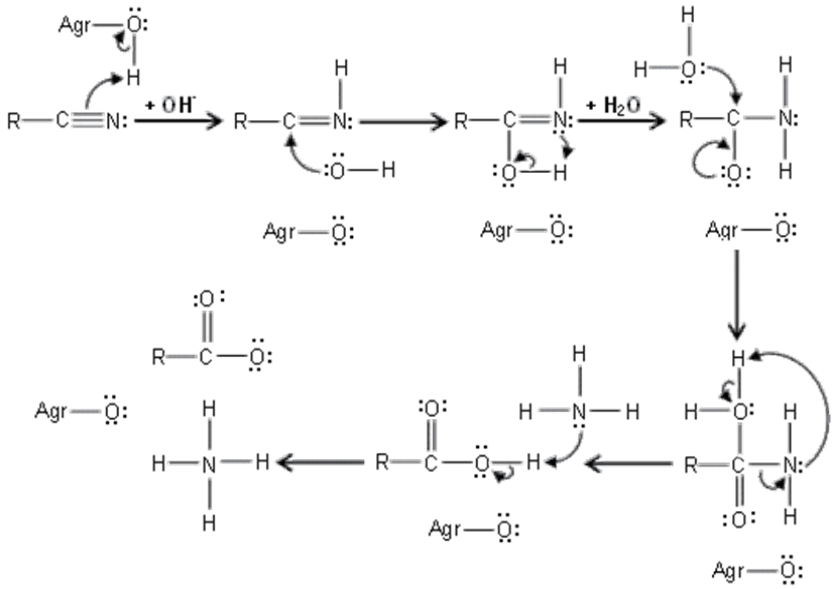

Figure 2. Pseudo-catalyst conversion of cyanide by free hydroxyl functional groups [61]

3. Biodegradation of cyanide by *Fusarium oxysporum* grown on *Beta vulgaris*

A number of different studies report on the application of cyanide degrading fungi. For instance, white rot fungi, *Trametes versicolor*, have been shown by Cabuk *et al*. [9] to tolerate cyanide concentration up to 130 mg F-CN/L, with complete degradation observed within 42 hours to produce minute quantities of ammonium-nitrogen (5.24 mg NH_4^+-N/L). Fourteen cyanide degrading fungi were examined by Pereira *et al*. [57] such as *Fusarium sp*. including *Aspergillus sp.* by Santos *et al.* [57, 62], and were found to tolerate cyanide concentration up to 520 mg F-CN/L. A list of other cyanide degrading species including degradation conditions are shown in Table 1.

There has been limited emphasis on the effect of carbon or nitrogen sources used in the biodegradation of cyanide. The viability of the agrowaste depends on the type of bioremediation required and the microorganism used. When the cultivating conditions are conducive, the minerals, proteins, carbohydrates and water in the agrowaste become easily accessible to the microorganisms [46]. Monosaccharides such as mannose, glucose and fructose present in the agrowaste can effectively support and/or enhance microbial growth [2]. Other overriding factors which directly influence cyanide degradation include exposure to direct sunlight, temperature and pH. Cyanide compounds are soluble in water, thus dissociate and evaporate easily at low pH (i.e. pH<9) while under high salinity, the solubility decreases. Also at neutral

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pH, weak-acid dissociable (WAD) cyanides such as copper or zinc cyanide complexes, if present in a high concentration, dissociate, releasing a cyano group. Similarly, the reduction in temperature reduces the activity of microorganisms used in bioremediation. A number of studies have proven that, at low temperature (below 10°C), growth of microorganisms is inhibited, resulting in low removal rates of contaminants such as ammonium-nitrogen, nitrates and cyanide [72, 29, 73].

In this study, the biodegradation of cyanide in the presence of heavy metals (arsenic, copper, lead, iron and zinc), using *Fusarium oxysporum* grown on *B. vulgaris* waste as the sole carbon source, without any buffer solution, was investigated. The effect of temperature and pH on cyanide degradation with minimal ammonium-nitrogen production was studied using a response surface methodology.

4. Materials and methods

The experiments were carried out in batch cultures. *B. vulgaris* waste was milled to ≤ 100 μm. A broth of 0.5 g of milled waste in 10 mL distilled water was autoclaved at 116°C for 15 min to prevent thermal breakdown of reducing sugars [51]. To the waste broth, wastewater (20 mL) with 1 mL of a spore solution (2.25 x 10^6 spore/mL) of *Fusarium oxysporum* was added to the *B. vulgaris* broth. The wastewater used had characteristics similar to the goldmine wastewater reported by Acheampong *et al.* [1] having metals such as arsenic, iron, copper, lead and zinc. The mixture was incubated for 48 hours in a rotary shaker at 70 rpm at the desired temperature and pH (- see Table 2). After this, KCN in distilled water, was added to make a final cyanide concentration of 500 mg CN^-/L in the mixture. Thereafter, the mixture was incubated for a further 72 hours at 70 rpm at the desired temperature (- see Table 2). All experiments were carried out in duplicate in airtight multiport round bottom Erlenmeyer flasks (n = 28; final volume of 51 mL). Cyanide (CN) (09701) and ammonium-nitrogen (NH_4^+-N) (00683) test kits (MERCK®) were used to quantify the residual free cyanide and ammonium-nitrogen concentrations using a NOVA 60 spectroquant. Free cyanide volatilised was accounted for using the mass balance equations below:

$$CN^-_s - \left(CN^-_r + CN^-_v\right) = CN^-_b \quad (1)$$

$$CN^-_v = CN^-_{vo} - CN^-_{vf} \quad (2)$$

where CN^-_s is the initial free cyanide concentration in the culture broth; CN^-_r is the measured residual free cyanide after incubation; CN^-_v is the volatilised free cyanide during incubation; CN^-_b is the bioremediated free cyanide; CN^-_{vo} is the initial free cyanide in control cultures (500 mg F-CN/L); and CN^-_{vf} is the final free cyanide in control cultures. The control was prepared under the same conditions as other cultures without the *Fusarium oxysporum*.

Microorganism	C-source	N-source	Temperature (°C)	pH	Reference
Fusarium oxysporum	***Beta vulgaris***	**KCN**	**30**	**11**	**This study**
Agrobacterium tumefaciens	Starch	KCN	-	7.2	[58]
Aspergillus awamori	*Citrus sinesis* extract	KCN	40	8.84	[62]
Baccillus pumilus	Glucose	KCN	40	8.5-9	[64]
Baccillus stearothermphilus	-	NaCN	27±2	7.8	[6]
Burkholderia cepacia	Fructose, glucose, mannose	KCN	30	10	[2]
Citrobacter sp., Pseudomonas sp.	Sugarcane molasses, glucose	$[Cu(CN)_4]^{2-}$, $[Zn(CN)_4]^{2-}$	35	7.5	[56]
Cryptococcus humicolus MCN2	Glucose	KCN	25	7.5	[36]
Eschericia coli	Glucose	KCN	30	9.2	[26]
Fusarium solani	Glucose	$K_2Ni(CN)_4$, KCN	25	7.0	[8]
Fusarium solani	Yeast	KCN	30	9.2-10.7	[19]
Fusarium oxysporum	Glucose	KCN	25	8.0	[57]
Fusarium oxysporum immobilised on sodium alginate	Formamide	Cyanides	25-30	8	[11]
Gloeocercospora sorghi, Stemphylium loti	Glucose	KCN	35, 28	5.3-5.7, 7.0	[48]
Klebsiella oxytoca	Glucose	KCN	30	7	[34]
Klebsiella oxytoca immobilised cell	Alginate and cellulose triacetate	KCN	30	7	[13]
Mixed culture of bacteria	Glucose	CN^-_{WAD}	22	7.0	[70]
Mixed culture of bacteria immobilised on ultrafiltration membranes	Phenol	Cyanides	25	-	[35]
Pseudomonas fluorescens	Glucose	Ferrocyanide	25	5	[21]
Pseudomonas fluorescens immobilised on calcium alginate	Glucose	Ferrocyanide	25-35	4-7	[20]
Pseudomonas fluorescens immobilised on zeolite	Zeolite	Tetra-cyano-nickelate (II)	30	-	[66]
Pseudomonas pseudoalcaligenes CECT5344	CH_3COONa	NaCN	30	9.5	[38]
Pseudomonas putida BCN3	Glucose	$[K_2[Ni(CN)_4]]$	30	-	[63]
Pseudomonas putida immobilised on sodium alginate	NaCN	NaCN	25	6.7	[7]
Pseudomonas putida immobilised on sodium alginate	NaCN, sodium alginate	NaCN, Cyanates and thiocyanates	25	7.5	[12]
Pseudomonas stutzeri AK61	-	KCN	30	7.6	[68]
Pseudomonas sp.(CM5, CMN2)	Glycerol	CN^-_{WAD}	30	9.2-11.4	[4]
Stemphilium loti		KCN	25	6.5, 7.5	[27]
Trametes versicolor	Citrate	KCN	30	10.5	[9]
Trichoderma sp.	Glucose	CN^-	25	6.5	[24]
Scenedesmus obliquus	NaCN	NaCN	-	10.3	[33]
Rhodococcus UKMP-5M	Glucose	KCN	30	6.6	[41]

Table 1. Cyanide degrading microbial species using different nutritional sources under different temperature and pH conditions

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Run	Temperature (°C)	pH
1	19.5	8.5
2	9	11
3	19.5	8.5
4	30	11
5	30	6
6	19.5	8.5
7	9	6
8	19.5	8.5
9	19.5	12.04
10	34.35	8.5
11	4.65	8.5
12	19.5	4.96
13	19.5	8.5
14	19.5	8.5

Table 2. Experimental variation of pH and temperature

The response surface methodology was used for the statistical design of the experiments to assess the influence of temperature and pH for optimal degradation of cyanide. A central composite design was used for the determination of optimal operating conditions with a minimum residual ammonium-nitrogen as one of the objectives. Design Expert software® version 6.0.8 (Stat-Ease Inc., USA) was used to generate the experimental runs.

Run	A	B	F-CN degraded (mg F-CN/L)		Residual NH_4^+ (mg NH_4^+-N/L)	
			Experimental value	Predicted value	Experimental value	Predicted value
1	0	0	239	238.86	210	219.14
2	-1	1	229	196.59	100	183.50
3	0	0	239	238.86	210	219.14
4	1	1	250	250.29	40	83.43
5	1	-1	135	167.62	320	222.79
6	0	0	239	238.86	210	219.14
7	-1	-1	127	126.92	128	70.86
8	0	0	239	239.14	210	200.86
9	0	1.414	263	285.75	210	117.41
10	1.414	0	196	172.77	100	135.11
11	-1.414	0	83	106.02	120	98.52
12	0	-1.414	201	178.03	30	136.30
13	0	0	239	239.14	210	200.86
14	0	0	239	239.14	210	200.86

A and B represent coded level of variables.

Table 3. Coded experimental design variables and the corresponding response

The results (Table 3) indicated a variation in responses measured. There was appreciable degradation of cyanide in Runs 9, 4, 1, 3, 6, 8, 13, and 14, with the highest cyanide degraded being 263 mg F-CN/L (Run 9) and the lowest (83 mg F-CN/L) being observed for Run 11. However, both cases had a high residual ammonium-nitrogen of 210 mg NH_4^+-N/L and 120 mg NH_4^+-N/L, respectively. Both Runs 9 and 11 were axial points. Run 9 with an extremely high pH resulted in high residual ammonium-nitrogen while Run 11 with an extremely low temperature was observed to have minimal microbial activity despite the presence of a suitable quantity of *B. vulgaris* used as a carbon source. A similar scenario had earlier been reported by Zilouei *et al.* [72] and Zou *et al*. [73], whereby a low temperature was found to inhibit the growth of microorganisms, thus resulting in low removal of contaminants (ammonium-nitrogen, nitrate and nitrite). On the other hand, Runs 1, 3, 4, 6, 7, 8, 13 and 14 had up to 99% correlation with the predicted values for cyanide degradation which indicated a high accuracy of the model (Equation 4) used for predicting cyanide degradation. However, only Runs 4 and 7, which showed minimal residual ammonium-nitrogen presence, can be used for optimisation for a pilot scale process.

5. Statistical model analysis

The statistical model summary clarifies the fitness of the mean and quadratic models for the two responses based on the Sequential Model Sum of Squares and Lack of Fit Test. The responses were analysed using ANOVA to assess the significance of the variables in the model. A quadratic model was found to give the best fit for the experimental results.

Factor	Coeff. Estimate	DF	Standard Error	95% CL Low	95% CL High	F Value	Prob > F	Significance
Intercept	239	1	10.03	215.27	262.73	11.41	0.0029	S
A	23.6	1	8.69	3.05	44.50	7.37	0.0300	S
B	38.09	1	8.69	17.54	58.63	19.21	0.0032	S
A^2	-49.87	1	9.05	-71.26	-28.49	30.40	0.0009	S
B^2	-3.62	1	9.05	-25.01	17.76	0.16	0.7005	NS
AB	3.25	1	12.29	-25.81	32.31	0.07	0.7991	NS

S = significant; NS = Not significant; CL = Confidence Level; DF = Degree of freedom; "Prob > F" less than 0,05 indicates the model term is significant while values greater than 0.1 indicates the model term is not significant; Std. Dev. = 24.58; R^2 = 0.8907; Adj. R^2 = 0.8127; Pred. R^2 = -0.1858; Adeq. Precision = 10.341

Table 4. ANOVA for F-CN Reponse Surface Quadratic Model

The predicted response (Y) for the biodegradation of free cyanide in terms of the coded values was:

$$Y = 239 + 23.6A + 38.09B - 49.87A^2 - 3.62B^2 + 3.25AB \quad (3)$$

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where *A* and *B* are the coded values of temperature and pH, respectively. When coefficients with significant effects were considered, Eq. (3) became;

$$Y = 239 + 23.6A + 38.09B - 49.87A^2 \quad (4)$$

A model reduction was appropriate since there were many insignificant model terms. Excluding these terms improved the model. The Model F-value of 11.41 for the cyanide biodegradation was significant; therefore, there was only a 0.29% chance that a "Model F-Value" this large could occur due to noise for the quadratic model. Statistically, an adequate ratio greater than 4 is desireable for measuring a signal to noise ratio; therefore, the adequate precision of 10.341 observed in this study indicates a passable signal that can be used to further navigate the design space. Figure 3 further justifies the fitness of the model with normality in the error term.

6. Representation of the response surface model

The interaction between independent variables can be studied by plotting three dimensional (3-D) curves of the response against the variables. It allows for the interpretation of experimental results and determination of optimal conditions. Elliptical contour shows the interaction between the independent variables is perfect while a circular contour indicates the variables are non-interactive [44, 47].

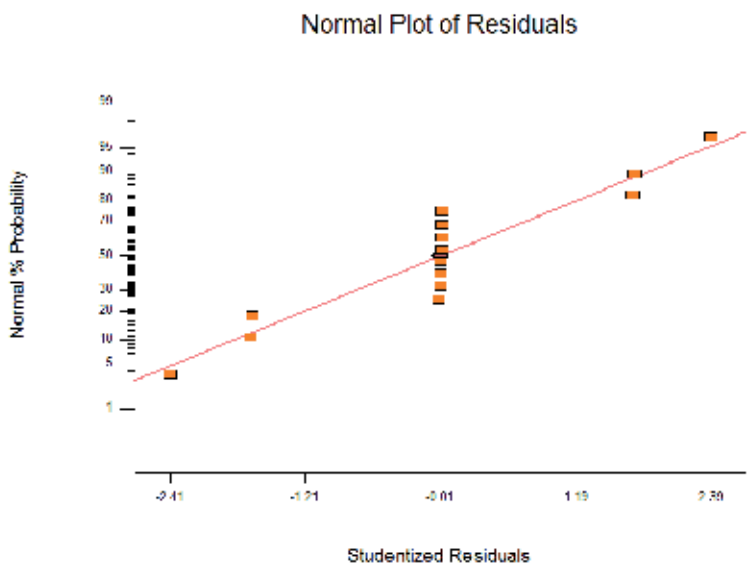

Figure 3. Normal probability plot of the residual F-CN

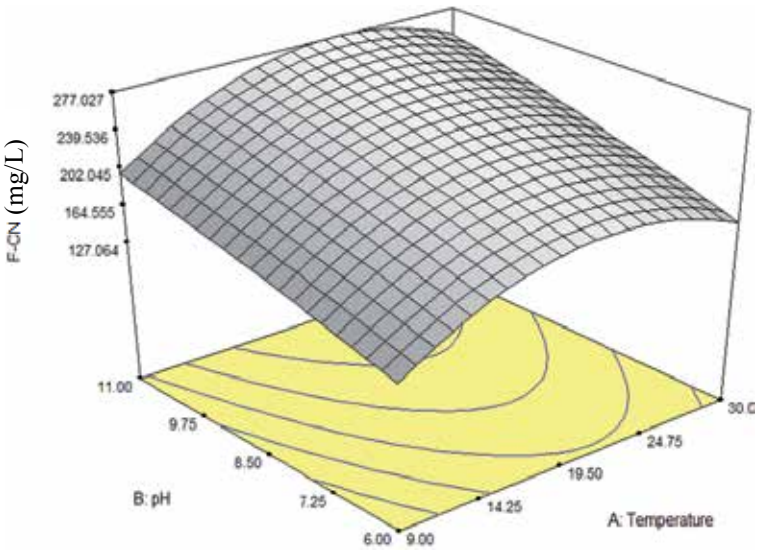

Figure 4. D plot showing interaction of independent variables on cyanide degradation

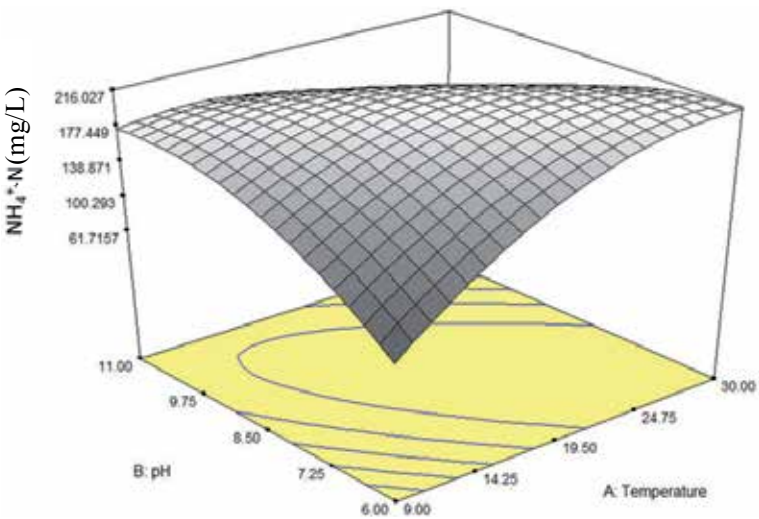

Figure 5. D plot showing interaction of independent variables on ammonium-nitrogen formation

7. Cyanide biodegradation optimisation

The optimisation was done using the Design-Expert software® numerical optimisation option where input factors were selected to achieve a desired perfomance. The numerical optimisation

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can maximise, minimise or achieve a targeted value: a single response; a single response subjected to upper and/or lower boundaries on other responses; and combinations of two or more responses. The desired goal for each variable and response is selected and the weight is chosen to show the degree of importance of individual goals. In this analysis, temperature and pH were set within range, cyanide degradation response was set at maximum while ammonium-nitrogen formation response was set at a minimum. The software gave three different solutions for this criteria with different desirability. The optimum point with the highest desirability was selected as shown in Fig.6 and 7. The optimal point with the maximum cyanide degradation of 250.436 mg F-CN/L and minimum ammonium-nitrogen formation of 74.285 mg NH_4^+-N /L was found to be at temperature of 30°C and pH of 11.

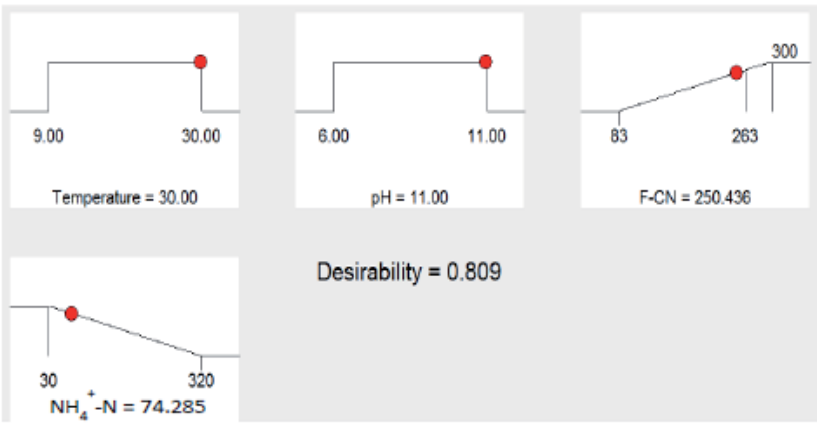

Figure 6. Desirability ramp for the numerical optimisation of cyanide degradation and ammonium-nitrogen formation

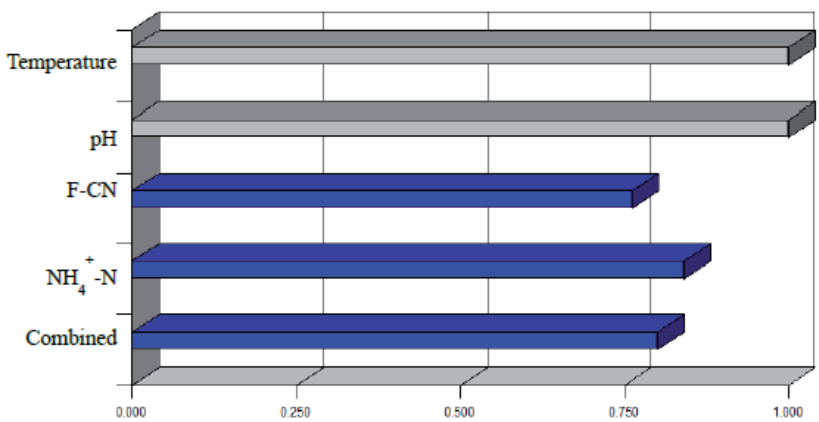

Figure 7. Desirability histogram for numerical optimisation of cyanide degradation and ammonium-nitrogen formation

8. Conclusion

Fusarium oxysporum cultures were grown on *B. vulgaris* waste to facilitate the biodegradation of cyanide, with the initial concentration of the cyanide being 500 mg CN^-/L. The wastewater used was similar to the effluent discharged into ponds by goldmines having metals such as arsenic, copper, lead, iron and zinc.

The response surface plot identified temperature as a more significant factor affecting both the cyanide degradation and ammonium-nitrogen formation. The ammonium-nitrogen produced can be used as a nitrogen source by the fungus.

The optimum condition for maximum cyanide degradation and minimum ammonium-nitrogen formation was found at temperature 30°C and pH of 11 where cyanide of 250.436 mg F-CN/L was degraded and ammonium-nitrogen of 74.285 mg NH_4^+-N/L was formed.

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Chapter 4

Origin of the Variability of the Antioxidant Activity Determination of Food Material

Irina Ioannou, Hind Chaaban, Manel Slimane and Mohamed Ghoul

Additional information is available at the end of the chapter

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1. Introduction

With the development of functional foods having beneficial effects for the health, the interest of scientists, consumers and industrialists in raw materials rich in antioxidants has increased considerably over last few years. Moreover, consumers require more accurate information on the composition of the food which they eat. Natural antioxidants such as phenolic compounds have been reported to possess beneficial bioactivities due to their capacity to act as antioxidant; anticarcinogenic, antibacterial, antimutagenic, anti-inflammatory, and antiallergic. These activities contribute to bringing a feeling of well-being to consumers. In fact, it was observed that the consumption of foods rich in antioxidants leads to the decrease of some diseases such as diabetes, cancer, cardiovascular or neuronal diseases [1]. To evaluate the antioxidant activity of raw materials several methods were investigated. These methods go through two steps. The first one is the extraction of the antioxidant compounds from the matrix of raw material and the second one consists in the determination of the antioxidant activity. For each step several alternatives are described in the bibliography. The data obtained showed a large variability depending on the method used. This renders the choice of an appropriate method a very sensitive task. The objective of this chapter is to make a critical study of the various methods of evaluation of the antioxidant activity of food raw materials. For this, in the first part, we will underline the different sources of variability from several examples of food raw materials. Then in the second part the different methods used for the extraction of antioxidants will be detailed. The third part will bring a complete view of the existing methods to measure antioxidant activity. The last part will deal with a general discussion on the meaning of the different values of antioxidant activity.

2. Identification of variability factors on the measurement of antioxidant activity

The scientific community is facing an accumulation of data on antioxidant activity. These data indicated a great variability depending on the method used, which does not render their comparison easy. To understand such variability, we analyzed several cases to highlight the factors that could be at the origin of the variability observed.

Case 1: Investigation of the antioxidant activity of pure components

The antioxidant activity obtained by different methods (ABTS, DPPH and ORAC) of 25 phenols was compared. The results obtained are summarized in table 1.

		ABTS	DPPH	ORAC
Flavonols	Q	1.8	0.9	4.2
	K	0.5	0.8	6.2
	M	1.5	1.8	3.6
	Q-R	0.6	1.0	4.6
	K-G	0.2	0.0	6.6
	M-R	6.6	2.0	6.0
Anthocyanins	C	2.0	0.5	4.4
	D	2.3	1.5	3.8
	C-G	1.9	0.6	7.3
	C-R	1.7	0.8	5.5
	C-GA	2.3	0.5	5.8
	D-G	3.5	1.4	5.9
Flavanones	HE NA	0.5	0.0	4.5
		0.0	0.0	5.6
Flavan-3-ols	CA	1.1	0.8	7.9
	ECa	1.3	1.0	5.1
	EGCa	1.2	1.5	3.1
	GCa	4.9	8.5	8.3
	EGCaG	2.0	3.7	3.4
Phenolic acids	GA CA EA	2.1	0.9	1.0
		1.3	0.9	5.3
		0.7	0.8	2.9

With: AA (ascorbic acid); C (cyanidin); C-G (cyanidin-3-O-glucoside); C-Ga (cyanidin-3-O-galactoside); C-R (cyanidin-3-O-rutinoside); Ca (catechin); CA (chlorogenic acid) ; D (delphinidin); D-G (delphinidin-3-O-glucoside); EA (ellagic acid); ECa (epicatechin); EGCa (epigallocatechin); EGCaG (epigallocatechin gallate); GA (gallic acid); GCa (gallocatechin); Gl (reduced glutathione); HE (hesperidin); K (kaempferol); K-G(kaempferol-3-O-glucoside); M (myricetin); M-R (myricetin-3-rhamnoside); NA (naringenin); Q (quercetin); Q-R (rutin); T (Trolox).

Table 1. Antioxidant values of phenolic compounds measured by ABTS, DPPH and ORAC methods [2]

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It appears that the values obtained of antioxidant activities are not of the same order of magnitude; the ORAC method gives the highest values followed by the ABTS and the DPPH method. Moreover, phenolic compounds having a high antioxidant activity with a given method may have low antioxidant activity with another method. For example, kaempferol 3-glucoside and quercetin have respectively an antioxidant value of 6.6 and 4.2 μmol Trolox with the ORAC method and only 0.2 and 1.8 with the ABTS method. The variability observed is due only to the method used. So, it seems that it is difficult to compare the numerical values of antioxidant activity provided by different methods of determination.

To analyse the effects of the extraction step and the variety of food, the antioxidant activities of extracts resulting from different raw materials such as onions and apples were investigated.

Case 2: Antioxidant activity of extracts from the food matrix

To highlight on the effect of the origin and the variety of food raw materials, [3] evaluated the total antioxidant capacity (TAC) by three methods (ABTS, FRAP and DPPH) using four varieties of onions with a similar procedure of extraction (methanol:water (70:30 v/v)). The activities obtained are summarized in table 2.

	ABTS (μmol Trolox/g FW)	**FRAP (μmol Trolox/g FW)**	**DPPH (μmol Trolox/g FW)**
White onion	11.82 ± 2.16	4.38 ± 0.40	3.04 ± 0.18
Yellow onion	15.22 ± 2.36	5.32 ± 0.59	4.56 ± 0.40
Red onion	28.18 ± 4.59	5.76 ± 0.47	5.20 ± 0.28
Sweet onion	10.56 ± 1.15	2.48 ± 0.19	1.42 ± 0.13

Table 2. Antioxidant activity of different onion varieties (Allium cepa).obtained with ABTS, FRAP and DPPH methods

These results indicate significant variations of the antioxidant activity depending on the variety tested. Antioxidant activity of the red onion is higher than that of white one. The magnitude of the variation is different according to the method used. For example, there is a factor of 2.4 between the red and white onion with ABTS, whereas this factor is only of 1.3 for FRAP and 1.7 for DPPH. For a given variety, the ABTS values are two times higher than FRAP or DPPH values, while values obtained by FRAP and DPPH are closer and sometimes do not indicate any difference between varieties. This behavior could be attributed to the fact that the active compounds in the origin of the antioxidant activity are not similar for the four varieties tested. This fact will have to be taken into account to compare the antioxidant activity obtained by different methods of determination.

To analyze the effect of the step of extraction, we gathered several results on the antioxidant activity of the golden delicious apple using different methods of determination (ABTS, FRAP and ORAC). The results obtained are given in table 3.

	ABTS	FRAP	ORAC
Ultrasound extraction with methanol	6.7	3.5	7.8
Extraction in plasma	8.3	4.4	9.15
Solvent extraction with methanol	40	_	_
Solvent extraction with methanol/ water (80%v/v)	4.62	_	_
Solvent extraction with acetone (70%), water (28%), and acetic acid (2%)	_	_	26.47

Table 3. Total antioxidant capacity of golden delicious apples (in μmol TE/ g FW) according to [4-7].

These results indicates that for a given method, the extraction procedure has a great impact on antioxidant activity values. For example with the ABTS method, using an extraction with methanol as solvent and assisted by ultrasound, this leads to 6.7 μmol TE/g FW; while the extraction with a mixture of methanol and water (80%v/v) or with acetone furnishes only 0.94 μmol TE/g FW. The measurement of bioavailability directly in plasma gives a value of 8.3 μmol TE/g FW. Different values are also obtained depending on the method of the extraction used with FRAP or ORAC protocols.

The analysis of the results of antioxidant activity of pure components and extracts from the food matrix indicates a broad variability in antioxidant values whatever the method used. This variability is also observed for a given method with the variety or the degree of maturation of the food raw material. This variability of the antioxidant activity determination can be attributed to three sources (i) factors related to food products such as the variety, and the growth method. (ii) Factors related to the extraction method such as pH, temperature, solvent, presence of an accelerator and (iii) factors related to the method used for the antioxidant activity determination.

3. Extraction techniques

To measure antioxidant activity of food raw materials, the active molecules must be extracted from the food matrix. The processes of extraction of the phenolic compounds are affected by several factors such as the pH, the temperature, the solvent used. Thus, the optimization of this step requires a judicious choice of the set points of these factors. However, in the bibliography few studies have been devoted to the optimization of these factors.

Moreover, these factors need to be adjusted according to the matrix of the raw material and the quantity of antioxidant molecules. To help in the choice of the most suitable method of the extraction, the main processes described in the literature are summarized in table 4. The advantages and the drawbacks of each process are also reported.

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Technique	Principle	Tool	Advantages	Drawbacks	Example of use
Solid-liquid extraction (SLE) [8]	Extraction with a solvent. Basically methanol based on maceration then filtration and evaporation of the solvent.	Appropriate solvent.	Simple. Popular.	Toxic solvent (methanol). High extraction time and low yield. Extracts not suitable to be added to food. Not safe for the environment.	Extraction of antioxidants from PistasciaLentiscus.
Ultra-Sound assisted extraction (UAE) [9,10]	Sonication of a sample-solvent mixture.	Ultrasounds with determined frequency and duration. Appropriate solvent.	More effective Reduction of the dependence on the extraction solvent Yields greatly enhanced with ethanol.	Less effective than microwave for example.	Extraction of antioxidants from rosemary leaves.
Microwave assisted extraction (MAE) [11-13]	Extraction of a sample-solvent using microwave energy.	Microwaves with determined power and duration. Appropriate solvent.	Reduced extraction time Minimized solvent volume Saved energy High extraction yields Increased solubility of phenolic compounds in solvents.	-	Tea polyphenols.
Pressurized Liquid Extraction (PLE) [14]	High temperature or pressure.	Appropriate solvent.	Faster extraction procedure Good recoveries of phenolic compounds from solid residues. Reduce waste generation, and improve sample throughput. Reduce extraction time.	-	Extraction of phenolic compounds from olive cakes.
Supercritical fluid extraction (SFE) [15]	Combination of low temperature and high	Supercritical fluid.	Low temperature used Reduced energy consumption	-	Extraction of peach almond oil.

Technique	Principle	Tool	Advantages	Drawbacks	Example of use
	pressure with a supercritical fluid.		Efficiency in solvent useRecycling possibility Prevention of oxidation reactions High product quality. Absence of solvent in solute phase. Flexible process.		
Aqueous two-phase extraction (ATPE) [16]	Extraction in an aqueous two-phase system.	Short chain alcohol/ hydrophilic organic solvents. Inorganic salts.	High extraction capacity. Mild conditions. Low cost. Short process time without back extraction. The potential to achieve the desired purification and concentration of the product in a single step.	No reports on the use of the ATPE to extract and purify anthocyanins.	Extraction of mulberry anthocyanins.

Table 4. Advantages and drawbacks of the main extraction methods used

Different processes of extraction of active compounds are available. However, the effectiveness of these processes is affected by several factors such as the nature of the solvent, the temperature or the extraction time. The presence of an accelerator of extraction such as microwaves or ultrasounds also plays significant role. The availability of the active molecule will be also taken into account. The analysis of the efficiency of the different processes described above indicates that the use of accelerators provides higher yields than the solid-liquid extraction (SLE) while allowing a low temperature to be maintained. The least advantageous method is the solid-liquid extraction due to the toxicity of solvent and the long time extraction in the majority of cases. The use of microwaves (MAE) as accelerator is highly acclaimed as an alternative method. The use of ultrasounds (UAE) also allows an enhancing of the extraction of active compounds at low temperatures but it leads to lower yields than with microwaves. Other accelerators can be used (ASE, PLE) but they need to increase the pressure and/ or the temperature, which can damage target molecules or alter their properties. Supercritical fluid extraction (SFE) does not use drastic conditions but the molecules extracted must to be soluble in liquid CO_2. The use of a co-solvent may be necessary if antioxidants are poorly soluble in CO_2. So, the difference in the efficiency of the different extraction methods used for antioxidant activity determination could be at the origin of the variability observed in the bibliography.

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Thus, the choice of a method of extraction needs to take into account the nature of the food matrix and the structure of the molecule to be extracted. The physico-chemical factors of the extraction must be also adjusted carefully. In conclusion there is a great need to standardize the methods of extraction by establishing different protocols and pay attention to different conditions.

4. In vitro methods for antioxidant activity measurement

An antioxidant is usually defined as a molecule which delays, prevents or removes oxidative damage to a target molecule [17], thus an antioxidant is assessed according to its ability to neutralize free radicals as for example in equation 1 to avoid oxidative degradations.

$$AO+FR\cdot \rightarrow AO\cdot +FR \quad (1)$$

AO: antioxidant molecule, FR·: free radicals

$$AH + R\cdot \rightarrow A\cdot + R \quad (2)$$

AH : antioxidant molecule, R·: free radicals

Free radicals are reactive oxygen species produced either through numerous biological reactions: mitochondrial respiratory chain or any inflammatory conditions, or from numerous environmental factors such as pollutants, U.V., alcohol, smoking, stress, drugs,... Free radicals are useful if they are in low quantity; they allow the elimination of old cells of the living organism by oxidation reactions or participating in the body's defense. However if they are too numerous, they attack other cells inducing a rapid aging of these cells which causes damage to living organisms. To avoid these reactions, antioxidants can neutralize free radicals and protect our cells. When antioxidant quantity is not enough to neutralize free radicals, it leads to the oxidative stress which has a great importance in the development of chronic degenerative diseases including coronary heart disease, cancer and the degenerative processes associated with aging.

Antioxidants can neutralize radicals by two different mechanisms. The final product will be the same but reactions occurring are different. Radicals can be deactivated either by hydrogen donation (Hydrogen Atom Transfer HAT) or by electron transfer (Single Electron Transfer SET). HAT and SET mechanisms may occur in parallel, the predominant mechanism being determined according to antioxidant structure and properties, solubility, partition coefficient, and system solvent [18]. A wide variety of one-dimensional methods have been developed to measure antioxidant activity in vitro. The methodological diversity is due to the use of a broad range of conditions for antioxidant activity measurement. This diversity has led to widely conflicting results that are extremely difficult to interpret.

4.1. Systems based on SET

SET-based methods involve two components in the reaction, i.e. the antioxidant and the oxidant. These methods measure the ability of an antioxidant to reduce any compound (metals, radicals) by electron transfer according to equations 3 and 4.

$$R\cdot + AH \rightarrow X^- + AH^+\cdot \quad (3)$$

$$M(III) + AH \rightarrow AH^+ + M(II) \quad (4)$$

SET reactions are pH dependent. Indeed, relative reactivity in SET methods is based primarily on deprotonation and the ionization potential of the reactive functional groups.. Ionization potential decreases when pH increases, so SET reactions are favored in alkaline environments. SET reactions are usually slow and can require a long time to reach their final state, so antioxidant capacity calculations are based on the decrease in product concentration rather than their kinetic.

- **ABTS (2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) assay**

ABTS assay is a spectrophotometric method which measures the ability to an antioxidant to scavenge a free radical cation ABTS▪+. This method was developed by [19] and adapted by [20] to generate directly the radical ABTS▪+ through a reaction between ABTS solution (7mM) with potassium persulfate (2,45 mM) in water. The reaction mixture, which is allowed to stand at room temperature for 12-16 h before use, produces a dark blue solution. Thus, the mixture is diluted with ethanol or phosphate buffered saline (pH 7.4) to a final absorbance of 0.7 at 734 nm (wavelength the most used) and 37 °C. The assay is based on the discoloration of ABTS▪+ during its oxidation by antioxidant compounds, thus reflecting the amount of ABTS radicals that are scavenged within a fixed time period (generally 6 min). The absorbance of the reaction mixture between radicals and antioxidants is compared to that of the 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). When Trolox is used as standard, this assay is also called Trolox Equivalent Antioxidant capacity (TEAC) assay.

The major advantages of this method are its simplicity to perform and its applicability in lipid and aqueous phases [21]. Thus this method has been widely used in testing antioxidant capacity in food samples. Moreover, the ABTS radical is stable over a wide pH range and can be used to study pH effects on antioxidant mechanisms [22]. This method can be automated and adapted to the use with microplates which allows the carrying out of this measurement with better precision and time.

A major disadvantage of this method is that only the rapid oxidation reactions can be measured because incubation time is often short (6 min). Thus, antioxidants whose constant rates of radical scavenging are low can be undervalued in comparison with their real antioxidant capacity. Moreover, imprecisions on ABTS values can be increased by the fact that variations can occur according to the preparation of ABTS▪+, and the medium temperature which has to be controlled.

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- **DPPH (2,2-diphenyl-1-picrylhydrazyl) assay**

DPPH is one of the oldest and most popular technique used to measure the antioxidant activity of a compound. This method was first described by [23] and subsequently modified by numerous researchers. This method measures the reducing ability of antioxidants toward DPPH▪. DPPH▪ is commercially available and does not have to be generated as for ABTS assay. The antioxidant effect is proportional to the disappearance of DPPH▪ in a methanolic solution. DPPH solution being purple, the absorbance of the mixture can be followed by spectophotometry at 515 nm. Assay time may vary from 10±20min up to 6h. Other techniques such as electron spin resonance (EPR) can be used [18].

Like ABTS, this method is simple and can be automated. However, values found by the DPPH method have to be considered as apparent antioxidant activities because (i) DPPH color can be lost via either radical reaction (HAT) or reduction (SET) as well as unrelated reactions, (ii) steric accessibility also influences the reaction, thus small molecules are favored because they have a better access to the radical site and other compounds such as carotenoids can interfer in the measurement of the antioxidant activity [24].

- **Ferric reducing ability of plasma (FRAP)**

The FRAP assay is different from the others as there are no free radicals involved but the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) is monitored. FRAP assay was initially described by [25] for measuring reducing power in plasma and subsequently adapted and modified by numerous researchers to measure antioxidant power of botanical extracts [26]. When an Fe^{3+}-TPTZ (2,4,6-tripyridyl-s-triazine) complex is reduced to Fe^{2+} by an antioxidant under acidic conditions, it forms an intense blue color with absorption maximum at 593nm. Thus the antioxidant effect can be followed by a spectrophotometer.

A major advantage of the FRAP assay is its simplicity, speed and robustness. The validity of this assay was proved in order to quantify samples with hydrophilic and lipophilic antioxidants. As for ABTS assay, only rapid reactions will be taken into account until the incubation time in this method is short (4-6 min). The FRAP assay measures only reactions following the SET mechanism, antioxidant hydrogen donator may go unmeasured by this assay. This method is thus used in parallel with others to determine the action mechanisms of antioxidants. Protein and thiol antioxidants, such as glutathione cannot be measured by the FRAP assay.

- **CUPric Reducing Antioxidant Capacity (CUPRAC) assay**

The CUPRAC assay has many similarities to FRAP, Cu is used instead of Fe. This assay is based on the reduction of Cu (II) to Cu (I) by the antioxidants present in the sample. Cu (I) forms a complex with neocuproine (2,9-dimethyl-1,10-phenanthroline) with a maximum absorbance at 450 nm. A dilution curve generated by uric acid standard is used to convert sample absorbance to uric acid equivalents [18]. Phenanthroline complexes have very limited water solubility and must be dissolved in organic solvents. Cuprac values are comparable to TEAC values, whereas FRAP values are lower. The CUPRAC assay has many advantages [27]. Indeed, the CUPRAC assay is more selective due to its lower redox potential. Sugars and citric acid cannot interfere in the assay because they are not oxidized in CUPRAC. The CUPRAC

reagent is much more stable than other radicals such as DPPH, ABTS. The redox reaction giving rise to a coloured chelate of Cu(I)-Nc is relatively insensitive to a number of parameters such as air, sunlight, humidity, and pH. The CUPRAC reagent can be adsorbed on a membrane to build an optical antioxidant sensor.

A variant of CUPRAC assay is Bioxytech using bathocuproine instead of neocuproine [18].

4.2. Systems based on HAT

The HAT-based methods involve a synthetic radical generator, oxidisable molecular probe and an antioxidant compound. This method measures the ability of an antioxidant to quench free radicals by hydrogen donation as in equation 2. Assays that are based on HAT mechanisms measure competitive kinetics [22].

Antioxidant with hydroxyl component OH donates an H atom to an unstable free radical to give a more stable radical. HAT reactions are solvent and pH independent and are usually quite rapid, typically completed in seconds to minutes. The presence of reducing agents, including metals, is a complication in HAT assays and can lead to erroneously high apparent reactivity [18].

- **Oxygen radical absorbance capacity (ORAC)**

The ORAC assay has been used widely in measuring the net resultant antioxidant capacity (or peroxyl radical absorbance capacity) of botanical and other biological samples.

The ORAC assay was developed by [28] for the determination of reactive oxygen species in biological systems. [29] modified the method using fluorescein (FL) as a more stable and reproducible fluorescent probe. This method exists under several adaptations but the principle always remains the same: using a fluorescent probe and AAPH (2,2′-azobis(2-amidinopropane) dihydrochloride) to generate peroxyl radicals. A HAT reaction occurs between antioxidant samples (or standard) and the peroxyl radicals generated by thermal degradation of AAPH. These reactions lead to a loss of fluorescence measured at 515 nm.

The final results (ORAC values) were calculated using the differences between blank and sample areas under the quenching curves of fluorescein, and were expressed as micromoles of Trolox equivalents (TE).

The ORAC method is superior to similar methods because it combines inhibition time and inhibition degree of free radicals. The ORAC using fluorescein is specific for antioxidants and is sensitive, precise and robust. This assay can model reactions of antioxidants with lipids in both food and physiological systems and it can be adapted to detect both hydrophilic and hydrophobic antioxidants with minor modifications. However, the need of a fluorometer, which may be not routinely available, is considered as a disadvantage of this method. The long analysis time has also been a major criticism even if this assay can be automated.

- **β -carotene bleaching test**

This assay was developed by [30] and modified by other researchers. This assay is based on the generation of a stable β –carotene radical from β –carotene peroxyl radical; the latter

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coming from lipids (linoleic acid for example) in the presence of ROS and O_2. Thus, the assay measures the ability to an antioxidant to quench β –carotene radical by donating hydrogen atoms. It results in the bleaching of the solution which can be followed with a spectrophotometer at 470 nm.

The main advantage of this assay is its applicability in both lipophilic and hydrophilic environments. Another advantage is that the carotenoid bleaching assay can detect either the antioxidant or pro-oxidant action of a compound under investigation. Lastly, the carotenoid bleaching assay can be automated by the use of microplates. However, a major limitation is that the discoloration of β-carotene at 470 nm can occur through multiple pathways, thereby complicating the interpretation of results. Other carotenoids such as crocin bleach only using the radical oxidation pathway but crocin is not commercially available. The use of molecules commercially available provide repeatable and reliable data between laboratories

- **Total peroxyl radical-trapping antioxidant parameter assay (TRAP)**

The total peroxyl radical-trapping antioxidant parameter (TRAP) assay was introduced by [31] to measure the total antioxidant status of human plasma. This assay monitors the ability of an antioxidant to interfere with the reaction between peroxyl radicals generated by AAPH (2,2'-azobis (2-amidinopropane) dihydrochloride) and the target. The oxidation is monitored by oxygen uptake measurement. Results are expressed as time necessary to consume all radicals in comparison with a standard (Trolox). Many modifications were realized on this assay to react with lipids, or to be followed by fluorimetry or to take into account interference from lipids and proteins in plasma [32]. Despite its simplicity, the TRAP assay leads to imprecise results because of difficulties to maintain the endpoint over the period of time. Several modifications were developed by using chemiluminescence methods [33].

5. Discussion — Conclusion

Many results on the determination of the antioxidant activity of purified molecules and / or food raw material have been published over the last few years. However, the obtained data present a broad variability even for a given method or a given molecule. To overcome these problems, some authors have proposed other alternatives by developing new methods, or new ways to process data and express the results. [34] proposed the « quencher method », where the antioxidant activity is directly measured from the solid sample without the extraction step. Free radicals are mixed with the food sample and a spectrophotometric method (ABTS, DPPH) was used. [35] developed the global antioxidant response (GAR) method which uses an in vitro approach with enzymatic digestion, designed to mimic digestion through the gastrointestinal tract aimed at releasing antioxidants in foods. [36] suggested a general method of standardization of estimations of total antioxidant activity (TAA) by extrapolating parameters to zero sample concentration based on a pseudo-first-order kinetics model. Accurate results were obtained in comparison with the ABTS method. Moreover, several papers deal with the standardization of the extraction procedures and the results analysis for a given method in order to minimize the observed variability [37]. However, it appears difficult to find a universal

method knowing that many kinds of antioxidants and radicals are present. Four general sources of antioxidant have been identified: (i) enzymes (superoxide dismutase, gluthatione peroxidase), (ii) large molecules (albumin, ferritin), (iii) small molecules (phenols, ascorbic acid, carotenoids) and (iv) and some hormones (estrogen, melatonin). Many kinds of free radicals can be found, for example O_2▪-, HO▪, NO▪, RO(O)▪, LO(O)▪. Moreover, the stability, the selectivity of the radicals and the reaction mechanisms can be also different. Thus, it is possible that no single method may be able to express the antioxidant capacity of different antioxidants taken independently or in a mixture [18]. Previous studies demonstrated that it is not appropriate to use one-dimensional methods to evaluate the antioxidant activity of multifunctional food such as fruits and vegetables, since they contain a large diversity of natural antioxidants.

The determination of antioxidant activity in the food matrix needs a sample preparation to extract the active molecules and then an accurate method of measurement and an expression of the results. (i) During sample preparation, precautions must be taken to avoid the loss of antioxidants due to the drastic conditions of extraction. A determination of all food constituents is necessary because a certain interference with antioxidants can occur. Antioxidant capacity values should only be compared where the method, the solvent and the analytical conditions are similar [38]. Indeed, some authors underlined that there is an effect of the solvent used for the extraction or used to solubilize antioxidants on the result of the antioxidant activity evaluation [39-42]. This is due to interference of the reaction mechanism and the solvent [38]. (ii) The method to measure the antioxidant activity must be chosen according to the nature of the active molecules present in the samples. Some methods described in part 3 are more appropriate for some kinds of antioxidants. For example the DPPH method is more adequate to lipophilic systems. Moreover, several assays must be carried out to determine a value of antioxidant activity. (iii) Results of antioxidant activity measurement can be expressed as EC50 (quantity of antioxidant necessary to assure 50% depletion of free radicals), tEC50 (time to reach 50% depletion of free radicals), tEC50 (time to reach 50% depletion of free radical) or AE (antiradical efficiency defined as the inverse of the product between EC50 and tEC50). Thus, taking these 3 parameters into account can be relevant to have a more comprehensive evaluation of antioxidant activity [38].

The determination of the antioxidant capacity by in vivo methods is not always feasible but it appears interesting because it simulates an environment closer to that really happening in biological systems. Methods using HAT reactions will be preferred to SET reactions because peroxyl radicals used in HAT assays are the predominant free radicals found in lipid oxidation and biological systems. To elucidate a full profile of antioxidant capacity against various ROS, the development of different methods specific for each ROS/ RNS may be needed. [18] proposed a comparison of different in vitro methods; conclusions given that ORAC, TRAP and LDL are considered to be the most biologically relevant assays [18] because the antioxidant capacity measured reflects closer the in vivo action of the antioxidants. So, it appears clearly that the antioxidant activity determination needs a standardization of the procedure used and a combination of at least two or three methods. The use of only one method does not reflect the antioxidant activity of food raw material due the variability of the molecules that act as antioxidant.

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Chapter 5

Identification of Putative Major Space Genes Using Genome-Wide Literature Data

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Additional information is available at the end of the chapter

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1. Introduction

Microgravity in life sciences is an important field of study, not only because of our interest in exploring and living in space for extended periods, but also for the potential insights it gives on earthbound health problems. With genome-wide array technologies, the study of microgravity effects on living organisms can be examined in much greater detail at the cellular and molecular levels which is key to elucidating the molecular mechanisms of this environmental factor. Microgravity is a main environmental risk factor of spaceflight [1, 2] and the adverse effects of microgravity have much in common with earthbound health problems related to low physical activity or reduced mechanical loading. Bone loss and muscle atrophy as well as immune system dysfunction are some of the main consequences common to both extended spaceflight and physical inactivity such as that associated with premature aging and degenerative disorders [3, 4]. Remarkable similarities have been noted between the clinical presentation of spinal cord injury and prolonged gravity unloading including atrophy in muscle and bone, cardiovascular disturbances, and alterations in renal, immune and sensory motor [5]. Microgravity research also holds promise in the area of tissue engineering. Microgravity simulation devices such as Rotating Wall Vessel (RWV) have been increasingly explored to generate 3-D organ mimics for liver and pancreatic islet transplantations [6-9]. Continued effort in microgravity research will deepen our understanding of space adaptation response and improve our ability to treat health-related problems, such as spinal cord injury, diabetes, osteoporosis, and premature aging. A better understanding of microgravity effects at the molecular level could help in the development of countermeasures that will protect astronauts from the deleterious effects of living in space as well as lead to the development of treatments for human diseases here on Earth.

Cellular environmental changes such as sudden gravity change is likely to alter the fundamental activities of genes and any change in the physiological function of a cell or an organism is most likely the result of changes in certain genes' expressions. Genes from many cell types have been shown to be sensitive to the microgravity environments (reviewed by Clement 2012). With the advent of high-throughput genomic technology such as microarrays, large scale genome-wide studies have been performed to assess the mRNA levels of cultured cells and organisms exposed to microgravity. This is an effective approach because the control of mRNA abundance of genes is effectively adapted by cells through controlling transcription (especially transcription initiation), nuclear pre-mRNA processing, mRNA transport, mRNA stability, etc. The cellular abundance of mRNAs is critical to gene function and protein production, which is intriguingly fine-tuned by non-coding regulatory RNAs such as miRNAs. Since the turn of the century, microarray studies have been increasingly used in space life sciences to assess the abundance of mRNAs in response to microgravity. The microgravity biotechnologies combined with microarray technology have been successfully used to study microgravity effect on gene expression on a wide variety of cell types. In a previous review, data was combined from all retrievable microarray-based microgravity research to identify the most frequently altered putative "major space genes" [10]. At that time we identified 26 microarray based microgravity studies in mammalian cells or tissue that had some form of published gene lists. In addition, we included the then available results (published gene lists) from four Xenopus studies. Candidate major space genes were defined as genes that appeared to have significantly altered expression levels in at least four studies. The resulting list of merely eight potential space genes were CD44, CTGF, CYR61, FN1, MT2, MT1, MARCKS, TUBA4A [4]

Since 2011, substantially more progress has been achieved in the literature because significantly more studies have been published with retrievable gene lists. The combination of a greater number of studies and a general increase in the availability of published gene lists, has enabled us to greatly expand our list of putative "major space genes" from the initial number of eight [4] to the present number of 129 at the same initial level of stringency, a gene's expression was found to be altered by microgravity in four or more studies. Thus, this paper is an extended review and meta-analysis of gene expression profiles to identify major space genes, with emphasis on findings on mammalian cells. To accomplish this, we first defined the method and scope of the current literature-based study to identify the putative major space genes from published data on microarray based microgravity studies in the literature. We proceeded to obtain our novel data at three different confidence levels for the putative major space genes. We further refined the criterion for putative major space genes to only include genes that were found to have altered expression patterns in five or more studies or model cell lines. This higher stringency of selection yielded a more focused group of 35 putative major space genes. Furthermore, we identified 13 genes as the most likely candidates for the major space genes because they have been reported most frequently (≥ 6 studies) as microgravity sensitive genes. We then proceeded to perform bioinformatics analysis at each of the three confidence levels of the putative major space genes. We will present and discuss the lists of candidate major space genes that are most frequently altered by microgravity environments. We also review and discuss recent advances in the area of microarray based microgravity research.

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2. Methods and results

The scope of the current study includes all the microarray based microgravity studies on gene expression regulations that have been documented in the literature. For the initial data collection, we started by doing a PubMed search with the terms such as "microarray and microgravity", "space flight and microarray" and "gene expression and microgravity". From these searches, we were able to identify 48 mammalian microarray studies of microgravity effect. Of these 48 studies there was some form of published gene list from 38 different cell lines in 35 microarray publications of mammalian cells exposed to microgravity, which provide the initial "materials" that this current study is based on. In this Methods and Results section, we present the methods and results together since they are intimately linked in the current approach. We present the methods and results in the following stages: First, the scope of the study data collection is tabulated in Table 1; second, the compilation of the "Master" gene list; Third, identification of the putative major space genes at three different levels of stringency; Fourth, bioinformatics analysis of these putative major space genes using Database for Annotation, Visualization and Integrated Discovery (DAVID) and Search Tool for Interacting Gene/Proteins (STRING).

2.1. Compilation of published gene expression data into a "Master" gene list

We collected information on microgravity sensitive genes from the literature into a tabular format so that the source of the reference, the model cell types, the types of microgravity, the duration of exposures, the platform of the gene expression analysis, magnitudes and directions of gene expression regulation, etc. were all included in the "Master" gene list. The source of data contributing publications used as the subject for our current study is shown in Table 1. The first step in the analysis of the collected data pool was to convert the collected published gene expression data into a format that can be compared directly. Since much of the comparison was across species, we chose to use gene symbols rather than accession numbers. This is mainly because accession numbers are different across species, but the gene symbols are typically the same. In addition, some of the gene lists included accession numbers and gene symbols, others included accession numbers and no gene symbols, and still others included gene symbols and no accession numbers. Therefore, we choose to use gene symbol for all further comparative analysis of these published data. Specifically, we used the DAVID Gene ID Conversion Tool [11] to convert all the differentially expressed genes in microgravity into the same format for comparison. To do this, we copied the accession numbers from each study and uploaded them into DAVID Gene ID Conversion Tool. Once uploaded, the accession numbers are automatically converted into the format chosen. In this case, we chose official gene symbols.

We then were able to assemble a "master" gene list from the 38 published gene lists (PGL) using gene symbols for direct comparison. Our main interest was to determine if a gene was differentially expressed in microgravity. For biological and technical repeats, the data were already averaged in the initial publications and the averaged data were presented in the PGL. There are also a few time-course studies using microarray profiling microgravity effects on

gene expression. If a gene was differentially expressed at any time point in a time-course study, it was included in the master list with its magnitude and direction of differential regulation. Even if a gene was differentially regulated in different directions among different time points, we counted it as a differentially regulated gene. Some of these differences in expression are discussed later in this paper.

This "Master" gene list is by no means a complete gene list since many of the publications in the scope of our current study do not include the full list of differentially regulated genes. Significantly, this master gene list provides the data necessary for the identification of putative major space genes at relatively high confidence levels.

Organism/Cell or Tissue Type	Microgravity	Array type	Citation
Human			
Renal	RWV/STS	Incyte	[12]
Renal	RWV/STS	Incyte	[13]
Liver	RWV	6K Human Array	[14]
Jurkat	STS	GeneFilter 20k array	[15]
Fibroblast	STS	in house	[16]
T-Cells	B	unknown	[17]
T-Cells	RPM	Affymetrix Human Genome Focus Array	[18]
T-Cells	RWV	Affymetrix Human U133A Array	[19]
Muscle	BR	Human AceGene Chip	[20]
Endothelial	RPM	unknown	[21]
Liver	RWV	Agilent 22k Human Microarray V2	[22]
Osteoblast	RPM	Atlas Glass Human 3.8 Microarray	[23]
Skin	RWV	Agilent 22k Human Microarray V2	[24]
Muscle	BR	MWG human 23k oligo array_version 3	[25]
Osteoblast	DL	Affymetrix Human U133 Plus 2.0 Array	[26]
Muscle	ULLS	unknown	[27]
Lymphoblastoids	ISS	Agilent 44k Whole Genome Microarray	[28]
Stem Cells	RWV	Affymetrix Human U133 Plus 2.0 Array	[29]
Lymphoblastoids	RWV	Illumina HumanWG-6 V4 BeadChip/RT2 miRNA PCR Array	[30]
T-Cells	RWV/ISS	Affymetrix Human U133 Plus 2.0 Array	[31]
Lymphoblastoids	ISS	Panorama Ab Microarray	[32]
Thyroid Cancer	RPM	Illumina HumanWG-6_V2_0_R3_11223189_A array	[33]
Endothelial	P	Illumina HumanWG-6_V2_0_R3_11223189_A array	[34]
Endothelial	RPM	Illumina HumanWG-6_V2_0_R3_11223189_A array	[35]

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Organism/Cell or Tissue Type	Microgravity	Array type	Citation
Endothelial	ISS	Affymetrix Human Gene 1.0 ST arrays	[36]
Thyroid	RPM	Illumina HumanWG-6_V2_0_R3_11223189_A array	[37]
Lymphocytes	RWV	Agilent Whole Genome Microarray/Agilent Human miRNA Microarray V2	[38]
Mouse			
2T3	RPM	Amersham CodeLink Uniset Mouse I Bioarray	[39]
2T3	RPM	Affymetrix GeneChip Mouse 430 2.0	[40]
Muscle	HLS	Agilent Mouse Oligo Array	[41]
Brain	HLS	AECOM Mouse 27k cDNA array	[42]
Muscle	STS/HLS	Affymetrix Mouse Expression 430 A Array	[43]
Osteoblast	RWV	Agilent Mouse Oligo Array	[44]
Osteoblast	DL	Affymetrix Mouse Genome 430 A 2.0 Array	[45]
Stem Cells	RWV	Roche Nimblegen	[46]
Stem Cells	ISS	Affymetrix Mouse Gene 1.0 ST	[47]
Thymus	STS	Affymetrix Mouse Gene 1.0 ST	[48]
Osteoclasts	RWV	Agilent whole genome 4X44K	[49]
Liver	RWV	Affymetrix Mouse Genome 430 2.0 Array	[7]
Fibroblast	RWV	Affymetrix Mouse Genome 430 A 2.0 Array	[50]
Glial	HLS	Illumina MouseRef-8 v.2 BeadChips	[51]
Rat			
Muscle	HLS	Atlas Rat 1.2 cDNA Array	[52]
Muscle	HLS	Affymetrix U34A Rat Genome Microarray	[53]
Muscle	STS	Atlas Rat 1.2 cDNA Array	[54]
Gastrocnemius	STS/HLS/D	Affymetrix U34A Rat Genome Microarray	[55]
Muscle	HLS	Atlas Rat cDNA Expression Array	[56]
PC12	RWV	In house	[57]
Stem Cell	RWV	CapitalBio Rat Genomic Array	[58]

RWV – Rotating Wall Vessel, HLS – Hind Limb Suspension

RPM – Random Positioning Machine, P- Parabolic Flight

D-Denervation, B-Balloon

STS-Space Shuttle, ULLS-Unilateral Lower Limb Suspension

DL-Diamagnetic Levitation

ISS - International Space Station

Table 1. Microarray Based Studies of Microgravity Effect on Mammalian Cells

2.2. Identification of putative major space genes with different levels of stringency

By compiling the published gene lists into the master list, it provided us with an accurate and convenient platform to identify putative major space genes at various levels of stringency using the simple "vote counting" method. At the very basic level, we identified 1199 genes that were differentially regulated in two or more of the documented studies. One level higher, we found 298 genes appeared to be affected by microgravity in three or more microarray-based microgravity studies. Furthermore, when we set the bar to four or more studies, we identified 129 genes (Table 2), which is in drastic contrast to the 8 genes found a few years ago using this same level of stringency. Because of the increase in the number of relevant studies, we were able to go beyond the level of four or more studies in the selection of putative major space genes which was the highest level possible in our previous report [4]. Just to reach one step further, we isolated 35 candidate major space genes in five or more studies (Table 2). Further still, we found 13 genes that were reported in six or more studies to be microgravity sensitive (Table 2). These two additional levels of higher stringency for the selection of putative major space genes enabled a significantly higher level of confidence. We performed further bioinformatics analysis on the differentially regulated genes of the top three stringency levels: gene lists of 129 genes (in ≥ 4 studies), 35 genes (in ≥ 5 studies), and 13 genes (in ≥ 6 studies), respectively.

Genes Differentially Regulated in 4 Studies				
ADAMTS1	CCT7	ETFA	MFNG	RPL29
ADORA2A	CD59	FOSL1	MMGT1	RPL9
ALDOA	CD9	FST	MMP1	RPLP0
ANPEP	CD93	GARS	MRPS35	SERPINE1
ANXA2	CDH1	GJB2	MX1	SGK1
ANXA3	CDV3	GNG10	NOTCH1	SLC16A3
AP1S1	CFLAR	GPNMB	NTN4	SNX7
AP3M1	CKS1B	HBEGF	PDGFRB	SPRY2
ASAP1	CLDN11	HERPUD1	PDIA4	SRGN
ASNS	CLIC3	ID1	PECAM1	TCP1
ATF3	CNBP	IGFBP6	PKIA	TFB2M
ATP5F1	CNIH	ITGAV	PLAT	TGM2
ATP6V0D1	COL8A1	JUNB	PLOD2	TLR4
BIRC3	CXCL2	KYNU	PLSCR4	TRIB3

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BNIP3L	DDIT3	LITAF	PRDX2	TXN
CAPN5	EEF1A1	LOC285741	PTX3	UQCRFS1
CBS	EFEMP1	LOC399942	RBM3	WISP2
CCL2	EGFL7	LOC643668	RPL10A	ZNF323
CCNC	EIF1B	LTBP2	RPL17	
Genes Differentially Regulated in 5 Studies				
ACP1	CD44	IGFBP3	LOX	TPM1
ADM	CDH5	IGFBP7	MMP10	TXNDC5
AKAP12	CMTM7	IL8	PHGDH	
CAV1	DDIT4	ITGA10	SFRP1	
CAV2	HSPA8	LIMCH1	TPI1	
Genes Differentially Regulated in 6 or more Studies				
CTGF	FN1	ITGB4	MT2	TXNIP
CYR61	FOS	KPNA2	MYC	
EGR1	HSPA1A	MT1	TUBA4A	

Table 2. Putative List of Major Space Genes differentially regulated in 4 or more studies

2.3. Bioinformatics analysis of the putative major space genes

In order to get a better understanding of the putative major space genes at the top three stringency levels, we subjected the genes listed in Tables 2 to further bioinformatics analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 [59, 60] and Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) [61, 62].

The DAVID analysis through gene enrichment allowed us to identify enriched Gene Ontology (GO) terms as well as statistically significant pathways. Each of the top three gene lists was uploaded to DAVID Functional tool (http://david.abcc.ncifcrf.gov) to identify the statistically significant KEGG Pathways as well as the frequency of genes belonging to a particular Gene Ontology. DAVID uses a modified Fisher Exact P-value for gene enrichment analysis and statistically determines the over-representation of functional gene categories in a gene list. P-values equal to or smaller than 0.05 are considered strongly enriched [59, 60]. We obtained the potential KEGG Pathways as well as enriched functional clusters as defined by DAVID [59, 60].

For the 129 genes differentially expressed in ≥ 4 microgravity studies, the pathway analysis resulted in eight pathways at P value ≤ 0.05. The KEGG Pathway analysis showed that largest number of enriched genes were in pathways directly related to various cancer. The 2nd largest pathway identified is focal adhesion (Table 3).

Term	Count	PValue	Genes
Bladder cancer	4	0.02	IL8, CDH1, MYC, MMP1
Focal adhesion	8	0.02	CAV2, CAV1, ITGAV, ITGB4, PDGFRB, ITGA10, BIRC3, FN1
Small cell lung cancer	5	0.028	CKS1B, ITGAV, BIRC3, MYC, FN1
ECM-receptor interaction	5	0.028	CD44, ITGAV, ITGB4, ITGA10, FN1
Ribosome	5	0.031	RPL17, RPL9, RPLP0, RPL10A, RPL29
Pathways in cancer	10	0.035	CKS1B, FOS, IL8, ITGAV, PDGFRB, CDH1, BIRC3, MYC, MMP1, FN1
Pathogenic Escherichia coli infection	4	0.043	LOC399942, TUBA4A, CDH1, TLR4
NOD-like receptor signaling pathway	4	0.053	CCL2, IL8, CXCL2, BIRC3

Table 3. KEGG pathway analysis of 129 putative space genes

We also conducted DAVID functional cluster analysis to determine functionally enriched gene sets from the list of 129 genes differentially regulated in ≥ 4 studies. We set the Stringency at the Highest and used a P-Value cut-off of ≤ 0.05 for inclusion of a term on the list. Based on these criteria, we generated a list with 40 functionally enriched GO categories (Table 4). Some of the top functional categories (based on P-Value) were regulation of apoptosis (17.8%), ion homeostasis (8.5%), cell motility (7.75%), and insulin-like growth factor binding (4.6%).

Term	Count	%	PValue
GO:0005520~insulin-like growth factor binding	6	4.65	1.85E-06
GO:0042981~regulation of apoptosis	23	17.8	4.54E-06
GO:0043066~negative regulation of apoptosis	14	10.9	2.57E-05
GO:0043065~positive regulation of apoptosis	13	10.1	6.73E-04
GO:0016477~cell migration	10	7.75	0.001106
GO:0051674~localization of cell	10	7.75	0.002297
GO:0048870~cell motility	10	7.75	0.002297
GO:0006873~cellular ion homeostasis	11	8.53	0.002595
GO:0007596~blood coagulation	6	4.65	0.002624
GO:0055082~cellular chemical homeostasis	11	8.53	0.002909
GO:0007599~hemostasis	6	4.65	0.00336
GO:0050801~ion homeostasis	11	8.53	0.004886
GO:0030005~cellular di-, tri-valent inorganic cation homeostasis	8	6.2	0.005339
GO:0032496~response to lipopolysaccharide	5	3.88	0.005751
GO:0002237~response to molecule of bacterial origin	5	3.88	0.008466
GO:0006469~negative regulation of protein kinase activity	5	3.88	0.008811
GO:0051412~response to corticosterone stimulus	3	2.33	0.009483

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Term	Count	%	PValue
GO:0030003~cellular cation homeostasis	8	6.2	0.009652
GO:0033673~negative regulation of kinase activity	5	3.88	0.009902
GO:0042325~regulation of phosphorylation	11	8.53	0.011735
GO:0051348~negative regulation of transferase activity	5	3.88	0.012339
GO:0030324~lung development	5	3.88	0.013689
GO:0051385~response to mineralocorticoid stimulus	3	2.33	0.014654
GO:0030323~respiratory tube development	5	3.88	0.01513
GO:0051174~regulation of phosphorus metabolic process	11	8.53	0.015164
GO:0019220~regulation of phosphate metabolic process	11	8.53	0.015164
GO:0055080~cation homeostasis	8	6.2	0.017558
GO:0060541~respiratory system development	5	3.88	0.018291
GO:0048754~branching morphogenesis of a tube	4	3.1	0.022669
GO:0006874~cellular calcium ion homeostasis	6	4.65	0.02823
GO:0009165~nucleotide biosynthetic process	6	4.65	0.029991
GO:0055074~calcium ion homeostasis	6	4.65	0.031203
GO:0001763~morphogenesis of a branching structure	4	3.1	0.031688
GO:0034654~nucleobase, nucleoside, nucleotide and nucleic acid biosynthetic process	6	4.65	0.034365
GO:0034404~nucleobase, nucleoside and nucleotide biosynthetic process	6	4.65	0.034365
GO:0006875~cellular metal ion homeostasis	6	4.65	0.036355
GO:0005840~ribosome	6	4.65	0.036807
GO:0045859~regulation of protein kinase activity	8	6.2	0.042448
GO:0055065~metal ion homeostasis	6	4.65	0.042745
GO:0043549~regulation of kinase activity	8	6.2	0.049406

Table 4. GO categories for the 129 space genes. Processed through DAVID with stringency set at highest

Next, we submitted the list of 35 genes that were differentially regulated in five or more studies to DAVID for bioinformatics analysis. The KEGG Pathway analysis identified focal adhesion and Extracellular Matrix (ECM)-receptor interaction pathways were the largest number of enriched genes (Table 5).

Term	Count	%	PValue	Genes
ECM-receptor interaction	4	11.43	0.004	CD44, ITGB4, ITGA10, FN1
Focal adhesion	5	14.29	0.007	CAV2, CAV1, ITGB4, ITGA10, FN1
Hypertrophic cardiomyopathy (HCM)	3	8.571	0.0432	ITGB4, ITGA10, TPM1
Dilated cardiomyopathy	3	8.571	0.0498	ITGB4, ITGA10, TPM1

Table 5. KEGG Pathways associated with 35 genes that are differentially regulated in 5 or more studies

We processed the same list of 35 genes through the DAVID Functional Clustering Tool using the highest stringency setting and generated a list with 31 enriched GO categories (Table 6). Some of the top categories are cell adhesion (22.9%), biological adhesion (22.9%), response to steroid hormone stimulus (20%), response to hormone stimulus (20%), response to endogenous stimulus (20%), regulation of apoptosis(20%), regulation of programmed cell death (20%), regulation of cell death (20%), and insulin-like growth factor binding (11.4%).

Term	Count	%	P Value
GO:0048545~response to steroid hormone stimulus	7	20	6.09E-06
GO:0005520~insulin-like growth factor binding	4	11	2.47E-05
GO:0009725~response to hormone stimulus	7	20	2.28E-04
GO:0009719~response to endogenous stimulus	7	20	3.87E-04
GO:0007155~cell adhesion	8	23	0.00126
GO:0022610~biological adhesion	8	23	0.00128
GO:0019838~growth factor binding	4	11	0.00178
GO:0005539~glycosaminoglycan binding	4	11	0.00403
GO:0016477~cell migration	5	14	0.00435
GO:0030247~polysaccharide binding	4	11	0.00525
GO:0001871~pattern binding	4	11	0.00525
GO:0051495~positive regulation of cytoskeleton organization	3	8.6	0.00535
GO:0051674~localization of cell	5	14	0.00634
GO:0048870~cell motility	5	14	0.00634
GO:0042981~regulation of apoptosis	7	20	0.01206
GO:0043067~regulation of programmed cell death	7	20	0.01263
GO:0010941~regulation of cell death	7	20	0.01284
GO:0006916~anti-apoptosis	4	11	0.01357
GO:0010638~positive regulation of organelle organization	3	8.6	0.01736
GO:0030005~cellular di-, tri-valent inorganic cation homeostasis	4	11	0.01756
GO:0055066~di-, tri-valent inorganic cation homeostasis	4	11	0.02011
GO:0030003~cellular cation homeostasis	4	11	0.02357
GO:0030324~lung development	3	8.6	0.02416
GO:0030323~respiratory tube development	3	8.6	0.02554
GO:0060541~respiratory system development	3	8.6	0.02839
GO:0055080~cation homeostasis	4	11	0.03198
GO:0014706~striated muscle tissue development	3	8.6	0.03393
GO:0060537~muscle tissue development	3	8.6	0.03711
GO:0051493~regulation of cytoskeleton organization	3	8.6	0.04324
GO:0044087~regulation of cellular component biogenesis	3	8.6	0.04674
GO:0030246~carbohydrate binding	4	11	0.04741

Table 6. 31 enriched GO categories generated from the list of 35 putative space genes.

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For the visualization of the association between the genes in the network, we performed further bioinformatics analysis using STRING (Search Tool for the Retrieval of Interacting Genes/ Proteins) [61, 62]. By using STRING we can examine co-occurrence, co-expression, and experimental evidence for relationships between the genes of interest. For our analysis, physical and functional interactions among the genes were determined using the high confidence score of 0.7. We uploaded the 129 genes that were differentially regulated in at least 4 of the studies to STRING and the resulting gene association network were shown in Figure 1. The blue lines indicate an association; the thicker the lines the higher the level of confidence. Most of the genes clustered near the center and with a strong association are among the 35 genes we identified as differentially regulated in five or more studies. For example, FN1 (identified in 6 or more studies) shows a strong association with MYC, EGR1 and CTGF all of which were also identified in 6 or more studies. FN1 also shows strong association with LOX, CD44, IGFBP3 and IL8 which are in 5 or more studies. FOS, which is another gene identified in 6 or more studies, shows strong association with MYC, EGR1, MMP10, and IL8 which are genes identified in 5 or more studies.

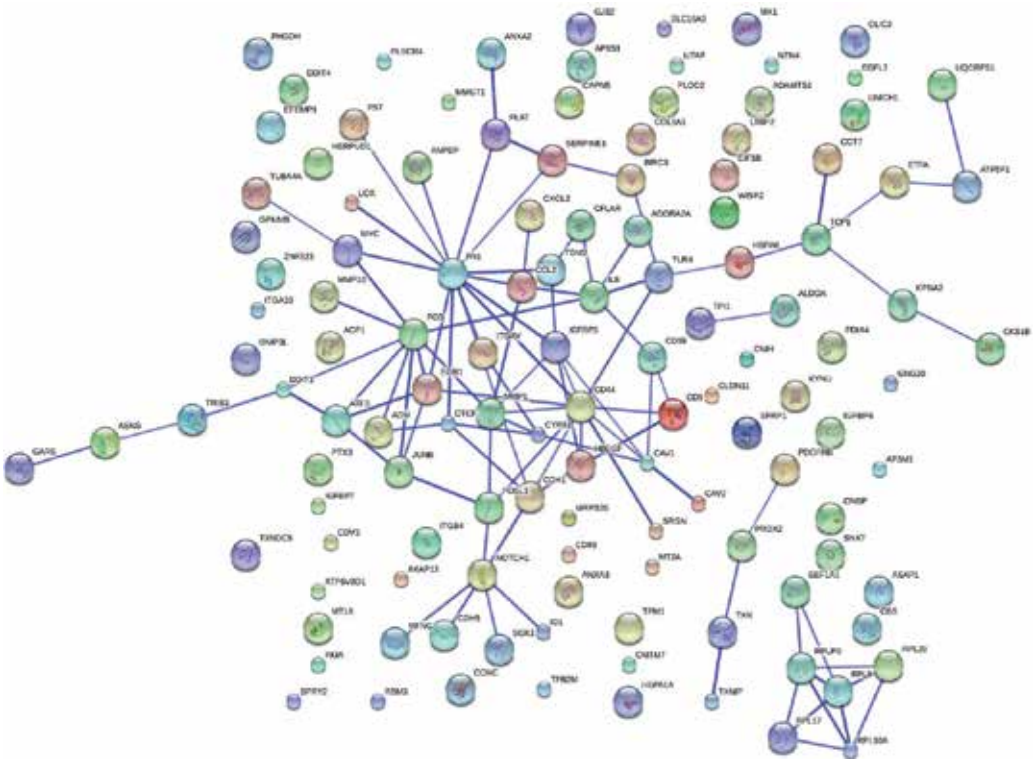

Figure 1. 129 genes that were differentially regulated in at least 4 of the studies were uploaded to STRING. This view shows the evidence of the association between genes. The thicker the line the higher the confidence level.

STRING analysis of the 35 genes differentially regulated in 5 or more studies more clearly show the strong association between FN1, EGR1, CTGF, LOX, MYC, FOS, IGFP3, and CD44 (Figure

2). Note that the genes FN1, EGR1, CTGF, MYC, FOS are among the genes that were differentially regulated in six or more studies, and therefore were identified to be the candidate major space genes at the highest confidence level in the present study.

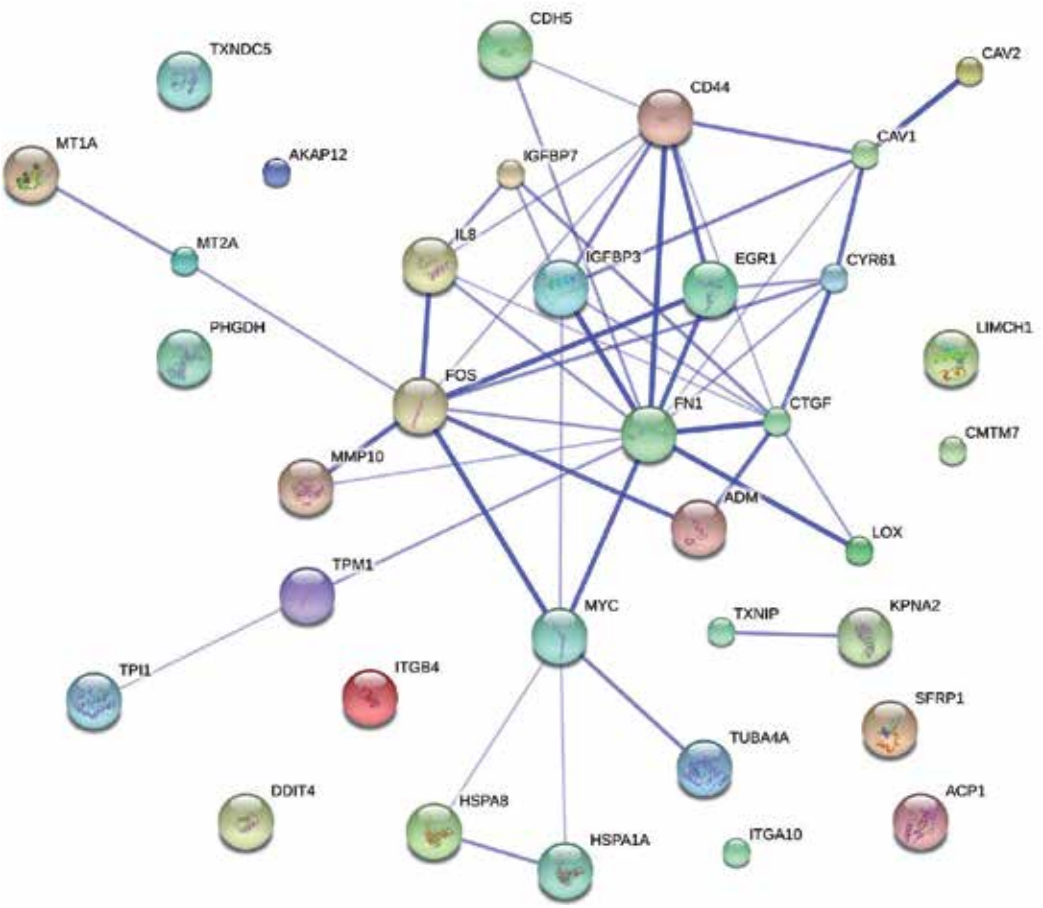

Figure 2. 35 genes that were differentially regulated in at least 5 of the studies were uploaded to STRING. This view shows the evidence of the association between genes. The thicker the line the higher the confidence level.

To further examine the nature of the top 13 genes that were identified in six or more studies to be gravity sensitive, we compiled them into a table which showed the species, cell types, types of microgravity, duration in microgravity and sources of references as well as the directions of the differentially regulated genes (Table 7). From this table we can see that none of the genes were consistently differentially regulated in the same direction. Genes that tend to co-express such as MT1 and MT2, CTGF and CYR61also seemed to co-express in the same direction in these studies. It is not clear why there is such a convergence in the expression patterns. Variables such as different cell types, different species, different forms of microgravity, duration of exposure, and different microarray platforms may be the contributing factors.

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Species	Cell	μg	Duration	CTGF	CYR61	EGR1	FN1	FOS	HSPA1A	ITGB4	KPNA2	MT1	MT2	MYC	TUBA4A	TXNIP	Citation
True Microgravity																	
Human	T-Cell	ISS	1.5h			-								-			[31]
Human	TSCE5	ISS	8d								-						[32]
Human	WTK1	ISS	8d								-						[32]
Human	WTK1	ISS	8d									-	-				[28]
Human	HUVEC	ISS	10d						-							+	[36]
Mouse	gastrocnemius muscles	ISS	11d 19h	+	+							+	+		-		[43]
Mouse	Thymus	ISS	13d									+	+				[48]
Mouse	Mesenchymal Stem Cells	ISS	200h								-					-	[29]
Rat	gastrocnemius muscles	STS	16d				-					+	+				[55]
Human	EA.hy926	PF	22s						-	-							[34]
Microgravity Emulation																	
Human	MG-63	ML	24h		-		+										[26]
Human	EA.hy926 Adherent	RPM	5d	-	-	-		-		-	+			+		-	[35]
Human	EA.hy926 Adherent	RPM	7d	+	-	-		-		-	+			+		+	[35]
Human	EA.hy926 3D aggregate	RPM	5d	-	-	-		-		-	+			+		-	[35]
Human	EA.hy926 Adherent	RPM	7d	-	-			+		-	-			-		+	[35]
Human	FTC-133	RPM	24h	+	+	+		+	+	+	+			+	+	+	[33]
Human	FTC-133 MCTS	RPM	24h	-	-	+		+	+	+	-			+	-	+	[33]
Mouse	RAW 264.7 OCL	RWV	24h							+							[49]
Human	Mesenchymal Stem Cells	RWV	24h	-			-										[29]
Human	HepG2	RWV	3D												-		[22]
Human	HEK	RWV	3-4D						+			+	+	-			[24]
Human	T-Cells	RWV	24h						+								[19]
Human	Renal	RWV	6d		+							-	-				[13]
Mouse	hepatocytes	RWV	4h													+	[7]

Species	Cell	μg	Duration	CTGF	CYR61	EGR1	FN1	FOS	HSPA1A	ITGB4	KPNA2	MT1	MT2	MYC	TUBA4A	TXNIP	Citation
Mouse	hepatocytes	RWV	24h					+									[7]
Mouse	calvarial osteoblasts	RWV	5d	-			-		-						-		[44]
Mouse	Dermal Fibroblast	RWV	2d/7d					-									[50]
Human	Muscle	BR	60d	+			+								+		[25]
Mouse	Soleus Muscle	HLS	7d				-										[56]
Rat	gastrocnemius muscles	HLS	16d		+												[55]

BR= bed rest HLS = Hind limb suspension ISS = International Space Station ML = Magneto Levitation PF = Parabolic flight RPM = Random Positioning Machine RWV = Rotating Wall Vessel STS = Space Shuttle. "+" Indicates up-regulation; "-" Indicates down-regulation.

Table 7. The top 13 putative space genes organized according to types of microgravity. The columns under the gene symbols show the direction of differential regulation in each cell type and microgravity condition.

3. Discussion

The Space Shuttle and the International Space Station (ISS) are engineering miracles [63]. However, the biological importance of the ISS remains mired in controversy over academic and commercial priorities and funding. Do microgravity models provide specialized biological conditions that can be exploited for translational application in the commercial healthcare sector? This is a matter of critical importance to national funding priorities, international competitiveness of the United States, and the health status of Americans and our allies [64]. Not humble or simple questions.

To quote Gene Kranz, the Johnson Space Center Flight Director best known for his leadership during the Apollo13 crisis: "Let's look at this from the point of view of status"[65]. To plan strategically, we need to understand the data we have, the timing of samples, the models studied, and the specifics of the analysis.

This article aims to summarize the status of genome-wide microarray studies in models of microgravity and the true microgravity of space: the data available, the timing of samples, the models studied, and the specifics of the analysis. Specifically, identifying the genes and pathways that may be of central importance in microgravity, may direct areas of commercial and health translation.

With genome-wide array technologies, it becomes possible to study microgravity effects on living organisms at the cellular and molecular levels and in much greater detail, which is key

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to elucidation of the molecular mechanisms of this environmental factor. Since 2011, there has been both an increase in the number of relevant publications and an increase in the quality of retrievable data. This has allowed us to expand our list of putative "major space genes" from the initial number of eight (from our initial attempt toward the identification of the major space genes using literature data [4]) to the present number of 129 genes, at the comparable confidence level. Because of the increase in the number of relevant studies, we were able to go beyond the level of four or more studies in the selection of putative major space genes which was the highest level possible in our previous report [4]. We proceeded to go two levels higher to identify genes found differentially regulated in five or more and six or more studies to be 35 genes and 13 genes, respectively (Table 2). These two additional levels of higher stringency for the selection of putative major space genes enable a significantly higher level of confidence. Our further bioinformatics analysis on the differentially regulated genes showed interesting connections among many of the putative major space genes and several key pathways.

Perhaps the most important insights gleaned from this analysis are the limitations of the current data, which in turn suggests vectors for future analysis [64]. The animal and cellular ground control models are diverse, and incompletely characterized for advantages and limitations and the durations of exposure to true microgravity or simulations, are broadly spread. The analysis platforms vary, but are similar in scope and sensitivity. Despite these limitations, some themes emerge. The effects of radiation are apparent, as are changes in redox potential in the response to microgravity. These are both pathways relevant to tumors.

We found thioredoxin-interacting protein (TXNIP) to be one of the putative space genes that was most frequently differentially expressed in microgravity (Table 2). TXNIP was up-regulated by 10.5 fold in human umbilical vein endothelial cells (HUVECS) in the ISS, making it the most significantly altered gene expression in that study of true microgravity. In microgravity emulated by the random positioning machine (RPM), TXNIP in endothelial cells was down regulated by more than 4-fold after five days and slightly up-regulated after 7 days [35]. However, the same group found TXNIP to be up-regulated in thyroid cancer cells exposed to emulated microgravity [33]. TXNIP is a tumor suppressor in thyroid cells [66]. Up-regulation of this tumor suppressor gene may explain why Grosse et al found that thyroid cancer cells became less aggressive when grown in emulated microgravity [33]..

Metallotionin I and II (MT-I and –II) are also among the top 13 putative major space genes affected (Table 2). These isoforms function primarily in metal ion homeostasis, scavenging of ROS, redox status, immune defense responses, cell proliferation and cell death [67, 68].

Changes in redox-related genes were also identified using fitness profiling of yeast deletion series grown in spaceflight and ground [69] using next generation sequencing. Techniques such as next generation sequencing technology, offer the potential for far more nuanced and detailed analysis of the whole genome, and secondary pathway analysis of the sequence data generated [69]. The genome-wide sensitivity profiles obtained from spaceflight were queried for their similarity to a compendium of drugs whose effects on the yeast collection have been previously reported. The effects of spaceflight have high concordance with the effects of changes in redox state, suggesting mechanisms by which spaceflight may negatively affect cell fitness.

The redox state of tumor cells is frequently disrupted and this is difficult to reproduce in ground-based cultures [70-72]. Hence, redox-dependent drug metabolism in tumors may be uniquely modeled in microgravity. Many of the genes most commonly associated with microgravity-related changes have been identified to have roles in cell cycling, which is critical for both carcinogenesis and responses to radiation damage. EGR1 (Early growth response protein 1; also referred to as Zif268, zinc finger protein 225; and NGFIA, nerve growth factor-induced protein A) is a tumor suppressor transcription factor for differentiation and mitogenesis. MYC encodes for a transcription factor with roles in cell cycle progression, apoptosis and cellular transformation [73]. Karyopherin alpha 2 (KPNA2) promotes tumorigenicity through up-regulation of c-MYC [74]. In endothelial cells grown in microgravity KPNA2 was shown to up-regulated in both adherent cells and multicellular conglomerates at 5 days in simulated microgravity, but down-regulated in multicellular conglomerates at 7 days [35]. HSP70 family members such as HSPA1A have been found to be critical to cellular homeostasis and cancer cell survival[75]. Integrin, Beta 4 (ITGB4) is the receptor for laminin and has been found to be up-regulated in thyroid cancer cells and MCTS grown on RPM for 24 hours [33].

Similar to the results of the current meta-analysis, Nislow et al. 2014 found spaceflight has subtle but significant effects on core cellular processes including growth control via RNA and ribosomal biogenesis, metabolism, modification and decay pathways. Furthermore, significant roles for DNA repair and replication, response to pH signaling, control of gene expression, and mitochondrial function were observed. The yeast chemogenetic analysis of spaceflight samples strongly implicates DNA and RNA damage as the major ground based analogs of spaceflight stress. Given the unique, and substantial radiation exposure in space, this is consistent with major radiation-mediated effects which may mimic cancer related effects.

Suppression of the immune system has been thought to be an important side-effect of microgravity exposure [1, 2, 31, 76]. Recently, a global gene expression analysis of human T cells after 1.5 h of stimulation by Con A and anti-CD28 in the LEUKIN spaceflight has identified immediate early genes whose transcription are inhibited in microgravity [31]. The transcription of immediate early genes is inhibited in T cells activated in microgravity, which may be involved in the molecular basis of spaceflight immunosuppression. NF-κB is known to regulate transcription in most mammalian cells and plays a key role in immune responses to antigens, cytokines, UV radiation, oxidized LDL, free radicals, etc. [77-81]. CREB, a cAMP-responsive transcription factor, regulates immune genes including IL-2, IL-6, IL-10, and TNF-α. CREB also promotes survival and proliferation to T-cells, monocytes, and macrophages [82]. EGR1 and MYC, which are among the 48 most significantly down-regulated by microgravity in the T cell activation study are identified as the putative major space genes in the current study (Table 2).

This analysis shows a commonality of gene changes and pathways between different microgravity models. As data is systematically accumulated, this type of analysis will allow even more meaningful analysis. A key question is whether the unique environment of the ISS induces biological changes of commercial translational value to enhance ground-based health care? In which areas does ISS provide a specific advantage over ground-based biological simulations to direct strategic planning of space based biological science? Within and between ground-based microgravity simulations, can we identify areas where specific techniques are

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best suited for health care applications? This approach will place space-based science at the center of academic medical center activity [83], and translate to commercial applications.

Abbreviations / Glossary

DAVID: Database for Annotation, Visualization and Integrated Discovery

EGR1: Early growth response protein 1

GO: Gene Ontology

HARV: High Aspect Rotating Vessel

HUVEC: Human Umbilical Vein Endothelial Cells

ISS: International Space Station

KPNA2: Karyopherin alpha 2

MT-1, -2: Metallotionin-I and –II

RCCS: Rotating Cell Culture System

ROS: Reactive oxygen species

RPM: Random Positioning Machine

RWV: Rotating Wall Vessel

STRING: Search Tool for the Retrieval of Interacting Genes/Proteins

TXNIP: Thioredoxin-interacting protein

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Chapter 6

Enzymatic Polymerization of Rutin and Esculin and Evaluation of the Antioxidant Capacity of Polyrutin and Polyesculin

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Additional information is available at the end of the chapter

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1. Introduction

Polyphenols have gained great attention due to their biological and pharmacological activities. Their anti-inflammatory, antioxidant, antimutagenic, anticarcinogenic and antiviral properties were studied in many *in vivo* and *in vitro* systems [1-7]. It seemed that these properties were potentially beneficial in preventing diseases and protecting genome stability. In fact, many of these properties were related to the antioxidant activities of polyphenols [7-10]. However, depending on their structure, the processability of these compounds was limited by their weak stability and low solubility in organic or aqueous solvents [11, 12]. With a view to improve these properties, derivatization of phenolic compounds by enzymatic polymerization was reported by several authors [13-15]. So, it is a useful alternative to chemical catalysis because it can be realized without less hazardous. The two principal enzymes family used in phenolic compounds polymerization process were the laccases and peroxidases. Horseradish peroxidases (HRP) are H_2O_2 dependent. HRP are used in several works to catalyze the polymerization of catechin [14, 16, 17], catechol [18], quercetin, rutin, daidzein 5, 6, 4′-trihydroxyisoflavone [16], 4-hydroxybiphenol [19, 20], 4-[(4-phenylazo-phenyimino)-methyl]-phenol [21], and phenols in various solvents, solvent-aqueous buffers mixture, buffers [22] and in ionic liquids at room temperature [23].

Laccase are also indicated as an efficient catalyst for polymerization of phenolic compounds [24]. Compared to HRP, laccase-catalyzed polymerization without the use of hydrogen

peroxide, as an oxidizing agent. Laccase from different origin (*Trametes versicolor*, *Myceliophthora, Agarucus bisporus, Ustilago maydis, Trametes pubescens, Pycnoporus coccineus, Pycnoporus sanguineus)* have been described for the polymerization of phenolic compounds as rutin [15, 25-29], esculin [28, 30], methoxyphenols, gallic acid, caffeic acid, vanillin, Kaempferol and quercetin [25].

As it has been mentioned previously one of the problem in the use of phenolic compounds, was their weak solubility. The first results of enzymatic polymerization reported that the obtained polymers of rutin and esculin were 4200-folds and 189-folds more water soluble than rutin and esculin, respectively [26, 28]. The solubility of polyrutin was also increased in dimethylformamide (DMF) and dimethyl sulfoxyde (DMSO) [15].

Enzymatic polymerization of phenolic compounds affected also their biological properties. These properties, including antioxidant activities, might be dependent on the molecular weight of the synthesized polymers and the type and the position of the linkages ($\overline{M_w}$, PDI, C-C or C-O bridges). Moreover, depending on the used method for determining antiradical activity (AAPH, DPPH,...) of polyphenols, results were controversial.

As an example, rutin polymerized by laccase from *Pycnoporus coccineus*, *Pycnoporus sanguineus* or *Myceliophthora* led to polymers with a better inhibition of AAPH radical, compared to its monomer [15, 27]. However, Anthoni *et al*. [26] reported that polyrutin, obtained by laccase from *Trametes versicolor* polymerization, had a weaker DPPH radical scavenging activity compared to rutin. This behavior could be due either to the used method of antioxidant activity determination or the degree of polymerization. Oligorutin fractions showed a higher ability of to reduce the genotoxicity induced by H_2O_2 and antimutagenic effect compared to monomeric rutin [28, 29].

For other phenolic compounds, like catechin, kaempferol, esculin and 8-hydroquinoline, polymerization enhanced inhibition effects against free radicals including-oxidation of low-density lipoprotein (LDL) [14] and DPPH radical [25].

Using xanthine oxidase inhibition test, it was well established that enzymatic polymerization of phenolic compounds (rutin, esculin, catechin and epigallocatechin gallate) increased antioxidant activity [14, 15, 26, 28, 30].

Furthermore, the polymerization of 3-methylcatechol by Kawakita et al. [31] led to the formation of polymers with high copper ions adsorption power.

The aim of this work was in one hand, to compare the effect of polymerization on the antioxidant activity of rutin and esculin (Figure 1) and in other hand, to discuss the structure-antioxidant activity relationship. Polyrutin and polyesculin were synthesized by laccase from rutin and esculin, respectively, and carefully separated in different fractions by diafiltration process. Antioxidant activity was evaluated by radical scavenging activity, iron chelating capacity, xanthine oxidase inhibition activity, cupric reducing capacity.

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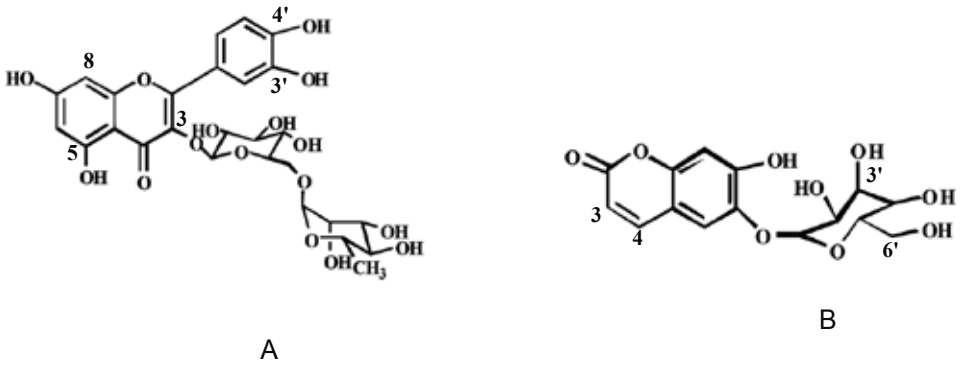

Figure 1. Molecular structure of rutin (A) and esculin (B)

2. Materials and methods

2.1. Chemicals

Laccase from *Trametes versicolor* (E.C. 1.10.3.2., 21.4 U mg-1), rutin hydrate (98%), esculin hydrate (98%), ascorbic acid, 2-deoxyribose, trichloroacetic acid (TCA), thiobarbituric acid (TBA), dimethylsulsulfoxide (DMSO), 2,2′-azino-bis(3methylbenzenothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2-2- diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2- carboxylic acid (Trolox) and hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich. All used solvents were HPLC grade from VWR.

2.2. Methods

2.2.1. Polymerization reaction

Polymerization reaction was carried out in the same operating conditions described by Anthoni et al [26, 28]. Rutin or esculin (50 g/L) was suspended in 1 L of a methanol/ water (30:70 v/v) reaction medium. Laccase solution (3 U/ mL) was added to the mixture. The reaction was stirred at 600 rpm, for 24 h for rutin and 72 h for esculin, at 20 °C. We noticed that rutin polymerization reaction didn't evolve beyond 24 h, whereas, esculin polymerization reaction continued till 72h. The Kinetic of polymerization reaction was followed with seize exclusion chromatography (SEC).

2.2.2. Polymers separation and lyophilization

Final reaction media enriched with rutin and esculin polymers was separated, by successive filtration processes on a 15, 5, 3 and 1 KDa membranes in diafiltration process (INSIDE CeRAM™), using a mixture of water/methanol (70:30 v/v) (5 L) as eluent, at 50°C. The transmembranaire pressure (ΔP) was fixed at 2 bars. The state permeate flux (F) was in the

range of 35 l/h/m^2.Then, the fractions were lyophilized (Christ Alpha 1-2 LD freeze dryer). Five fractions were thus obtained and characterized (Table 1).

	Fractions	
	Rutin	**Esculin**
Permeate on Mb 1KDa	R1	E1
Retentate on Mb 1KDa	-	E2
Permeate on Mb 3 KDa	R2	-
Retentate on Mb 3 KDa	-	E3
Permeate on Mb 5 KDa	R3	-
Retentate on Mb 5 KDa	R4	E4
Retentate on Mb 15 KDa	R5	E5

Table 1. Fractions of polyrutin and polyesculin obtained after separation, Mb : membrane; KDa : Kilo Dalton

2.2.3. Seize exclusion chromatography analysis (SEC)

Relative masses of polymers were evaluated by size exclusion chromatography (SEC) (HPLC LaChrom, UV 280 nm LaChrom L-7400, Tosoh TSKgel α 3000 column, 60 °C). Dimethylformamide (DMF) with 1 % LiBr was used as a mobile phase (0.5 mL/min). Molecular mass calibration was obtained using standards of polystyrene and polystyrene sulfonate. The obtained data allowed the determination of number-average molecular mass ($\overline{Mn}$), weight-average molecular mass ($\overline{M_w}$), weight-average molecular mass index (I_M) and polydispersity (PDI) as described by Faix et al. [32].

2.2.4. UV analyzes

The UV spectra of rutin, esculin solutions and their obtained polymers fractions were determined using a UV6000LP spectrometer (Spectra System, Thermofinnigan).

2.2.5. FTIR analysis

The IR analyses were conducted by ATR-FT-IR spectroscopy using a FT-IR spectrometer Tensor 27 (Bruker). The analysis was carried out on monomers and polymers lyophilized powders.

2.2.6. Radical scavenging on ABTS $^{+\cdot}$

The assay was conducted according to protocols presented by Re et al. (1999) and van den Berg et al. (2001) [33, 34]. To generate the ABTS $^{+\cdot}$ radical, the ABTS stock solution (7 mM) and potassium persulphate (2.45 mM) in water were allowed to stand in the dark at room temperature for 12-16 h before use. For the reaction, 10 µl of each sample at various concentrations

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(from 800 to 0.25 μM) was added to 990 μl of diluted $ABTS^{+\cdot}$ (absorbance 0.7 at 734 nm) and the absorbance was recorded every min. A standard curve was prepared using a series of concentrations of trolox (from 0 to 15 μM) with 990 μl of diluted $ABTS^{+\cdot}$ solution. The radical scavenging capacity of tested samples was calculated based on the trolox standard curve and expressed as the trolox equivalent antioxidant capacity (TEAC) and as IC_{50}.

2.2.7. Radical scavenging activity on DPPH·

The free radical scavenging capacity of the esculin and rutin and their polymers was determined with 2,2-diphenyl-1-picryl-hydrazyl as described by Bruda et Oleszek [35]. A solution of 1 ml of monomers or polymers (from 10^2 to 4 10^4 μM, concentrations were calculated from $\overline{M_w}$) in methanol, was mixed with 2 ml of DPPH (10 mg/L in methanol/water, 80:20, v/v). A reference sample was prepared by adding 1 ml of methanol in 2 mL of DPPH solution. Monomers and polymers absorbance for each concentration was evaluated at 527 nm, after 15 min, at 23 °C. The antiradical activity was calculated as a percentage of DPPH discoloration using the following equation (1).

$$Antiradical\ activity = \left(1 - \frac{Absorbance\ of\ the\ sample - Absorbance\ of\ polymers}{Absorbance\ of\ the\ reference}\right) * 100 \quad (1)$$

The results were expressed as IC_{50} and TEAC according to the calibration curve (from 0 to 5 μM).

2.2.8. Inhibitory effect on deoxyribose degradation

Inhibitory effects of tested compounds on deoxyribose degradation were determined by measuring the competition between deoxyribose and theses compounds for the hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system (referred to non site-specific assay) or Fe^{3+}/ascorbate/H_2O_2 system (referred to site-specific assay which could indicate the hydroxyl scavenging power of tested molecules by iron chelating power) according to the method described by Halliwell et al. [36] with slight modifications.

The tested sample was added to the reaction mixture containing deoxyribose (10 mM), Fe(III) chloride (10 mM), EDTA (1 mM), and H_2O_2 (10 mM), ascorbic acid (1 mM), 1mM H_2O_2 and 50 mM potassium phosphate buffer (pH 7.4). The mixture was incubated for 1 h at 37°C, TBA (1%) and TCA (2.8%) were added to the above mixture, and then heated for 90 min on water bath at 80 °C. The absorbance at 532 nm was then measured against a blank containing deoxyribose and buffer. For site-specific hydroxyl radical scavenging activity, the procedure was similar to the above method, except that EDTA was replaced by the equivalent volume of buffer. The gallic acid was used as a standard. The percentage of deoxyribose degradation inhibition was calculated using the equation (2).

$$PI(\%) = \left(1 - A_s / A_c\right) * 100 \quad (2)$$

where A_C is the absorbance of negative control and A_S the absorbance of sample solution.

Results of deoxyribose assay in the presence and the absence of EDTA are expressed as IC_{50} and as TEAC.

2.2.9. Xanthine oxidase inhibition assay

The tested samples were solubilized in phosphate buffer (pH 7.5, 50 mM), except rutin which was dissolved in a minimum of DMSO (5 µl) and then in buffer. The assay was conducted as described by Kong et al. [37]. Tests solutions were prepared by adding 1600 µL of buffer, 300 µL of tested solutions (from 4 10^{-6} to 10^{-3} M), 1000 µL of a solution of xanthine (0.15 mM) and 100 µL of a solution of xanthine oxidase (0.2 U/mL). The reaction was monitored for 6 min at 295 nm. Two samples were prepared, the first without tested solutions to determine the total uric acid production, and the second without enzyme to measure the absorbance of tested solutions at 295 nm for the range of concentrations. Results were expressed as the final concentration that results in half-maximal enzyme velocity (IC50) and calculated by standard curve regression analysis and as TEAC according to the calibration curve (from 1 10^{-3} to 5 10^{-1} µM).

2.2.10. Cupric reducing antioxidant capacity (CUPRAC)

The cupric reducing antioxidant capacity was determined according to the method of Apak et al. [38] To each tube containing 20 µl of tested substrate concentration, we added $CuCl_2$ to a final concentration of 3.12 mM, ethanolic neocuproine solution and NH_4Ac buffer solution (pH=7) to final concentrations of 2.34×10^{-3} M and 312 mM, respectively. The total volume was then adjusted with distilled water to 2 ml and mixed well. Absorbance against a reagent blank containing all reagents except $CuCl_2$ and neocuproine was measured at 450 nm after 1h. The results were expressed as equivalent of Trolox according to the calibration curve (from 10 to 10^3 µM).

3. Results

3.1. Polymers synthesis, separation and characterization

Kinetics of esculin and rutin polymerization were monitored by SEC-UV at 280 nm. Once the polymerization was achieved, polymers were separated, by successive diafiltration process. Weight-average molecular mass $\overline{M_w}$, polydispersity (PDI) and weight average molecular mass index (I_M) of obtained fractions (R1-5 and E1-5) were summarized in table 2. These results indicated clearly that the polymerization of the two substrates was occurred and led to polymers of rutin and esculin with high molecular weight (Figure 2).

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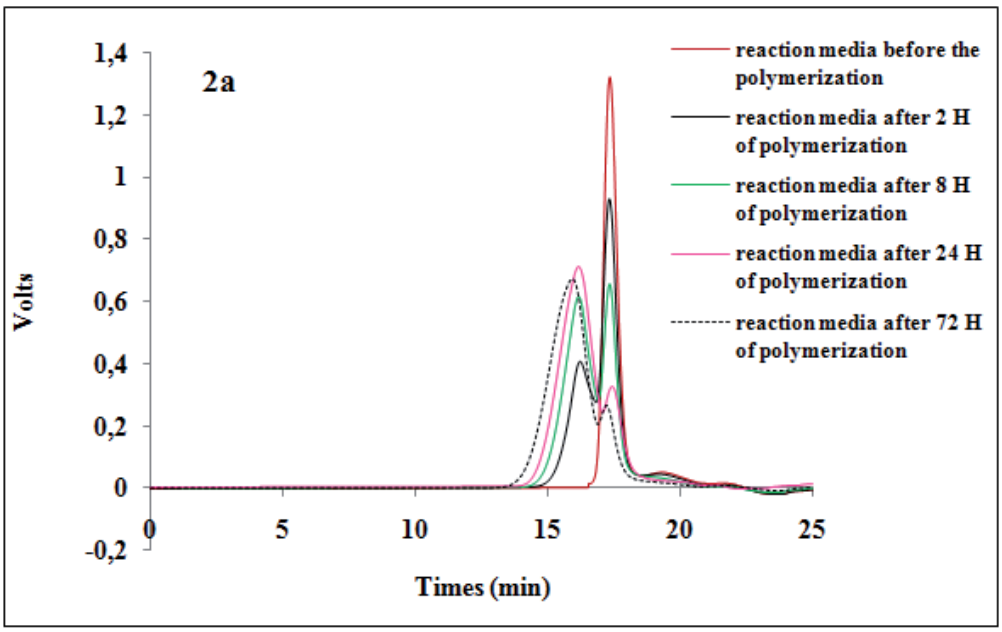

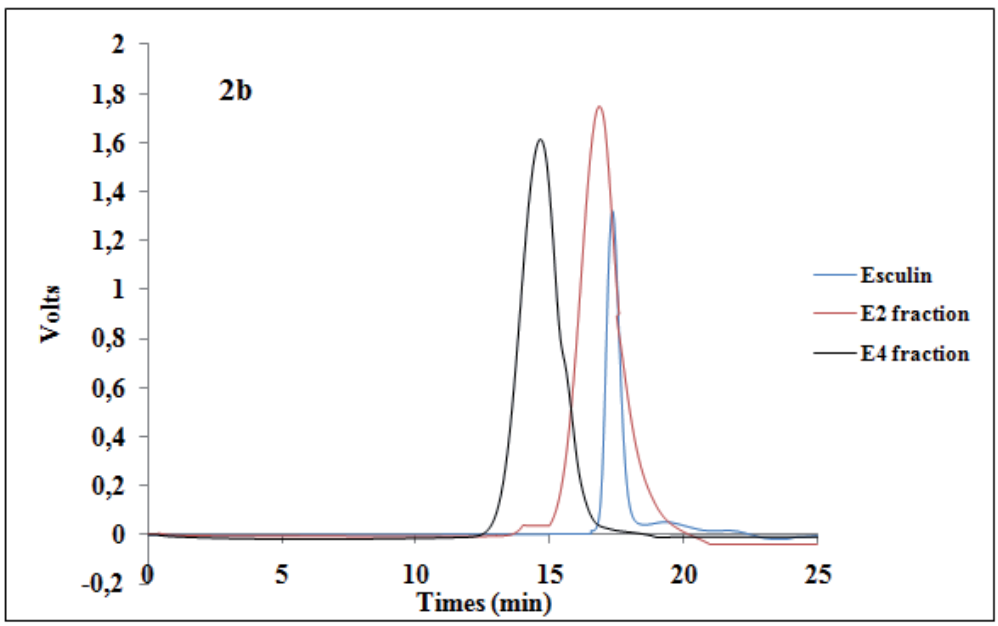

Figure 2. Kinetic of esculin polymerization determined by SEC-UV using dimethylformamide (DMF) with 1 % LiBr as a mobile phase (0.5 ml/min) (2a). SEC-UV analyses of esculin and polyesculin fractions E2 and E4 using dimethylformamide (DMF) with 1 % LiBr as a mobile phase (0.5 ml/min) (2b).

Fractions	$\overline{M_w}$ (g /mol)	PDI	I_M
Rutin (R)	611.21 ± 80.54	1.0024 ± 0.012	1 ± 0.0
R1	2127.42 ± 67.12	1.17 ± 0.03	3.48 ± 0.14
R2	4301.8 ± 102.72	1.37 ± 0.07	7.05 ± 0.16
R3	5069.93 ± 116.2	1.36 ± 0.04	8.30 ± 0.18
R4	7106.54 ± 96.62	1.35 ± 0.08	11.64 ± 0.14
R5	8331.85 ± 146.24	1.42 ± 0.12	13.65 ± 0.22
Esculin (E)	339.36 ± 43.46	1.009 ± 0.09	1 ± 0.0
E1	688.12 ± 40.66	1.31 ± 0.11	2.02 ± 0.12
E2	1021.33 ± 48.51	1.48 ± 0.06	3.009 ± 0.14
E3	3042.1± 86.24	1.39 ± 0.13	8.96 ± 0.25
E4	5080.43 ± 70.96	1.41 ± 0.07	14.97 ± 0.20
E5	6973 ± 68.1	1.54 ± 0.10	20.54 ± 0.20

Table 2. Weight-average molecular mass ($\overline{M_w}$), polydispersity (PDI) and weight-average molecular mass index (I_M) of obtained polyrutin (R1-R5) and polyesculin (E1-E5) fractions.

3.2. UV and FTIR investigations

The UV-visible spectrum of rutin, in methanol/water (30/70 v/v), presented two maxima of absorption at 282 and 359 nm due to the π-π* transition of the aromatic electrons. For polyrutin fractions (R1, R3 and R5) the 359 nm band was larger and presented a hypsochromic shift of 5 nm. Such results could be due to the implication of the B ring of rutin in the formation of polymers. In fact, Anthoni *et al.* [26] and Marckam [39] observed a similar behavior. The latter stated that the presence of a substitution on the 5, 7 and 4′ positions of the phenolic rings led to a hypsochromic shift.

The UV spectra of esculin and polyesculin fractions E2, E3, E4 and E5 presented the same peaks with a maximum of absorption at 345 nm while the peaks correspondent to E5 were broader than those of esculin, which could be attributed to conjugated oligomeric structure [15, 40]. The same profile was reported by Anthoni et al. for the esculin polymerization [30].

FTIR spectra of rutin and polyrutin fractions (R1, R3 and R5) (Figure 3), showed a new peaks at 1220 cm^{-1} and at 1465 cm^{-1}.The peak at 1220 cm^{-1} indicated the formation of new ether bonds C-O. The signal at 1465 cm^{-1} could be attributed to a bond C-C while the absence of a peak at 1747 nm on the R1 spectra compared to rutin spectra could be explained by the disappearance of C-H bonds. These results showed that obtained polyrutin fractions were formed through C-C and C-O linkages. In fact, many authors reported that flavonoid polymers were composed of phenylene units and/or oxyphenylene units [24, 26, 41]. Uzan et al. [27] reported that the nucleophilicity of the aromatic A-ring seemed to play a major role as the reactive hydroxylated ring in coupling reactions for the formation of a new bond. They suggested that polymerization of rutin by *Pycnococus* laccases led to formation of polymers through C-C and C-O bonds and

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more precisely through C8-C8, C6-O4′ and C8-C5′ linkages. A study of the polymerization of quercetin by Bruno et al. [12], with Horseradish peroxidase (HRP), showed that the highest occupied molecular orbital (HOMO) was concentrated on the catechol group. Therefore, these authors expected the polymerization reaction to take place in the two more negative carbons of that group 2′ and 5′.

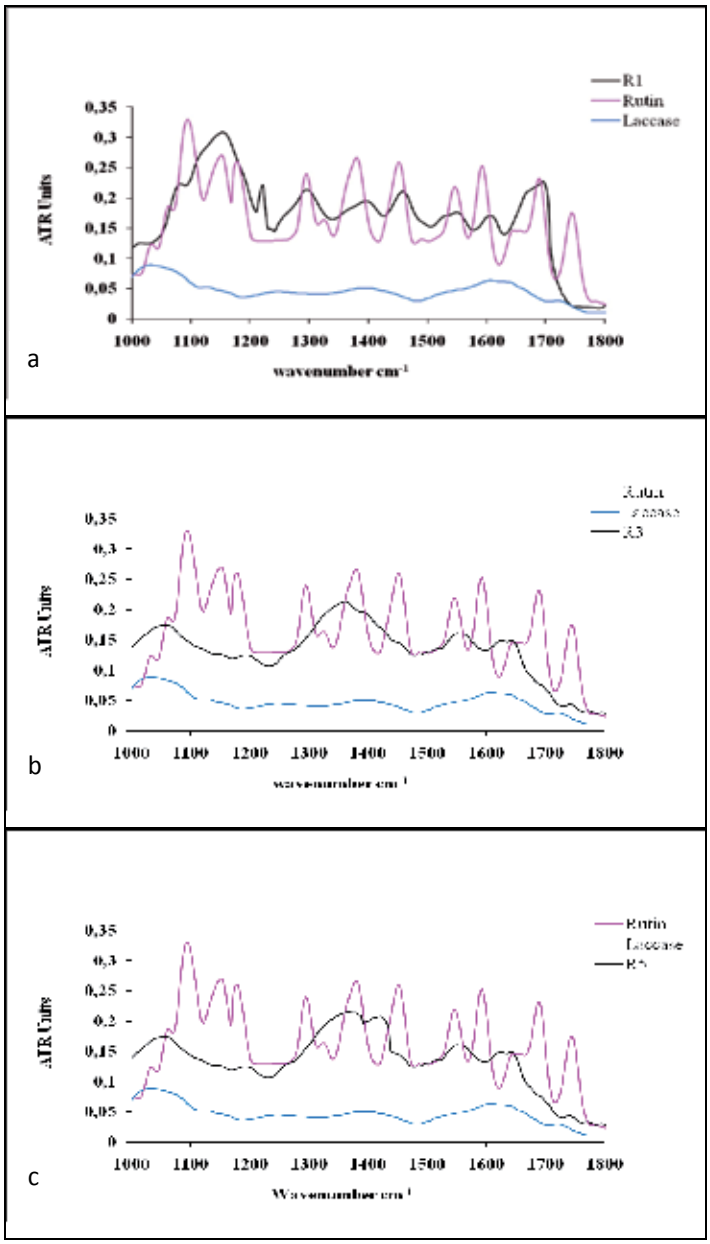

Figure 3. FTIR spectrum of rutin fractions and laccase, R1 (a), R3 (b) and R5 (c).

As for rutin, FTIR spectra of polyesculin fraction E2, E3, E4 and E5 showed a new peak at 1400 cm^{-1} compared to the spectra of esculin. This could be due to a formation of C-C bonds. In fact, Anthoni et al [30] reported the formation of C-C and C-O linkages, involving both the phenolic and the glucosidic part of the coumarin during the esculin polymerization. Moreover, an *in silico* structure investigation of oligoesculin by the same authors suggested the preferential formation of C8-C8 linkage between esculin units during the polymerization reaction.

The obtained and reported data of UV and FTIR suggested that different linkages (C-C, C-O) could be achieved depending to monomer, enzyme and operating conditions (pH, temperature, medium). This might affects the antioxidant activity of the polymer.

3.3. Evaluation of antioxidant activity of rutin and polyrutin fractions

Different methods were used to evaluate the antioxidant activity (free radicals scavenging activity, iron chelating capacity, xanthine oxidase inhibition activity and cupric reducing capacity) of esculin, rutin and their derivatives. Results were summarized in Table 3.

	ABTS		DPPH		Hydroxyl radical		XO inhibition		Iron chelation		CUPRAC
	IC_{50} (µM)	TEAC (µM)	IC_{50} (µM)	TEAC (µM)	IC_{50} (µM)	TEAC (10^{-2} µM)	IC_{50} (µM)	TEAC (10^{-3} µM)	IC_{50} (µM)	TEAC (10^{-3} µM)	TEAC (µM)
R	320±12	3.89±0.2	1.1±0.1	113.4±13.5	18.6±1.6	101±0.08	962±16	5±0.1	58.3±5	1±0.1	315±18
R2	440±14	2.83±0.25	3.9±0.2	62.0±8.2	25.7±1.75	75±0.09	119.02±14	47±4	49.7±6	1.1±0.1	411±27
R3	540±22	2.31±0.42	18±0.9	56.9±5.25	30±1.5	62±0.06	29.74±7	190±2	38.3±1	1.5±0.2	483±13
R5	640±24	1.95±0.48	38±0.2	43.2±6.75	38.32±1.9	49±0.05	14.12±1.5	400±16	36.5±4	1.6±0.1	527±29
E	450±2	0.003±0.1	9200±9	0.021±0.005	5600±64	0.3±0.01	779±33	7±0.5	6800±9	0.8±0.1	29±1.5
E2	110±19	0.1±0.02	500±43	3.9±0.4	1600±13	1.1±0.05	301±21	18±0.3	2100±5	2.7±0.2	89±4
E3	30±1	0.41±0.05	500±32	3.9±0.3	353±21	5.3±0.07	160±9	35±1.5	650±25	9±0.1	328±10
E4	9±0.5	0.83±0.08	480±25	3.7±0.5	150±8	12.5±0.5	154±14	36±1	423±12	10±0.5	538±4
E5	1±0.1	1.23±0.4	480±39	3.7±0.3	70±4	26.9±1.5	141±6	40±0.8	180±5	30±1.2	898±34

Table 3. Antiradicals, xanthine oxidase inhibition, iron chelating and CUPRAC activities of rutin, esculin and their polymer fractions. Results are represented by the means ± SD of three experiments. TEAC: Trolox equivalent antioxidant capacity. IC50: The half maximal inhibitory concentration; ABTS: 2,2'-azino-bis(3methylbenzenothiazoline-6-sulfonic acid) diammonium; DPPH: 2-2- diphenyl-1-picrylhydrazyl; XO: xanthine oxidase. CUPRAC: Cupric reducing antioxidant capacity.

3.3.1. Free radicals scavenging activity of rutin and polyrutin fractions

Results in Table 3 showed that IC_{50}, related to polyrutins, increased progressively versus $\overline{M_w}$. The fraction R5, presenting the highest $\overline{M_w}$, led to highest IC_{50} values (640, 38 and 38.32

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μM) compared to IC_{50} values obtained in presence of rutin (320, 1.1 and 18.6 μM) respectively for ABTS, DPPH and hydroxyl radicals. These results suggested that higher is the $\overline{M_w}$ lower is the antiradical activity. The low antiradical activity of polyrutin fractions observed in this study was in accordance with that reported by other authors [15, 26, 27].

3.3.2. Xanthine oxidase inhibition activity of rutin and polyrutin fractions

For XO inhibition activity (Table 3), the IC_{50} values of polymer fractions appeared to be lower than the IC_{50} value of rutin (962 μM). The results indicated that IC_{50} decreased when the $\overline{M_w}$ arised, which traduced the better ability of polyrutins to inhibit XO compared to monomeric rutin. The fraction R5 illustrated the highest XO inhibition power, 68-folds better than monomeric rutin. The strong XO inhibition observed for polyrutin fractions was in accordance with other studies dialled in enzymatic flavonoid polymerisation [14, 15, 25, 26, 42].

3.3.3. Iron chelating properties of rutin and polyrutin fractions

All polyrutin fractions exhibited higher degree of iron chelating ability (Table 3). This activity grow with the increase of $\overline{M_w}$. The polyrutin fraction R5 presented the highest iron chelating power with an IC_{50} value of 36.5 μM compared to 58.3 μM, in presence of the monomer.

3.3.4. CUPRAC of rutin and polyrutin fractions

The cupric ion (Cu^{2+}) reducing abilities of rutin and polyrutin fractions (R2, R3 and R5) were shown in Table 3. It appeared that the cupric ion (Cu^{2+}) reducing powers of different tested compounds were in the following order R5 (TEAC of 527 μM)>R3 (TEAC of 483 μM)>R2 (TEAC of 411 μM)> rutin (TEAC of 315 μM), meaning that cupric ion (Cu^{2+}) reducing ability increased with the increase of $\overline{M_w}$.

3.4. Evaluation of antioxidant activity of esculin and polyesculin fractions

3.4.1. Free radicals scavenging activity of esculin and polyesculin fractions

Polyesculin fractions presented lower IC_{50} values than those of monomeric esculin which indicated their stronger antiradical activity (Table 3). Polyesculin fraction E5 was the most potent one. It was respectively for ABTS, DPPH and hydroxyl radicals 450, 19 and 80 folds more active than esculin (450, 9200, 5600 μM). Unlike rutin, the antiradical activities increased with $\overline{M_w}$ when ABTS and hydroxyl radical methods were used. However, for DPPH the IC_{50} remained constant, about 480 μM, for all tested fractions. So, DPPH scavenging activity seemed to be independent to the degree of polymerisation.

3.4.2. Xanthine oxidase inhibition activity of esculin and polyesculin fractions

Results in Table 3 showed that for all polyesculin fractions, IC_{50} were lower than that of the monomer (779 μM). This activity was linked to $\overline{M_w}$ and decreased as $\overline{M_w}$ increased. The fraction E5 presented the lowest IC_{50} and therefore the highest XO inhibition activity, 5-folds higher than monomer.

3.4.3. Iron chelating properties of esculin and polyesculin fractions

Polyesculin fractions exhibited high degree of iron chelating activity, according to the site-specific hydroxyl radical-scavenging assay (Table 3). Results showed that iron chelating capacity was high as the $\overline{M_w}$ increases. The best iron chelating power was observed in the presence of the E5 fraction (IC_{50}=180 μM), which was 37-folds better than esculin (IC_{50}=6800 μM).

3.4.4. CUPRAC of esculin and polyesculin fractions

Table 3 indicated that polyesculin fractions presented higher TEAC than esculin. This activity rose as the $\overline{M_w}$ increased. Therefore, the best cupric reducing antioxidant capacity was seen with the E5 fraction which was 30-folds more active than esculin.

3.5. Structure-antioxidant activity relationship

The structure-antioxidant activity relationship of monomeric flavonoids and coumarins was well investigated. According to many authors [43] free hydroxyl groups on C4', C3' and C7 played a major role in antiradical activity of rutin and esculin. However, few data are available about the behaviour of these activities with polymerization. In this work we observed a decrease of polyrutin antiradical activities with $\overline{M_w}$ increase. This decrease could be attributed to the loss of these groups during the rutin polymerization reaction.

For high iron chelating power and CUPRAC, hydroxyl groups on C5, C3 and the 4 oxo (for flavonoids) and hydroxyl groups and catechol moiety (for coumarins) were essential. So, high iron chelating and cupric reducing antioxidant capacities observed with polyrutin and polyesculin fractions suggested that these groups were not implicated in the linkage occurred in rutin polymerization reaction [44-47].

For high xanthine oxidase inhibition activity, several works reported the importance of the presence of a double bond between C2 and C3 and free hydroxyl groups on C5 and C7 [26, 48-50]. High inhibition of the xanthine oxidase obtained in the presence of polyrutin and polyesculin fractions implicated that these groups are not affected during the polymerization reaction.

4. Conclusion

Polyphenolic polymers of rutin and esculin were synthesized using a laccase from *Trametes versicolor*. These polymers were fractioned by diafiltration process.

The analyses of rutin polymers by FTIR showed the presence of new C-C and C-O bonds and the desperation of a C-H bond on monomer. These results suggested that polyrutin were synthesized through phenylene and oxyphenylene units. For polyesculin fraction, FTIR analyses indicated the presence of only C-C bond.

Free radical scavenging activity of rutin was decreased by the enzymatic polymerization while polyesculin fractions showed a high antiradical activity compared to monomeric esculin. This

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behaviour suggested that the antioxidant activity depend on the position of linkage through the polymerization reaction. For esculin, it seemed that the polymerization didn't affect groups implicated in the antioxidant activity. This could explain the high antioxidant activity values observed for polyesculin.

Both polyrutin and polyesculin fractions exhibited a high XO inhibition activity, iron chelating and cupric reducing antioxidant capacities.

Abbreviations

AAPH: 2,2′-azobis (2-amidinopropane)dihydrochloride ; ABTS: 2,2′-azino-bis(3methylbenzenothiazoline-6-sulfonic acid) diammonium salt; ATR: Attenuated Total Reflectance; $CuCl_2$: Copper (II) chloride; DMF: dimethylformamide; DMSO: and dimethyl sulfoxyde; DPPH: 2-2-diphenyl-1-picrylhydrazyl; EDTA: Ethylenediaminetetraacetic acid; FTIR: Fourier Transformed InfraRed analysis; H_2O_2: Hydrogen peroxide; HOMO: Highest occupied molecular orbital; HRP: Horseradish peroxidises; I_M: weight average molecular mass index; LDL: Low-density lipoprotein; LiBr: Lithium bromide; $\overline{Mn}$: Number-average molecular mass; $\overline{M_w}$: Weight-average molecular mass; NH_4Ac: Ammonium acetate; NH_4Ac: Ammonium acetate; TBA: Thiobarbituric acid; TCA: Trichloroacetic; TEAC: Trolox equivalent antioxidant capacity; Trolox: 6-hydroxy-2,5,7,8-tetramethylchroman-2- carboxylic acid; XO: Xanthine oxidase

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Chapter 7

The Use of Lactic Acid Bacteria in the Fermentation of Fruits and Vegetables — Technological and Functional Properties

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Additional information is available at the end of the chapter

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1. Introduction

The relationship between food and health has been investigated for many years, and therefore, the development of foods that promote health and well-being is a key research priority of the food industry [1]. Fruits and vegetables are an essential part of human nutrition. Unfortunately, the daily intake of fruits and vegetables is estimated to be lower than the recommendation of the World Health Organization (WHO) [2], who suggest a dietary intake of 450 and 500 g of fruits and vegetables, respectively. Vegetables are strongly recommended in the human diet because they are rich in antioxidants, vitamins, dietary fibres and minerals. The majority of vegetables consumed in the human diet are fresh, minimally processed, pasteurised or cooked by boiling in water or microwaving, and vegetables can be canned, dried, or juiced or made into pastes, salads, sauces, or soups. Fresh vegetables or those that have been minimally processed have a particularly short shelf-life because they are subjected to rapid microbial spoilage. In addition, the above cooking processes can cause a number of potentially undesirable changes in physical characteristics and chemical composition [3,4].

Therefore, these drawbacks could be reduced by novel technologies, such as new packaging systems, high-hydrostatic pressure processing, ionisation radiation and pulsed electric fields [5-7]. The use of natural antimicrobial preservatives is considered to be the simplest and most valuable biological technique to keep and/or enhance the safety, nutrition, palatability and shelf-life of fruits and vegetables [5]. Lactic acid fermentation of vegetables, currently used as the bio-preservation method for the manufacture of finished and half-finished foods, is an important biotechnology for maintaining and/or improving safety, nutritional, sensory and

shelf-life properties of vegetables. Three technology options are usually considered for lactic acid fermentation of vegetable: spontaneous fermentation by autochthonous lactic acid bacteria, fermentation by starter cultures that are added into raw vegetables, and fermentation of mild heat-treated vegetables by starter cultures [18]. For thousands of years, microorganisms have been used to produce and preserve foods through the process of fermentation. Fermented foods have been adopted in various ways depending on the properties of the available raw materials and the desired features of the final products [8-10]. Food produced by traditional methods has become popular among consumers who know that their food is manufactured from high quality raw materials, without preservatives and other synthetic additives that are characterised by unique flavour values [11].

2. Fermentation from a biochemical point of view

Bourdichon et al. [12] describe the fermentation process as "a metabolic process of deriving energy from organic compounds without the involvement of an exogenous oxidising agent". Fermented foods are subjected to the actions of microorganisms or enzymes. Fermentation plays different roles in food processing, such that desirable biochemical changes have occurred [13]. The fermentation process is very important in the improvement of technological properties of preservation, such as a relative cost-effectiveness and low energy requirements, which are essential for ensuring the shelf-life and microbiological safety of the product [8]. The major roles of fermentation are considered to be the following:

1. preservation of food: the formation of inhibitory metabolites, such as organic acid (lactic acid, acetic acid, formic acid, propionic acid), ethanol, bacteriocins, etc., often in combination with a decrease in water activity (by drying or the use of salt) [14-15].
2. improving food safety through the inhibition of pathogens [16,17] or the removal of toxic compounds [18].
3. improving nutritional value: biological enrichment of food substrates with proteins, essential amino acids, essential fatty acids and vitamins [19,20].
4. organoleptic food quality: enrichment of the diet through the development of a diversity of flavours, aromas, and textures in food substrates [21-24].
5. decrease in cooking times and fuel requirements [25].

Interest in the biopreservation of food has created a demand for more natural and minimally processed food, with particular interest in naturally produced antimicrobial agents [26].

3. Lactic acid bacteria (LAB) in food fermentation and new natural antimicrobial compounds

LAB have traditionally been associated with food fermentation. LAB are generally considered beneficial microorganisms, with some strains even considered to promote good health

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(probiotic), and their extensive historical use contributes to their acceptance as being GRAS (generally recognised as safe) for human consumption [27]. LAB are used as natural or selected starters in food fermentation and exert health benefits through the antimicrobial effect produced from different metabolic processes (lactose metabolism, proteolytic enzymes, citrate uptake, bacteriophage resistance, bacteriocin production, polysaccharide biosynthesis, metal-ion resistance and antibiotic resistance) [28,29,9]. Spontaneous fermentation typically results from the competitive activity of a variety of autochthonous and contaminating microorganisms, which may lead to a high risk for failure. Both from a hygiene and safety perspective, the use of starter cultures is recommended, as it leads to rapid inhibition of spoilage and pathogenic bacteria while yielding processed fruit with consistent sensory and nutritional quality [30].

Interest in the biopreservation of food has prompted the quest for novel antimicrobial compounds from different natural origins. The LAB of genera such as *lactobacilli* and *lactococcus* are amongst the most important known members that have probiotic activity [31]; these bacteria produce antimicrobial peptides most frequently referred to as bacteriocins [32,33]. Bacteriocins ensure the stability of fermented plant products, reduce microbial contamination during fermentation, inhibit the growth of moulds and delay microbiological spoilage of baked goods [34].

LAB have strong inhibitory effects on the growth and toxin production of other bacteria. This activity can occur due to the following factors: competition for available nutrients; decrease in redox potential; production of lactic acid and acetic acid and the resulting decrease in pH; production of other inhibitory primary metabolites, such as hydrogen peroxide, carbon dioxide or diacetyl; and production of special antimicrobial compounds, such as bacteriocins and antibiotics [35].

Each of these properties, particularly when combined, can be used to extend the shelf-life and safety of food products [36].

Amongst the various technologies, lactic acid fermentation may be thought of as a simple and valuable biotechnology for maintaining and/or improving the safety, nutritional, sensory and shelf-life properties of fruits and vegetables [37,38]. Overall, LAB are a small part (2–4 $\log_{10}$ CFU g^{-1}) of the autochthonous microbiota of raw vegetables, and their cell density is mainly influenced by the vegetable species, temperature and harvesting conditions [37].

Interest in the use of LAB fermentation of vegetable products stems largely from the nutritional, physiological and hygienic aspects of the process and their corresponding implementation and production costs [39].

LAB fermentation represents the easiest and most suitable way to increase the daily consumption of nearly fresh fruits and vegetables.

4. Unique features of fermented fruits and vegetables

Buckenhuskes and colleagues [40] generally agreed that fermented plant products are the "food of the future". The following factors support this idea: products can be marked as

"natural" or "biological"; desirable flavour compounds are enhanced while negative flavour compounds (for example, glucosinolates) are destroyed; handling and storage (without cooling) is simple; easy methods exist for the pre-handling of raw material before further processing; desired metabolites (lactic acid, amino acids) are enriched; and the process results in the detoxification of pathogens [41]. Fruits and vegetables preserved using LAB with antimicrobial properties are perceived as suitable products for the human diet [42].

Dieticians and physicians recommend fermented fruits and vegetables due to the health-promoting properties of these foods. Fermented fruits and vegetables are low-calories foods because they contain considerably lower quantities of sugars compared to their raw counterparts. Fermented vegetables are a source of dietary fibre, which impedes the assimilation of fats and regulates peristalsis in the intestines; they are also a valuable source of vitamin C, B-group vitamins, phenolics and many other nutrients present in the raw material. Lactic acid may also lower gut pH, thereby inhibiting the development of putrefactive bacteria [42].

Many types of fermented fruit and vegetable products exist in the world: sauerkraut, cucumber pickles, and olives in the Western world; Egyptian pickled vegetables in the Middle East; and Indian pickled vegetables, Korean kim-chi, Thai pak-sian-don, Chinese hum-choy, Malaysian pickled vegetables and Malaysian tempoyak. Lactic acid-fermented cereals and tubers (cassava) include Mexican pozol, Ghanaian kenkey, Nigerian gari; boiled rice/raw shrimp/raw fish mixtures such as Philippine balao-balao and burong dalag; lactic-fermented/leavened breads such as sourdough breads in the Western world; Indian idli, dhokla, khaman and Sri Lankan hoppers; Ethiopian enjera, Sudanese kisra and Philippine puto; and Chinese sufu/tofu-ru [9,43].

Commercial distribution of these fermented products lags far behind that of fermented meat and dairy products due to a lack of standardised manufacturing protocols; in addition, their ingredients are subject to limiting and unpredictable weather and geographic conditions [44]. The lactic acid fermentation of vegetables currently has industrial significance only for cucumbers, cabbages and olives [45]. Several other varieties of vegetables cultivated mainly in Southern Italy or, more generally, in the Mediterranean area, such as carrots, French beans, marrows, artichokes, capers and eggplants, may benefit from increased safety, nutritional, sensory and shelf-life properties through standardised industrial lactic acid fermentation [46].

5. The use of microorganisms in our diet opens new opportunities

Either as traditional fermented foods or as novel approaches, the rationalised use of microorganisms in our diet could reveal new opportunities. Low dietary quality is an important factor that limits adequate nutrition in many resource-poor settings. Bioavailability is a key aspect of dietary quality with respect to the adequacy of micronutrient intake [47]. Prebiotic food ingredients encourage the growth of probiotic bacteria. The appropriate combination of prebiotics and probiotics manifest in a higher potential for synergistic effects 48]. Probiotic foods are fermented products that contain a sufficient number of a certain live microorganism to favourably modify the intestinal microbiota of the host [49]. Recently developed probiotics

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tend to be milk-based, although in recent years other substrates have been explored for new probiotic formulations. Amongst these substrates, cereals are becoming one of the most promising alternatives to milk due to their ability to support the growth of probiotic bacteria and their protection against bile resistance [50].

According to Kim et al. [51], cabbage (including the Chinese cabbage), pH-adjusted tomato (pH 7.2), carrot and spinach media give relatively higher fermentability than other vegetables because they have more fermentable saccharides. The tomato (*Lycopersicon esculentum* L.) is one of the most popular and extensively consumed vegetable crops worldwide. The nutritional significance of lycopene, a carotenoid with potent antioxidant activity, has been reported, and accumulating evidence has shown an inverse correlation between the consumption of tomato products rich in lycopene and the risk of several types of cancer and cardiovascular disease [52-54]. Approximately 90 % of the lycopene in dietary sources is found in the linear, all-*trans* conformation, while human tissues mainly contain *cis*-isomers. It has been suggested that the *cis*-isomers of lycopene are better absorbed than the all-*trans* form because they are shorter, have greater solubility in mixed micelles, and have a lower tendency to aggregate [55]. Studies have shown that lycopene levels in plasma increase only after the consumption of red tomato paste and purified lycopene [54]. It has also been revealed that the absorption of lycopene is greater from processed tomatoes than from fresh tomatoes because processing breaks down the tomato cell matrix and makes the lycopene more available [56,57].

The red colour of tomatoes is a result of the degradation of chlorophylls and the increased biosynthesis of carotenoids [58]; thus, a tomato's colour is related to its maturity and post-harvest treatment. Colour is therefore an important attribute indicating the quality of tomato fruit, and it is used in the food industry to predict the colour of finished products. Additionally, the application of instrumental colour measurement to objectively define the colour of tomatoes is an important research topic [59,60]. It was reported that the colour coordinates of a product could relate to its concentration of lycopene and other carotenoids [61,62].

There is an increasing consumer demand for high quality meat products that taste good and are both nutritious and easy to prepare. The diverse nutrient composition of meat makes it an ideal environment for the growth and propagation of meat spoilage microorganisms and common food-borne pathogens. It is therefore essential to apply adequate preservation techniques to maintain its safety and quality [63]. The processes used in meat preservation are principally concerned with inhibiting microbial spoilage, although other methods of preservation seek to minimise additional deteriorative changes in colour and oxidation [64]. The most investigated new preservation technologies for fresh meat involve non-thermal inactivation, such as high hydrostatic pressure (HHP), novel packaging systems, including modified atmosphere packaging (MAP) and active packaging (AP), natural antimicrobial compounds and biopreservation. Storage life is extended and safety is increased by using natural or controlled microflora, including the extensively studied LAB and their antimicrobial products, such as lactic acid and bacteriocins. Bacteriocins are a heterogeneous group of antibacterial proteins that vary in their spectrum of activity, mode of action, molecular weight, genetic origin and biochemical properties [65].

The destruction of the total BLIS (bacteriocin-like inhibitory substances) activity after treatment with proteinase K, trypsin, pepsin and chymotrypsin indicates that antimicrobial

substances produced by the tested LAB possess a proteinaceous nature. They might be bacteriocins because protease sensitivity is a key criterion in the classification of antimicrobial substances as BLIS [66]. In our previous studies, BLIS produced by *Lactobacillus sakei* KTU05-6 and *P. pentosaceus* KTU05-9 were designated as sakacin 05-6 and pediocin 05-9 [67]. We proposed that due to their broad inhibition spectrum, the presence of BLIS and organic acids in tested LAB is an indication that these bacteria can be used widely in the food industry as bio-preservatives.

Consumer interest for diverse fermented foods has increased in recent years because of the positive perception of their beneficial impact on health. Hence, there is an evident need to find novel methods and new food preservation agents from natural origins. Biopreservation refers to extending the shelf-life and enhancing the safety of foods using microorganisms or their metabolites [68]. In this aspect, LAB are very good candidates [69].

The food matrices in vegetables offer promising potential as sources and carriers of probiotic strains [70]. Vegetables are fundamental sources of water-soluble vitamins (vitamin C and group B vitamins), provitamin A, phytosterols, dietary fibres, minerals and phytochemicals [71] in the human diet. LAB are a small part (2.0–4.0 $\log_{10}$ CFU g^{-1}) of the autochthonous microbiota of raw vegetables [37]. Under favourable conditions of anaerobiosis, water activity, salt concentration and temperature, raw fruits and vegetables may be subject to spontaneous lactic acid fermentation. In some cases, alcoholic fermentation takes place concomitantly [72].

Tomatoes are a rich source of a variety of nutritional compounds, especially key antioxidant components, such as the carotenoid lycopene, vitamin C, and a range of polyphenols. The possible protective characteristics of these antioxidants are of great interest, and consumers have already become aware of their potential importance. A survey of the literature revealed that a great deal of research has been conducted on the biochemical composition of tomatoes and their products [73]. Lycopene, a natural carotenoid found in tomatoes, has been reported to possess various health benefits, such as preventive properties against cardiovascular disease and cancer [74].

6. Lactic acid fermentation of tomatoes: effects on *cis*/*trans* lycopene isomers, β-carotene concentration and the formation of L(+) and D(-)-lactic acid

The production of L-lactic acid and D-lactic acid isomers during the fermentation of different tomato varieties (var. Ronaldo and var. Cunero) by the bacteriocin-producing LAB *Lactobacillus* and *Pediococcus* spp. have been investigated. The influence of lacto fermentation on the lycopene and β-carotene contents and their relation to the colour characteristics of fermented tomato products were also investigated [75]. Tomato var. Cunero and Ronaldo, the LAB strains were used in this investigation. Tomato var. Cunero and Ronaldo were obtained from the Lithuanian Institute of Horticulture (Babtai, Lithuania) harvested in 2011. Pure cultures of *Lactobaccilus sakei KTU05-6*, *Pediococcus acidilactici KTU05-7* and *Pediococcus pentosaceus KTU05-8*, characterized as a bacteriocin producing strains [76] are from collection of Kaunas University of Technology (Kaunas, Lithuania) [75].

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The LAB strains were propagated in nutrition media (moisture content 72 %), prepared by mixing extruded rice flour (100 g) and tap water. After addition of pure LAB cell suspension (5 g, 10.2 $\log_{10}$ colony-forming units (CFU) g^{-1}) the mixture was incubated at optimal temperatures (30 °C for *L. sakei*, 32 °C for *P. acidilactici* and 35 °C for *P. pentosaceus*) for 24 h. For comparison purpose control product was prepared using spontaneous fermentation of rice flour without bacterial inoculum at 30 °C for 48 h. Enumeration of LAB was carried out by plating the diluted samples onto MRS agar at 30 °C for 48 hours. Products obtained after propagation of individual LAB in rice media were used for fermentation of tomato pulp [75].

A rapid and specific Megazyme assay kit for simultaneous determination of L- and D-lactic acid (Megazyme Int., Bray, Ireland) in foods was used as reported by De Lima et al. [77] in this investigation. Extraction of carotenoids and carotenoid analysis by Reverse Phase Liquid Chromatography (RP-HPLC) were used [75] and the colour characteristics of fermented and untreated tomato pulp were evaluated of the surface using CIEL*a*b* [78].

6.1. The effect of selected fermentation media on LAB viability

As reported in the literature, the behaviour of different LAB depends on substrate composition, where bacteria in different substrates are able to produce different metabolites or increased biomass [79]. For maximum health benefits, it is important to have a significant number of viable LAB present in the probiotic product [80].

Extruded rice flour, a current product of the cereal processing industry, was found to show good fermentability. Counts of viable bacteria cells were measured between 6.62 and 8.50 $\log_{10}$ CFU g^{-1} after 48 h of analysed LAB cultivation in selected media (Table 1) [75]. The lowest biomass of bacteria was found in the spontaneously fermented rice media (5.57 $\log_{10}$ CFU g^{-1}). According to the obtained results, rice flour is a suitable medium for LAB cultivation to produce a functional food while most likely maintaining the other functional properties of rice. These results are in agreement with Trachoo et al. [81], who showed a biomass increase of lactobacilli over 2.5 $\log_{10}$ CFU mL^{-1} during 24 h in a germinated rice broth [75].

Samples	Extruded rice			Tomato products		
	LAB count	pH	TTA	LAB count	pH	TTA
P.p.	8.51±0.05d	3.37±0.01a	8.2±0.2b	6.61±0.03c	3.50±0.01a	6.4±0.3b
P.a.	6.62±0.03b	3.40±0.01a	8.2±0.2b	4.54±0.04b	3.71±0.01b	6.8±0.2c
L.s.	7.75±0.03c	3.42±0.01a	8.3±0.2b	4.83±0.03b	3.70±0.01b	7.1±0.2d
SF	5.57±0.02a	3.73±0.01b	7.2±0.2a	2.83±0.02a	3.92±0.01c	5.6±0.2a

The numbers are means followed by standard deviations (n = 3).

Means within a column with different superscript letters are significantly different ($p < 0.05$).

Samples: tomato products fermented with: P.p. – *P. pentosaceus,* P.a. – *P. acidilactici,* L. s. – *L. sakei;* SF – spontaneous fermented.

Table 1. The influence of fermentation media on LAB cell counts ($\log_{10}$ CFU g^{-1}), pH and TTA values.

L. sakei, *P. acidilactici* and *P. pentosaceus* were found to be capable of sufficient rapid utilisation of tomato pulp for cell synthesis and organic acid production. They reduced the pH to 3.5–3.7 and increased the TTA to as high as 6.4. The viable cell counts reached 6.61 $\log_{10}$ CFU g^{-1} after 48 h of fermentation. In either case, tomato products treated with spontaneous fermentation had pH values that were higher by 7.2% and TTA values that were lower by 17.3 % than products treated with lactofermentation (Table 1) [75].

Acid production depends on the concentration of viable bacteria able to utilise the available carbohydrate sources in the substrate [82]. The viable LAB cells in the fermented tomato products were found to be lower on average by 30% (lactofermentation) or 49.2% (spontaneous fermentation) compared to the rice media (Table 1); however, three LAB counts measured after 48 h of fermentation varied between 4.54 and 6.61 $\log_{10}$ CFU g^{-1}. To achieve health benefits, probiotic bacteria must be viable and available at a high concentration, typically approximately 6 $\log_{10}$ CFU g^{-1} of product [80]. According to Sindhu and Khetarpaul [83], probiotic fermentation of indigenous food mixtures containing tomato pulp increases the acidity and improves the digestibility of starch and protein. Our results support the hypothesis that rice media contain the essential nutrients to support the growth of lactobacilli and can be directly used as a fermentation substrate of LAB. The obtained biomass levels are above the minimum required for a probiotic formulation.

Classic lactic acid vegetable fermentation is a microbial process that involves heterofermentative and homofermentative LAB, generally *Lactobacillus* and *Pediococcus* [82]. At a pH between 3.5 and 3.8, vegetables will be preserved for a long period of time [83]. Tomatoes treated by lactofermentation could be recommended as useful and safe products for human nutrition. Furthermore, fermented tomatoes could serve as a healthy product for vegetarians and consumers who are allergic to dairy products [75].

6.2. The production of L- and D-lactic acid during lactofermentation of tomato pulp

Our results showed that all the analysed LAB produced a mixture of L- and D-lactic acid (Figure 1), and the highest amounts of each form were determined in tomato products treated by spontaneous fermentation (7.18±0.03 and 7.67±0.11 mg/100 g, respectively). As reported by Hartman [85] and Li and Cui [86], *Lactobacilli amylophilus, L. bavaricus L. casei*, *L. maltaromicus*, and *L. salivarius* predominantly yield the L-isomer. Strains such as *L. delbrueckii*, *L. jensenii*, and *L. acidophilus* yield D-lactic acid or mixtures of both forms. LAB such as *L. pentosus*, *L. brevis* and *L. lactis* can ferment glucose into lactic acid through homolactic fermentation. The fermentation of rice with two strains of *L. delbrueckii* yielded 3.23 and 5.04 mg/100 g of D-lactic acid [87].

The concentration of D-lactic acid in fermented tomato products was measured between 4.05±0.05 and 6.34±0.04 mg/100 g, and the concentration of L-lactic acid ranged from 4.26±0.04 to 7.19±0.08 mg/100 g (Figure 1). The results of our study indicate that compared to spontaneous fermentation, the use of *P. pentosaceus* allowed a reduction in the content of D-lactic acid in tomato products by 11.8% (Figure 1). Fermentation with *P. acidilactici* and *L. sakei* reduced the content of the latter isomer at a higher level (on average by 40.6%).

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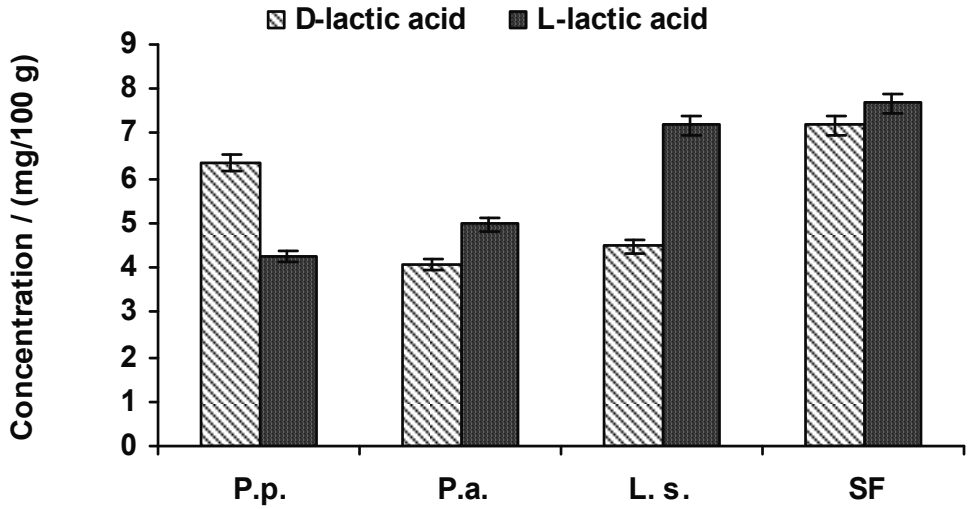

Figure 1. Concentrations of L- and D-lactic acid in fermented tomato products. Samples: fermented with LAB: P.p. – *P. pentosaceus*, P.a. – *P. acidilactici*, L.s. – *L. sakei*; SF – spontaneous fermented

In summary, *P. pentosaceus* can produce D-rich lactic acid (L/D ratio 0.64), while the other strain, *L. sakei*, produces L-rich lactic acid (L/D ratio 1.61). Fermentation with *P. acidilactici* and spontaneous fermentation gave almost equal amounts of both lactic acid isomers (L/D ratio 1.17 and 1.07, respectively).

By evaluating our knowledge of the potential toxicity of D-lactic acid in terms of nutrition, we can report that tomato products prepared using a pure culture of LAB were found in all cases to be safer than those treated with spontaneous fermentation. The level of D-lactic acid in pure LAB-fermented tomato products was significantly lower (p<0.05) than that in those spontaneously fermented (Figure 1). Based on these results, *L. sakei KTU05-6* could be selected as the L-lactic acid bacteria and is recommended for the fermentation of tomatoes [75].

6.3. Trans/cis lycopene and β-carotene contents in fermented tomato products

The results from our analysis of lycopene and β-carotene contents in fermented tomato products are presented in Figure 2. The highest concentration of total carotenoids (on average 6.83 mg/100 g) were measured in a var. Cunero sample fermented with *P. pentosaceus* and in a var. Ronaldo sample fermented with *L. sakei*. However, fermentation with the latter bacteria increased the total level of carotenoids by 41.1 and 33.6%, respectively, compared to untreated samples. Compared to untreated tomatoes, fermentation with *P. acidilactici* reduced the concentration of total carotenoids by 3.6% in the samples of var. Cunero and var. Ronaldo (3.96 and 4.61 mg/100 g, respectively), which was accompanied by a reduction in β-carotene content (Figure 2) [75].

On average, the fermented tomato samples of var. Cunero had 24.7 % lower β-carotene and 11.5% higher lycopene content compared to untreated tomatoes. In contrast, the β-carotene

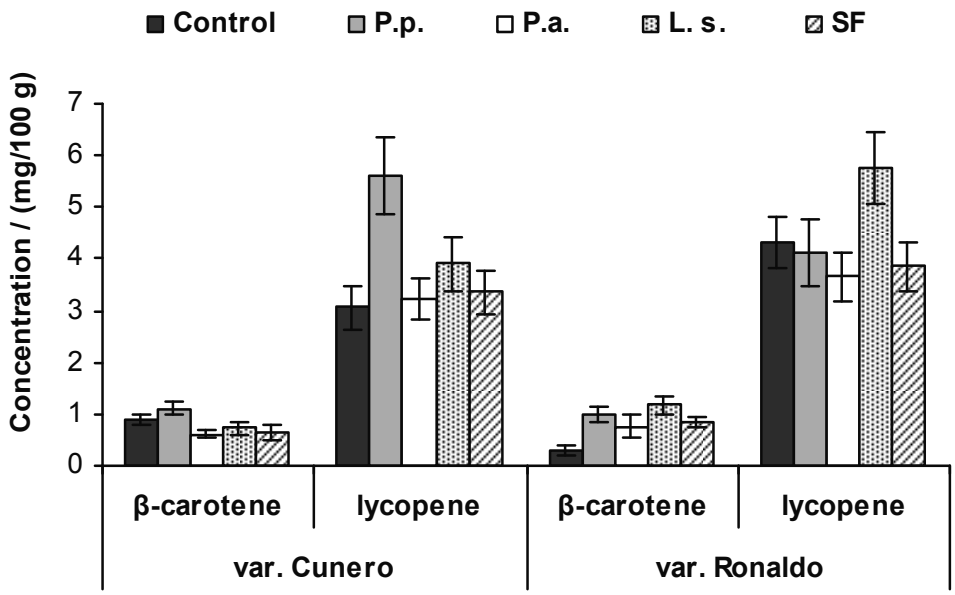

Figure 2. Carotenoid contents in untreated and fermented with different LAB tomato products. Samples: Control – untreated tomato pulp; tomato pulp fermented with: P.p. – *P. pentosaceus*; P.a. – *P. acidilactici* MI807; L.s. – *L. sakei*, SF – spontaneous fermented.

concentrations in all the fermented tomato products of var. Ronaldo were generally higher, with an average increase of 69.4% compared to untreated tomatoes (Figure 2) [75].

A 24.8% increase in lycopene content was reached in the var. Ronaldo samples after fermentation with *L. sakei.* Spontaneous fermentation or treatment by *P. pentosaceus* reduced the concentration of lycopene by 11.0 and 4.4%, respectively, compared to the control sample (Figure 2).

According to these results, lactic acid fermentation generally had a positive effect on the lycopene and total carotenoid contents of the fermented tomato products. The β-carotene contents were influenced not only by which LAB was used but also by the variety of tomato. As reported in the literature, compositional variation of lycopene in tomatoes occurs as a consequence of varietal differences, climate conditions, agricultural variables, stage of maturity, harvesting and post-harvest handling and conditions during storage [75]. Other researchers reported lycopene values within the range of 3.1–7.7 mg/100 g for different tomato cultivars [88]. However, Camara et al. [89] reported a lycopene concentration of 6–15 mg/100 g for whole fresh tomato fruit [89], which is higher than the results of this investigation. Lycopene content may be directly affected by the pH of the fruit, as the low pH of red tomatoes accumulates more lycopene [90].

Our analysis of all-*trans* and *cis*-lycopene showed that the amounts of both isomers depended significantly on the tomato variety and were slightly affected by the LAB strain used for fermentation (Figure 3). The fermented tomato products of var. Ronaldo had all-*trans*- and *cis*-

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lycopene contents that were higher on average by 25.9 and 62.6%, respectively, compared to the tomato products of var. Cunero [75].

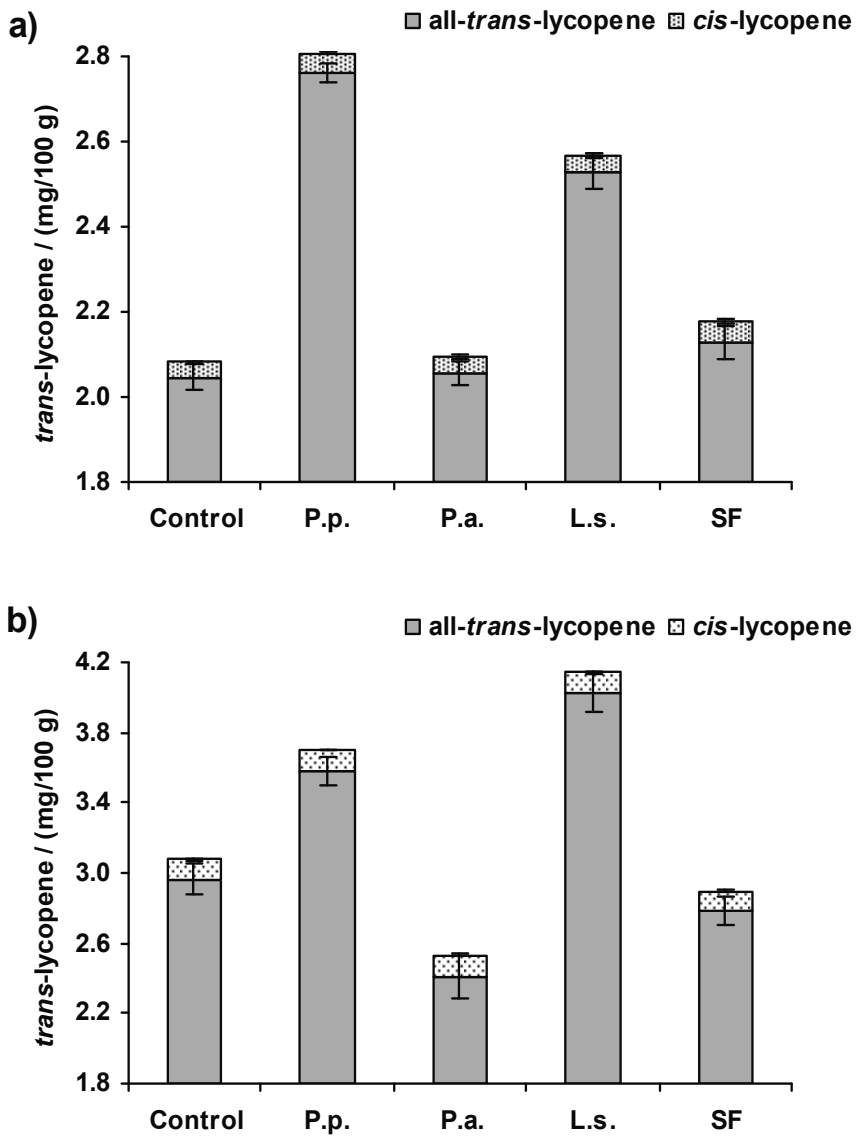

Figure 3. Content of all-*trans*- and *cis*-lycopene in fermented tomato of var. Cunero (a) and var. Ronaldo (b) products. Samples: Control – untreated tomato pulp; tomato products fermented with: P.p. – *P. pentosaceus*, P.a. – *P. acidilactici*, L. s. – *L. sakei*; SF – spontaneous fermented.

The control samples of var. Ronaldo had 3.3-fold higher *cis*-lycopene (3.4 mg/kg) compared to var. Cunero (11.3 mg/kg) (Fig. 3). Fermentation by *P. pentosaceus* or *L. sakei* increased the *cis*-lycopene contents on average by 30.6 and 8.5%, respectively in the products of var. Cunero and var. Ronaldo. A lower increase in *cis*-lycopene was noticed during fermentation of the var. Cunero tomatoes with *P. acidilactici* as well as during spontaneous fermentation (an average increase of 9%). Similarly, lactofermentation using *P. acidilactici* and *L. sakei* increased the *cis*-lycopene contents by 5.8% on average in the tomato products [75].

The fermentation of var. Cunero and var. Ronaldo tomatoes by *P. pentosaceus* and *L. sakei* produced an average of 22.2% more all-*trans*-lycopene compared to the controls (204.6 and 296.0 mg/kg, respectively) (Figure 3) [75].

The *cis*/*trans* ratio of var. Cunero and var. Ronaldo tomatoes were 1.67 and 3.81, respectively. The highest *cis*/*trans* ratio was found in the var. Cunero samples fermented by *L. sakei* (2.08), following that of var. Ronaldo samples fermented by *P. acidilactici* (4.90) and spontaneous fermentation (4.09) [75].

It is known from the literature that in human subjects, lycopene from *cis*-isomer-rich tomato sauce is more bioavailable than that from all-*trans*-rich tomato sauce [91]. Because of the positive effect of lactofermentation on the *cis*/*trans* lycopene ratio, fermented products of the var. Ronaldo tomato, fermented with *P. acidilactici* or *L. sakei*, could be recommended as more biologically accessible products with greater functional value.

6.4. Colour characteristics of fermented tomato products

The results from our analysis of the red (a*) and yellow (b*) colour coordinates of fermented tomato products are presented in Table 2. No relation was found in the var. Cunero samples between the yellow colour coordinate (b*) and total carotenoid, lycopene or β-carotene contents ($p > 0.05$) (Table 2). However, the red colour coordinate (a*) slightly correlated ($R^2 = 0.672$) with β-carotene content.

In contrast, a weak relation was noticed between colour coordinate b* of var. Ronaldo and total carotenoid or β-carotene contents ($R^2 = 0.581$ or $R^2 = 0.596$, respectively) (Table 2). In addition, samples of this variety showed a strong relation between colour coordinate b* and lycopene content ($R^2 = 0.825$, $p = 0.03$). No significant relations were observed between a* and β-carotene or lycopene contents ($p > 0.05$) (Table 2) or between total carotenoids and the colour tone (h^o) or colour purity (C) values of the var. Cunero and var. Ronaldo samples (Table 3) [75].

The best estimation for β-carotene content was obtained using the b* chromaticity value from the whole fruit measurements or the transformed a^{*2} value from the pure measurements [91]. Neither model, however, could explain more than 55% of the variation in β-carotene levels, suggesting that chromaticity values may not be appropriate for estimating tomato β-carotene content. It has been stated that the inspection of different chromaticity values and regression models suggest that colorimeter readings may not be highly useful for estimating β-carotene content in the tomato fruit [92].

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Samples	var. Cunero			var. Ronaldo		
	a*	b*	a*/b*	a*	b*	a*/b*
K	13.97±1.3c	15.47±0.8a	0.903	13.84±0.9c	16.71±1.1b	0.828
P.p.	14.57±1.1d	16.46±1.3b	0.885	15.15±0.8e	18.28±0.9c	0.829
P.a.	11.41±0.9a	17.29±1.3bc	0.660	13.38±1.1b	19.37±1.3d	0.691
L. s.	13.03±1.1b	15.09±1.3a	0.864	13.85±0.5c	15.54±0.7a	0.891
SP	13.44±1.3b	19.13±1.4d	0.703	14.26±1.2d	19.54±1.3d	0.730
a; b and a/b correlation with total carotenoid content						
^{R}	0.3905	0.04763	0.2673	0.001697	0.5808	0.5985
p	0.2597	0.7243	0.3723	0.9476	0.1342	0.1248
a; b and a/b correlation with lycopene content						
R^2	0.3186	0.02779	0.1973	0.004398	0.8248	0.7373
p	0.3215	0.7887	0.4537	0.9156	0.0329	0.0624
a; b and a/b correlation with β-carotene content						
^{R}	0.6718	0.1955	0.6326	0.001697	0.5808	0.5985
p	0.0894	0.4560	0.1077	0.9476	0.1342	0.1248

The numbers are means followed by standard deviations (n = 3). Means within a column with different superscript letters are significantly different ($p < 0.05$).

Samples: control – untreated tomato pulp; tomato products fermented with: P.p. – *P. pentosaceus,* P.a. – *P. acidilactici L.,* s. – *L. sakei;* SF – spontaneous fermented; R^2 – correlation coeficient.

Table 2. Colour coordinates (a*, b*) of tomato var. Cunero and var. Ronaldo samples and their correlations between total carotenoids, lycopene and β-carotene contents

Samples	var. Cunero		var. Ronaldo	
	C	h^o	C	h^o
Control	22.01±2.3b	47.03±3.1a	21.97±2.4b	46.85±2.4a
P.p.	21.93±1.5b	48.34±3.2bc	23.75±1.9c	50.11±1.9c
P.a.	20.77±1.7a	56.60±2.7de	23.44±1.8c	55.30±2.9d
L.s.	20.04±1.3a	49.46±2.4c	20.99±1.6ab	48.52±1.4b
SP	23.24±2.1c	55.20±1.7d	24.35±1.3cd	53.66±2.3d
Correlation with total caratenoid content				
^{R}	0.00000565	0.2332	0.4974	0.2043
p	0.9970	0.4099	0.1834	0.4448

The numbers are means followed by standard deviations (n = 3). Means within a column with different superscript letters are significantly different ($p < 0.05$).

Samples: control – untreated tomato pulp; tomato products fermented with: P.p. – *P. pentosaceus,* P.a. – *P. acidilactici L.,* s. – *L. sakei;* SF – spontaneous fermented; R^2 – correlation coeficient.

Table 3. Colour tone (h^o) and purity (C) of tomato var. Cunero and var. Ronaldo samples and their correlation with total carotenoid contents

The overall results indicate that lycopene content could be measured simply and quite accurately across a wide range of tomato genotypes using chromaticity values taken from fruit puree [91]. In contrast, Liu et al. [93] reported that treating tomatoes with a daily light treatment enhances exocarp lycopene accumulation with minimal effect on the colour. Arias et al. [59] also observed that the b* characteristic was not appropriate for predicting the lycopene content of tomatoes.

According to the obtained results, colour tone (h^o) and purity (C) are not suitable indicators of the total carotenoid content in the evaluation of tomato products. We postulate that measuring the yellow coordinate (b*) could be a simple and non-destructive method for predicting lycopene concentration in tomato products [94].

7. The use of tomato additives fermented with *Pediococcus pentosaceus* KTU05-9 and *Lactobacillus sakei* KTU05-6 to improve the quality of ready-to-cook minced meat products

The influence of lactic acid fermentation with BLIS-producing lactobacilli (*Pediococcus pentosaceus* KTU05-9, *Lactobacillus sakei* KTU05-6) on the parameters of tomato powder and the impact of fermented tomato products on the acceptability, colour characteristics and carotenoid content of ready-to-cook minced pork meat products (RCMP) have been investigated [95]. In this experiment used tomato powder was obtained from "Obipectin AG" (Bischofszell, Switzerland). The lactic acid bacteria (LAB) P. pentosaceus KTU05-9 and Lactobacillus sakei KTU05-6, previously isolated from spontaneous rye sourdoughs [96] revealed antimicrobial activity against undesirable microorganisms in the food industry by producing organic acids and BLIS [97,98,67] were used for tomato powder fermentation. The LAB were stored at –70 °C and cultured at temperatures of 35 °C (KTU05-9) or 30 °C (KTU05-6) for 48 h in MRS broth (CM0359, Oxoid Ltd, Hampshire, UK) supplemented with 40 mmol L^{-1} fructose and 20 mmol L^{-1} maltose. Solid state fermentation of tomato powder was used [95].

The cell growth results observed at 48 h of fermentation in tomato media are presented in Figure 4. We found the highest amount of LAB in samples treated with *L. sakei* (8.15 $\log_{10}$ CFU g^{-1}). The spontaneously fermented samples yielded 6.69 $\log_{10}$ CFU g^{-1} of LAB. The lowest amount of LAB was found in samples fermented with *P. pentosaceus* (4.58 $\log_{10}$ CFU g^{-1}).

Different substrates may affect microorganism growth and metabolism [99]. High viable counts are necessary to obtain the desired acid production and pH reduction, which affects the organoleptic properties and shelf-life of the products while preventing contamination. However, the success of fermented products does not rely solely on the ability to provide enough LAB cells; in addition, the consumer must find these organoleptic properties acceptable, which is related in many cases to the organic acid content. We found the lowest pH after 48 h of fermentation in samples fermented with *P. pentosaceus* (pH = 4.1) (Figure 5). Samples fermented with *L. sakei* or through spontaneous fermentation had a pH of 4.16 [95].

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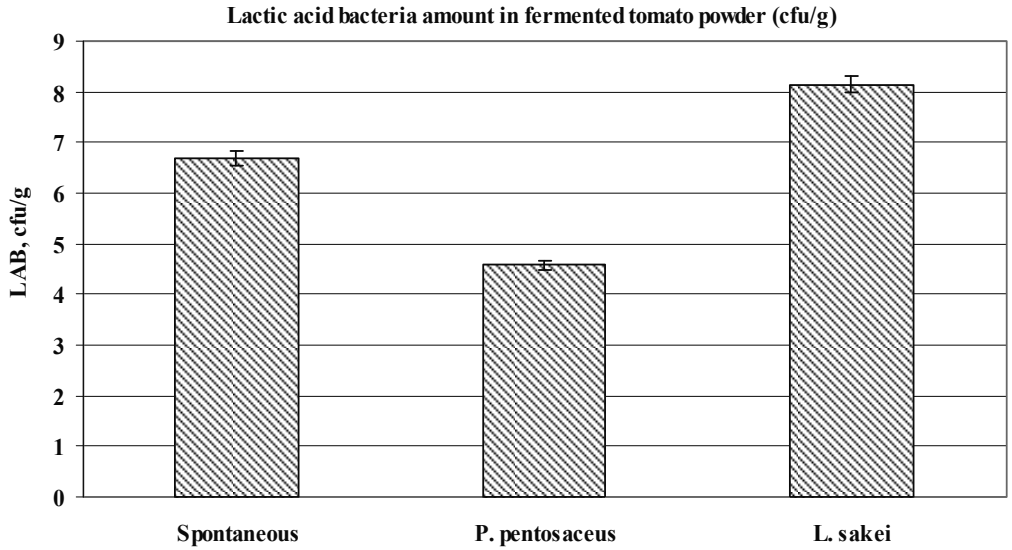

Figure 4. Lactic acid bacteria (LAB) amount (cfu/g) in fermented tomato powder (Samples: Spontaneous – tomato powder fermented spontaneous; P. pentosaceus - tomato powder fermented with *P. pentosaceus;* L. sakei – tomato powder fermented with *L. sakei*; $p < 0.05$).

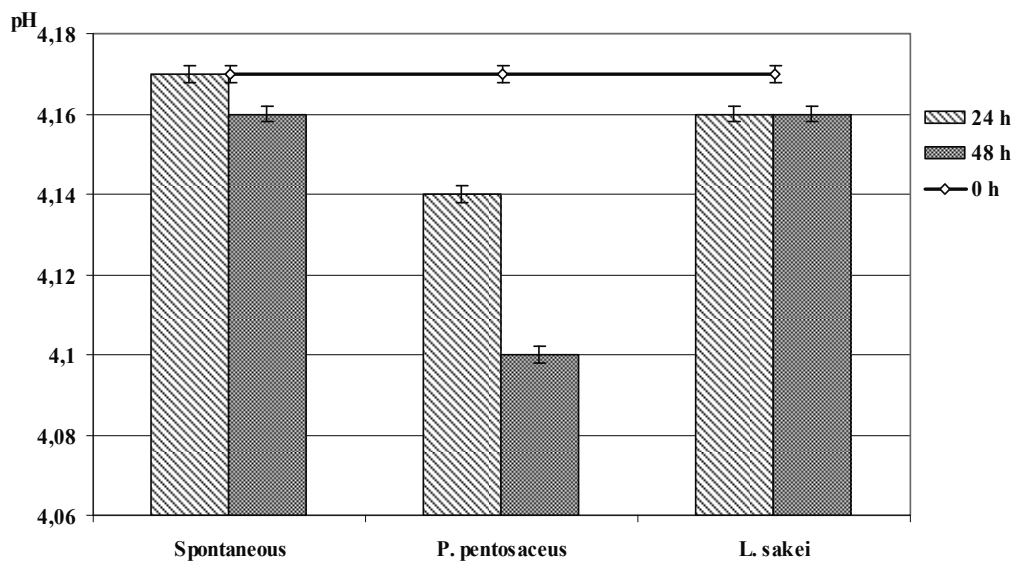

Figure 5. pH of fermented tomato powder (Samples: Spontaneous – tomato powder fermented spontaneous; P. pentosaceus - tomato powder fermented with *P. pentosaceus;* L. sakei – tomato powder fermented with *L. sakei*).

7.1. Colour parameter relation with carotenoid content in fermented tomato products

By influencing consumer choice and preferences, colour is an important quality attribute in the food and bioprocessing industries. Food colour is governed by the chemical, biochemical, microbial and physical changes that occur during growth, maturation, post-harvest handling and processing. Measuring the colour of food products has been used as an indirect measure of other quality attributes, such as flavour and pigment contents, because it is simple, fast and correlates well with other physicochemical properties [100]. We found that fermentation influenced the colour characteristics and carotenoid content of tomato products (Figure 6) [95]. The highest concentration of carotenoids was found in samples fermented with LAB starters (*P. pentosaceus, L. sakei*). Spontaneous fermentation also increased the content of carotenoids in the tomato samples, but not as effectively (the total carotenoid content in the spontaneously treated samples was 54.78 mg/100 g). A strong and significant relation was found between colour tone (ho) and lycopene content and between colour tone (h°) and total carotenoid content ($R^2 = 0.9045$; $p = 0.0489$ and $R^2 = 0.9035$; $p = 0.0495$, respectively). We found correlations ranging from 0.8922 to 0.5091 between others colour characteristics and β-carotene, lycopene and total carotenoid content, but they were not significant [101].

The beneficial effects of lycopene on health have been reviewed [102-104]. According to our results, fermentation with *L. sakei* and *P. pentosaceus* increases the carotenoid concentration in tomato products by two-fold. We did not study the effects on tomato product fermentation using different LAB starters, and more research is needed to explain the mechanism of increasing carotenoids in fermented tomato products [95].

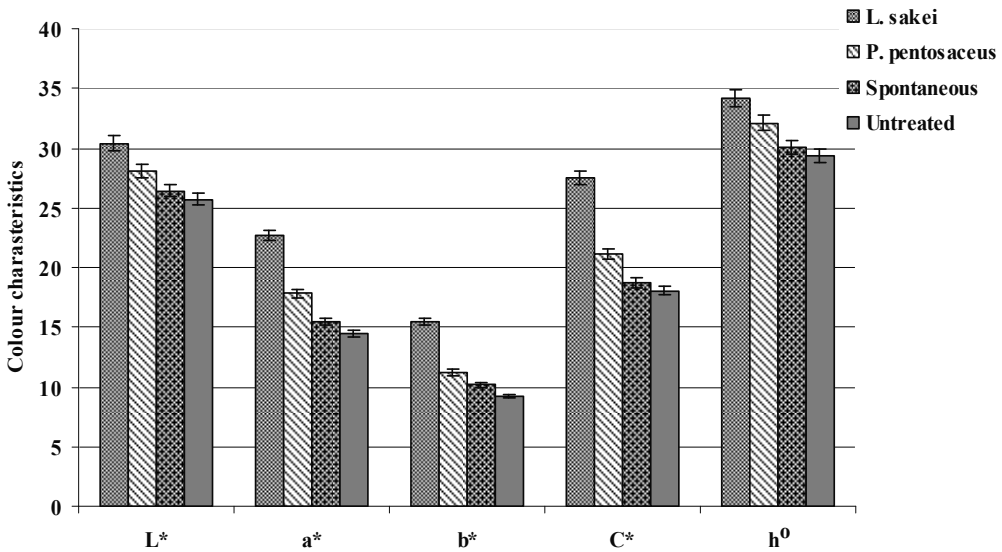

Figure 6. Colour characteristics and carotenoids content (mg/100 g) of fermented and untreated tomato products (Samples: Untreated – untreated tomato powder; Spontaneous – tomato powder fermented spontaneous; P. pentosaceus - tomato powder fermented with *P. pentosaceus*; L. sakei – tomato powder fermented with *L. sakei*; $p < 0.05$).

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7.2. The influence of fermented tomato additives on the acceptability of Ready-to-cook Minced Meat Products (RCMP)

We found significant differences in the acceptability of RCMP with and without 10 or 30 % tomato powder treated with different LAB (*L. sakei* KTU05-06, *P. pentosaceus* KTU05-09) or spontaneous fermentation (Figure 7) [95]. The highest RCMP acceptability was found with 10 % L. sakei fermented tomato powder (an average score of 9.38). Control samples (without additives) were found to be less acceptable (an average score of 5.86) compared to samples with 10 % fermented tomato additives. Ready-to-cook minced pork meat products with 30 % additive were found to be less acceptable than samples with 10 % additive. Compared to samples with 30 % additive, the most acceptable samples were those without fermented tomato products (an average score of 7.72) [95].

7.3. The influence of fermented tomato additives on the colour characteristics of Ready-to-cook Minced Meat Products (RCMP), and the influence of carotenoid content on thermal-treated and untreated RCMP

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) previously endorsed the use of lycopene (both natural and synthetic) as a food colour at its eighth, eighteenth, and twenty-first meetings [106-108] but was not able to establish an Acceptable Daily Intake (ADI) due to the limited information available. At its sixty-seventh meeting, JECFA agreed that both synthetic lycopene and lycopene extracted from *Blakeslea trispora* are acceptable as food colours and established a group ADI of 0-0.5 mg/kg bw/day for both preparations [109]. Adding tomato, tomato products or lycopene to meat could lead to products with health benefits. Few studies have been reported regarding the use of tomato products or lycopene in meat products. Candogan [110] reported on the use of tomato paste in beef patties, while Deda, Bloukas, and Fista [111] investigated its use in frankfurters. Calvo et al. [112] reported on the use of lycopene from tomato peel in dry fermented sausages. However, we could not find data on tomato product fermentation with different LAB starters or how fermented tomato products influence RCMP quality parameters [95].

We found that the addition of tomato products significantly affected ($p < 0.05$) all colour parameters (Table 4) of the final product (thermal-treated and untreated). The controls had the highest ($p < 0.05$) lightness and the lowest ($p < 0.05$) redness and yellowness as a consequence of lower hue angle and saturation index. These tendencies were found for both thermal-treated and untreated products [95].

High variation in the colour parameters of fermented meat products has been reported [113-115]. These variations could be due to the calibration plate used in the determinations, the composition of the meat products, the size of the meat particles and the ripening time.

Furthermore, the addition of tomato products affects the carotenoid content of RCMP (Table 5) [95]. We found that thermal treatment decreases the carotenoid concentration in RCMP. After thermal treatment, we found 23.71 and 52.03 % less ß-carotene (in samples with 10 % spontaneously treated products and in samples with 30 % L. sakei-fermented tomato products, respectively). Additionally, 10.78 and 50.00 % less lycopene was found in samples with 30 %

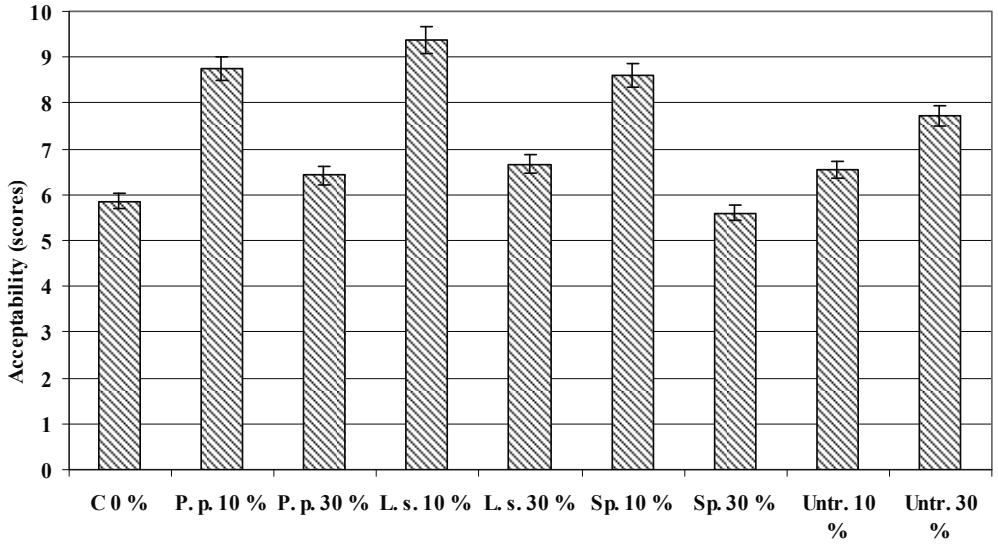

Figure 7. Acceptability of ready-to- cook minced meat products (RCMP) (Samples: C0 % - RCMP without tomato products; P.p. 10 % - RCMP with 10 % with P. pentosaceus fermented tomato products; P.p. 30 % - RCMP with 30 % with P. pentosaceus fermented tomato products; L.s. 10 % - RCMP with 10 % with L. sakei fermented tomato products; L.s. 30 % - RCMP with 30 % with L. sakei fermented tomato products; Sp. 10 % - RCMP with 10 % spontaneous fermented tomato products; Sp. 30 % - RCMP with 30 % spontaneous fermented tomato products; Untr. 10 % - RCMP with 10 % untreated tomato powder; Untr. 30 % - RCMP with 30 % untreated tomato powder; $p > 0.05$)

spontaneously treated products and in samples with 30 % untreated tomato products, respectively, and as a consequence, the highest loss of total carotenoid content was found in samples with 30 % untreated tomato product (49.25 %) [95].

RCMP samples	L*	a*	b*	C	h°
Thermal treated					
P. p. 30 %	45.64±0.11^{a}	19.09±0.21^{a}	28.16±0.69^{a}	34.02±0.71^{a}	55.87±0.96^{a}
P. p. 10 %	52.76±0.18^{c}	12.8±0.81^{b}	23.05±0.52^{b}	29.02±0.32^{b}	63.83±0.78^{b}
L. s. 30 %	47.96±0.20^{c}	19.27±0.93^{a}	29.93±0.71^{a}	35.60±0.23^{a}	57.22±0.63^{a}
L. s. 10 %	48.45±0.19^{c}	9.31±0.74^{c}	21.89±0.64^{b}	23.79±0.18^{c}	66.96±0.88^{b}
Sp. 30 %	44.54±0.23^{a}	12.01±0.63^{b}	20.33±0.55^{b}	19.02±0.13^{c}	54.32±0.49^{a}
Sp. 10 %	43.51±0.11^{a}	8.21±0.32^{b}	17.75±0.39^{c}	16.57±0.42^{d}	52.65±0.55^{a}
Untr. 30 %	42.10±0.25^{a}	7.56±0.41^{c}	14.24±0.44^{c}	14.99±0.54^{d}	53.66±0.86^{a}
Untr. 10 %	42.14±0.17^{b}	4.32±0.37^{d}	12.32±0.60^{d}	15.01±0.30^{d}	52.75±0.97^{a}
C 0%	60.07±0.43^{d}	2.41±0.30^{d}	11.84±0.22^{d}	12.99±0.21^{e}	82.71±0.60^{d}
Thermal untreated					
P. p. 30 %	46.03±0.25^{a}	17.27±0.51^{b}	26.90±0.74^{a}	31.97±0.65^{a}	57.30±0.40^{d}
P. p. 10 %	50.64±0.19^{b}	12.90±0.72^{a}	28.96±0.83^{a}	31.70±0.82^{a}	65.99±0.93^{c}

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RCMP samples	L*	a*	b*	C	h°
L. s. 30 %	45.63±0.63[a]	18.08±0.61[d]	27.60±0.52[a]	32.99±0.74[b]	56.77±0.68[b]
L. s. 10 %	50.9±0.79[a]	12.14±0.21[a]	28,50±0.48[a]	30.98±0.65[a]	66.93±0.88[c]
Sp. 30 %	47.23±0.28[a]	15.23±0.34 [a]	25.36±0.39[b]	29.59±0.54[c]	62.39±0.45[a]
Sp. 10 %	45.21±0.41[a]	14.02±0.19 [a]	21.45±0.41[d]	28.96±0.91[c]	61.33±0.46[a]
Untr. 30 %	46.27±0.52[a]	13.25±0.16[a]	21.55±0.57[d]	28.69±0.58[c]	61.45±0.41[a]
Untr. 10 %	44.25±0.39[a]	11.03±0.11[c]	19.56±0.59[d]	26.98±0.62[d]	60.84±0.93[a]
C 0%	58.38±0.48[c]	3.93±0.18[e]	15.33±0.78[e]	25.63±0.61[d]	81.18±0.54[e]

Samples: C 0 % - RCMP without tomato products; P.p. 10 % - RCMP with 10 % with *P. pentosaceus* fermented tomato products; P.p. 30 % - RCMP with 30 % with *P. pentosaceus* fermented tomato products; L.s. 10 % - RCMP with 10 % with *L. sakei* fermented tomato products; *L.s.* 30 % - RCMP with 30 % with *L. sakei* fermented tomato products; Sp. 10 % - RCMP with 10 % spontaneous fermented tomato products; Sp. 30 % - RCMP with 30 % spontaneous fermented tomato products; Untr. 10 % - RCMP with 10 % untreated tomato powder; Untr. 30 % - RCMP with 30 % untreated tomato powder.

Means in column with common letter are not different ($p > 0.05$).

Table 4. Colour coordinates (a*, b*), L* - lightness, colour tone (h°) and purity (C) of thermal treated (10 min in 100 °C temperature water) and untreated ready- to-cook minced meat products (RCMP)

Samples	ß–carotene	Lycopene	Total carotenoids content
	mg/100 g		
Thermal untreated			
Untr. 10%	0.19±0.02[a]	0.48±0.06[a]	0.67[a]
Untr. 30%	0.25±0.01[b]	1.46±0.10[b]	1.71[b]
Sp. 10%	0.69±0.02[c]	1.02±0.09[b]	1.71[b]
Sp. 30%	0.97±0.02[c]	2.13±0.15[c]	3.1[c]
L. s. 10%	1.23±0.09[d]	3.59±0.21[d]	4.82[c]
L. s. 30%	1.95±0.08[e]	9.66±0.17[e]	11.61[e]
P. p. 10%	1.01±0.07[d]	3.67±0.21[d]	4.68[d]
P. p. 30%	1.76±0.03[e]	10.32±0.11[e]	12.08[e]
Thermal treated			
Untr. 10%	0.10±0.02[a]	0.24±0.07[a]	0.34[a]
Untr. 30%	0.15±0.04[a]	0.93±0.05[b]	1.08[b]
Sp. 10%	0.49±0.07[b]	0.91±0.06[b]	1.40[b]
Sp. 30%	0.74±0.04[c]	1.75±0.09[c]	2.49[c]
L. s. 10%	0.59±0.06[b]	2.15±0.13[c]	2.74[c]
L. s. 30%	1.47±0.08[d]	7.32±0.14[d]	8.79[d]

Samples	ß–carotene	Lycopene	Total carotenoids content
	mg/100 g		
P. p. 10%	0.76±0.06[c]	2.62±0.20[c]	3.38[c]
P. p. 30%	1.17±0.10[d]	6.31±0.26[d]	7.48[d]

Samples: C0 % - RCMP without tomato products; P.p. 10 % - RCMP with 10 % with *P. pentosaceus* fermented tomato products; P.p. 30 % - RCMP with 30 % with *P. pentosaceus* fermented tomato products; L.s. 10 % - RCMP with 10 % with *L. sakei* fermented tomato products; *L.s.* 30 % - RCMP with 30 % with *L. sakei* fermented tomato products; Sp. 10 % - RCMP with 10 % spontaneous fermented tomato products; Sp. 30 % - RCMP with 30 % spontaneous fermented tomato products; Untr. 10 % - RCMP with 10 % untreated tomato powder; Untr. 30 % - RCMP with 30 % untreated tomato powder.

Means in column with common letter are not different (p> 0.05).

Table 5. ß–carotene, lycopene and total carotenoids content in thermal treated and untreated *ready- to-cook minced meat products (RCMP)*

8. Conclusions

Lactic acid fermentation represents the easiest and most suitable way to increase daily consumption of nearly fresh fruits and vegetables. The health and safety of the products can be aided by the development of starter cultures. Progress in the field of antimicrobial LAB strains with multi-functional properties, including the degradation of mycotoxins, can be engineered to significantly improve the quality, safety and acceptability of plant foods.

Tomato processing resulted in several important changes in carotenoid concentration and lycopene isomer profile. Treatment with LAB breaks down the tomato cell matrix and makes carotenoids more available, yielding a higher level of total carotenoids. Moreover, tomatoes subjected to lactic acid fermentation results in high lycopene bioavailability accompanied by increased *cis*-lycopene content. According to our results, *P. pentosaceus* and *L. sakei* may be useful for the preservation of tomatoes. Such products could be recommended as being more biologically accessible with higher functional value.

The results of our tomato product colour analysis offered the possibility of evaluating the level of lycopene using the yellow colour characteristic of tomato products; this method was reproducible and accurate enough to substitute for the chemical extraction determinations and may be a useful tool for the tomato industry.

The direct use of *Pediococcus pentosaceus* KTU05-9 and *Lactobacillus sakei* KTU05-6 for tomato product fermentation increases the carotenoid content in tomato products, which is a beneficial additive that improves the colour, functional value and acceptability of ready-to-cook minced meat products. Ready-to-cook minced meat products that have been enriched with carotenoids, which lend good sensory quality and are produced to contain a high level of lycopene and ß-carotene, can increase the intake of carotenoids in the diet. This is the first time that selected lactobacilli-fermented tomato products have been used as source of lycopene and ß-carotene for food, and more research is needed to explain the mechanism of carotenoid increase in fermented tomato products.

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All the tested LAB produced a mixture of L- and D-lactic acid, with the latter isomer at a lower level. Because of the potential toxicity of D-lactic acid in food, we report that the tomato products prepared using pure cultures of tested LAB were found in all cases to be safer than those treated by spontaneous fermentation.

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Chapter 8

Growing Uses of 2A in Plant Biotechnology

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Additional information is available at the end of the chapter

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1. Introduction

The combination, 'pyramiding' or 'stacking' of multiple genes in plants is a fundamental aspect of modern plant research and biotechnology. The most widely adopted stacked traits (herbicide tolerance and insect protection) provide growers with benefits of increased crop yield, simplified management of weed control and reduced insecticide use. The global acreage of stacked traits or more precisely genetically modified organisms bearing stacked traits is expected to increase rapidly in the near future, with the introduction of nutritional and/or industrial traits to satisfy the needs of consumers and producers [1]. Several approaches have been used to stack multiple genes into plant genomes and then to coordinate expression [2-4]. Stacking approaches include sexual crossing between plants carrying distinct transgenes [5,6], sequential re-transformation [7], and single-plasmid [8] or multiple-plasmid co-transformation [9]. These strategies, however, suffer from the inherent weakness that co-expression of the heterologous proteins is unreliable.

Due to limited genomic coding space, many viruses encode more than one protein from a single mRNA transcript. Internal ribosome entry site (IRES) sequences serve as a launching pad for internal initiation of translation, allowing expression of two or more genes from a single transcript [reviewed in 10]. A number of IRES motifs from plant [11] and animal [12] viruses have been used to direct the expression of multiple recombinant proteins in plants and plant cells [13,14]. However, widespread use of IRES motifs in plant biotechnology is limited: they are not small (~600 base pairs), adding to the size of the transgene; translation efficiency of a gene placed after the IRES is much lower than that of a gene located before the IRES [14]. One promising gene/protein strategy adopted by some viruses to ensure a balance of proteins *in vivo* is to express polyprotein precursors with cleavable linkers between the proteins of interest [15]. Several groups demonstrated the potential of this approach by co-expressing two proteins

separated by the tobacco etch virus (TEV) NIa protease recognition sequence (heptapeptide cleavage recognition sequence ENLYFQS) together with the NIa proteinase [16-18]. The utility of the NIa protease is limited due to the presence of a nuclear-localizing signal (NLS) within the protease and the amount of energy necessary to express the 49 kDa protease. It is also possible to use linker sequences that are putative substrates of known endogenous plant proteases [19].

To bypass the need for an endogenous or recombinant accessory protease acting on the translated polypeptide product a different approach involves the use of self-processing viral 2A peptide bridges [reviewed in 20, 21]. The designation "2A" derives from the systematic nomenclature of protein domains within the polyproteins of picornaviruses. In foot-and-mouth disease virus (FMDV) and some other picornaviruses the oligopeptide 2A region of the polyprotein manipulates the ribosome to "skip" the synthesis of the glycyl-prolyl peptide bond at its own carboxyl terminus leading to the release of the nascent protein and translation of the downstream sequence [22]. Under the monikers of "Skipping", "Stop-Carry On" and "StopGo" translation, it allows the stoichiometric production of multiple, discrete, protein products from a single transgene [23,24]. Several recent review articles have amply covered the role of 2A biotechnology in animal systems [20, 25]. This summary-review will provide an up-to-date overview of 2A and cover the wider application of 2A-polyproteins to the expression of multiple proteins in plants.

2. The 2A story — The end of the beginning

2.1. The co-translational model of 2A-mediated "cleavage"

FMDV, like other members of the family *Picornaviridae*, is a non-enveloped RNA virus which contains a single-stranded, positive-sense RNA molecule of approximately 8500 nt that functions as an mRNA [26]. This (+) RNA encodes a high molecular mass polyprotein that undergoes co-translational processing to yield the structural proteins (1A, 1B, 1C and 1D, commonly known as VP4, VP2, VP3 and VP1 respectively) which comprise the viral capsid, and the non-structural proteins (2A, 2B, 2C, 3A, 3B, $3C^{pro}$, and $3D^{pol}$) that control the viral life cycle within host cells [27]. The 2A oligopeptide is only 18 amino acids (aa) long (-LLNFDLLKLAGDVESNPG-) defined by the co-translational "cleavage" at its C-terminus and a post-translational cleavage at its N-terminus, mediated by the virus-encoded proteinase $3C^{pro}$ [28]. Analysis of recombinant FMDV polyproteins [29] and artificial polyprotein systems in which 2A was inserted between two reporter proteins [22] showed that just 2A, plus the N-terminal proline of the downstream protein 2B was sufficient for highly efficient co-translational "cleavage" (Figure 1, Panel A). Quantification of products using *in vitro* cell-free translation systems showed the product upstream of 2A accumulated in a molar excess over that downstream – at variance with a proteolytic model of 2A which predicts a 1:1 stoichiometry of the cleavage products [23, 30, 31]. We and others have shown that 2A is not a proteinase, nor a substrate for a host-cell proteinase, but an autonomous element mediating a co-translational "recoding" event [27, 29]. From these observations we proposed a model of the 2A

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reaction based on hydrolysis of the nascent chain from ribosome-associated tRNA at the peptidyl-transferase centre [23, 24, 30]. For in-depth reviews of the model see [24, 32, 33].

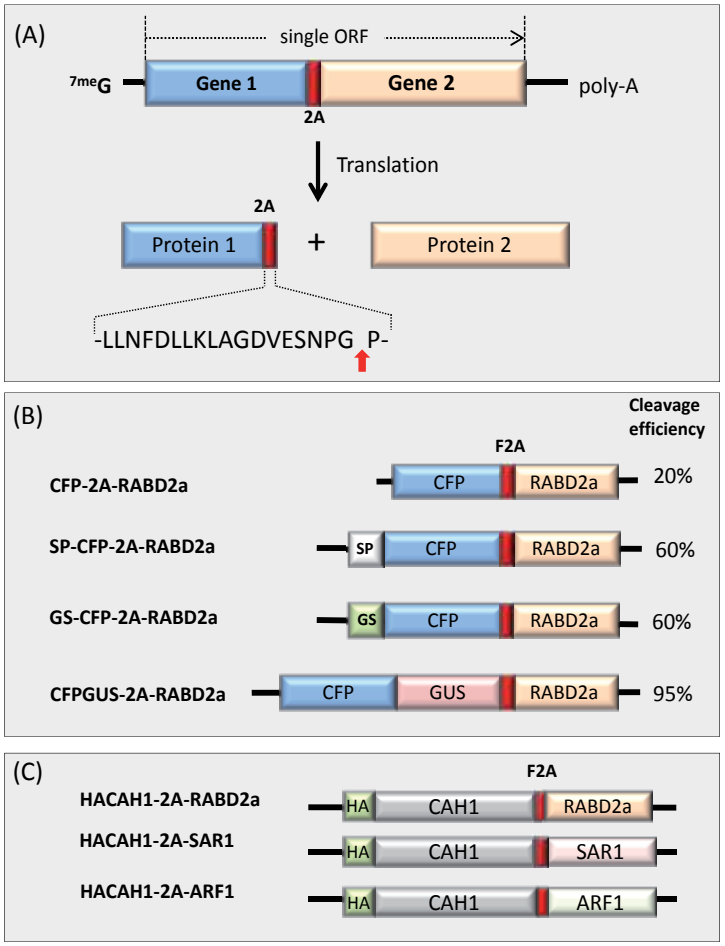

Figure 1. Schematic overview of 2A function and gene fusion constructs. Panel A: Two individual polypeptides can be generated from one transcript using F2A to link the individual genes. Panel B: F2A cleavage efficiency. All constructs shared a common core consisting of CFP-F2A-RABD2a. Panel C: Sequences encoding HA-tagged CAH1 were fused in-frame to wild type and mutant versions of genes encoding the GTPases RABD2a, SAR1, and ARF1 linked by F2A-the pre-protein has the endomembrane targeting sequence. These synthetic polyproteins were efficiently cleaved when transiently expressed in protoplasts and *in planta*. CFP, enhanced cyan fluorescent protein; GUS, β-glucuronidase; SP, ER signal peptide of CAH1 protein; GS, Golgi targeting signal of N-acetylglucosaminyl transferase 1; RABD2a, *Arabidopsis* RABD2a GTPase; SAR1, *Nicotiana tabacum* SAR1p; ARF1, *Arabidopsis* ADP-ribosylation factor 1; HA, hemagglutinin epitope tag; CAH1, *Arabidopsis* α-CAH1 (adapted from [60]).

2A comprises two parts, an N-terminal region (without sequence conservation) predicted to form an alpha helix, and a C-terminal motif,-DxExNPG, followed by a proline required for the reaction. Recently it was shown that the synonymous codon usage of this conserved motif is biased [34]. The amino acids E,S,N,P,G,P tend to use GAG, TCC, AAC, CCT, GGG and CCC

respectively. The results also indicate that the synonymous codon usage of the 2A peptide has no effect on 2A activity. In summary, our results indicate the conserved –DxExNPG motif within the peptidyl transferase centre (PTC) of the ribosome is restricted and it forms a tight turn, shifting the ester bond between the C-terminal glycine and $tRNA^{Gly}$ (in the P site of the ribosome) into a conformation which rules out nucleophilic attack by prolyl-$tRNA^{Pro}$ (in the A site)-no peptide bond is formed. Although no stop codon is involved, eukaryotic translation release (termination) factors 1 and 3 (eRF1/eRF3) release the nascent protein from the ribosome [35-37]. Due to its mode of action, the 2A peptide has been described as a "***c**is*-acting **hy**drola**s**e **el**ement" (CHYSEL) [32]. Our model of this translational recoding event predicts two outcomes, either ribosomes terminate translation, or, translation of the downstream sequences resumes. Skipping induced by 2A sequences gives approximately equal expression of the proteins upstream and downstream of the 2A site as measured by: i) CAT and GUS enzyme activity [38]; ii) cell free translation *in vitro* and Western blot [22, 23, 31, 39, 40]; iii) GFP/FACS with antibiotic resistance [41]; iv) co-fluorescence reporting [42, 43]; v) fluorescence resonance energy transfer (FRET) analysis [44] and vi) protein segregation in transgenic animals [45, 46]. Since these sequences act co-translationally, artificial polyprotein systems may include signal sequences to localize different protein translation products to discrete sub-cellular sites.

2.2. 2A and 2A-like sequences

Probing databases for the presence of the "signature" motif (-DxExNPGP-) showed that "2A-like" sequences were present in several genera of the *Picornaviridae* (aphtho-, cardio-, erbo-, tescho and certain parechoviruses), single-stranded RNA insect viruses (iflaviruses, dicistroviruses, tetraviruses), double-stranded RNA viruses of the *Reoviridae* (type C and non-ABC rotaviruses, cypoviruses) and penaeid shrimp viruses [47, reviewed in 20,48]. Previously we demonstrated the activity of "2A-like" sequences within non-long terminal repeat retrotransposons (non-LTRs) of *Trypanosoma brucei*, *T.cruzi*, *T.vivax*, and *T.congolense* [31, 49] and more recently within the non-LTRs of a wide range of multicellular organisms: *Xenopus tropicalis* (African claw-toed frog, vertebrate), *Branchiostoma floridae* (Amphioxus, Florida lancelet, cephalochordate), *Strongylocentrotus purpuratus* (purple sea urchin, echinoderm), *Aplysia californica* (California sea slug, mollusc), *Crassostrea gigas* (Pacific oyster, mollusc), *Lottia gigantean* (Owl limpet, mollusc) and *Nematostella vectensis* (sea anemone, cnidarian) [50]. Presently, *in silico* searches have identified the 2A motif in a range of putative retrotransposon domains (Table 1).

Chimeric polyproteins incorporating 2A have been widely tested in eukaryotic systems, including mammalian [22], plant [38], insect [51], yeast [39] and fungal cells [52].The 2A system does not work in prokaryotic cells-the reported proteolysis activity of 1D-2A in *Escherichia coli* cells [53] was not detected in equivalent constructions in our laboratory showing "cleavage" specificity for eukaryotic systems alone [54]. The unique activity of 2A peptides has led to their use as tools for co-expression of two (or more) proteins in biomedicine and biotechnology [reviewed in 20, 21, 55]. The most widely used 2A sequence is derived from the FMDV (hereafter referred to as "F2A") [42]. Other 2A peptides used successfully include "T2A" from *Thosea asigna* virus (TaV), "E2A" from equine rhinitis virus (ERAV) and "P2A" from porcine

Cellular 2A	Name	2A sequence
Non-LTR retrotransposons		
Trypanosoma spp		
T.brucei	*Ingi*	-RSLGTCKRAISSIIRTKMBVSGDVEENPGP-
T.cruzi	*L1Tc*	-QRYTYRLRAVCDAQRQKLLLSGDIEQNPGP-
Strongylocentrotus purpuratus	STR-32_SP	-NSSCVLNIRSTSHLAILLLLSGQVEPNPGP-
(purple sea urchin)	STR-51_SP	-SRPILYYSNTTASFQLSTLLSGDIEPNPGP-
	STR-61_SP	-GARIRYYNNSSATFQTILMTCGDVDPNPGP-
	STR-69_SP	-CRRIAYYSNSDCTFRLELLKSGDIQSNPGP-
	STR-197_SP	-KHPILYYTNGESSFQIELLSCGDINPNPGP-
Crassostrea gigas (Pacific oyster)	*CR1-1_CGi*	-SRHIVVYNFYLQFFMFLLLLCGDIEVNPGP-
Lottia gigantean (owl limpet)	*CR1_1_LG*	-TLLNDTFSSILYYCFILIIRSGDIELNPGP-
Aplysia californica (California sea slug)	*Ingi-1_AC*	-PGFFLGGQHNPAWLARLLILAGDVEQNPGP-
Retrotransposons (unpublished)	Accession number	
Rhipicephalus pulchellus (Zebra tick)	JAA55454.1	-SFVFTSLYADIVHCLCSLLLSGDVELNPGP-
	JAA55744.1	-HCCLSIVVQCCDVIRSLLLLAGDIETNPGP-
Biomphalaria glabrata (freshwater snail)	AC233255.1	-KWKFSVRDSRIKYLSLLILIAGDVESNPGP-
Caenorhabditis elegans (roundworm)	Z49911.1	-LCETPSLPHTTFLKRKLLVRSGDVESNPGP-
	AL132860.1	-LCETPSLPHTTFLKRKLLVRSGDVESNPGP-
	F0081226.1	-LCETPSLPHTTFLKRKLLVRSGDVESNPGP-
Chlorella variabilis (Green alga)	EFNS2199.1	-LRLPCSCSTTALIKRMKLLLSGDVEENPGP-

The –DxExNPGP- motif conserved among 2A/2A-like sequence is shown in red.

Table 1. Active 2A cellular sequences.

teschovirus-1 (PTV-1) [Table 2]. Comparing the *in vitro* activity of different 2As inserted between GFP and GUS, we have shown that $T2A_{20}$ has the highest cleavage efficiency followed by $E2A_{20}$, $P2A_{20}$, and $F2A_{20}$ [31]. In 2A peptide-linked TCR:CD3 constructs, Szymczak and colleagues demonstrated that $F2A_{22}$ and $T2A_{18}$ have higher efficiency than $E2A_{20}$ [44]. In human cell lines, zebrafish and mice, cleavage and targeting of NLS-EGFP and mCherry-CAAX to the nucleus and plasma membrane, respectively, was the most efficient in $P2A_{19}$-linked constructs followed by $T2A_{18}$, $E2A_{20}$ and $F2A_{22}$ [56]. To allay public fears and opposition to plants carrying a transgenic viral sequence, efficient 2A-like cellular sequences could be used (Table 1) [Unpublished Data].

Virus	Abbreviation	2A/2A-like sequence	References
Picornaviridae			
Foot-and-mouth disease virus	FMDV	-PVKQLLNFDLLKLAGDVESNPGP-	[38,44,83,99,106,150]
Equine rhinitis A virus	ERAV	-QCTNYALLKLAGDVESNPGP-	[44]
Porcine teschovirus -1	PTV-1	-ATNFSLLKQAGDVEENPGP-	[45]
Tetraviridae			
Thosea asigna virus	TaV	-EGRGSLLTCGDVESNPGP-	[44,46]

The –DxExNPGP-motif conserved among 2A/2A-like sequence is shown in red.

Table 2. Examples of 2A/2A-like sequences used in biomedicine and biotechnology

2.3. Intracellular protein targeting Of 2A constructs

For effective technologies, some synthesized proteins must be transported across membranes and directed towards other sites in order to function. Protein targeting occurs either co-translationally (targeting to endoplasmic reticulum [ER], Golgi, vacuole, plasma membrane) or post-translationally (targeting to nucleus, mitochondria, chloroplast, etc) and is orchestrated by distinct signal sequences encoded within the polypeptide [42]. In plants, the original FMDV-2A sequence was tested in various artificial polyproteins using reporter genes chloramphenicol acetyltransferase (CAT), β-glucuronidase (GUS) and green fluorescent protein (GFP) expressed in transgenic tobacco plants. This preliminary series of studies suggested that 2A cleaves proteins properly in plant cells [38, 57] and directs protein targeting to different cellular compartments *via* either co-or post-translational mechanisms [58]. Subsequently, Samalova and co-workers questioned its use in plant systems, suggesting that the 2A sequence was dispensable for efficient cleavage of polyproteins carrying a single internal signal peptide – it appears signal peptide cleavage by signal peptidase was responsible for processing the polyprotein. The use of a self-cleaving 2A was required when both halves of the fusion were translocated across the ER membrane, however, the upstream product was mis-sorted to the vacuole. Furthermore, it was shown that the FMDV 2A peptide resulted in low rates of polypeptide separation in plant cells when placed downstream of common fluorescent proteins (GFP and RFP derivatives) [43].

The *Arabidopsis* carbonic anhydrase (CAH1) is one of the few plant proteins known to be targeted to the chloroplast *via* the secretory pathway – the pre-protein has the endomembrane targeting sequence. The need for post-translational modifications, such as N-glycosylation, for proper folding, and to enhance stability and/or function of these proteins probably explains the use of this alternative trafficking pathway [59]. Recently, the FMDV 2A co-expression system was re-assessed to study the effects of three Ras-like small GTPase proteins, RAB2a, ARF1, and SAR1 on CAH1 protein trafficking in plant cells [60]. Members of this superfamily share several common structural features and act as molecular switches that regulate many aspects of plant vesicular transport [61,62]. Rabs regulate virtually all steps of membrane traffic from the specification of membrane identity to the accuracy of vesicle targeting [63]. ARF1 has been shown to play a critical role in COPI-mediated retrograde trafficking, while SAR1 is involved in COPII-mediated ER-to-Golgi protein transport [reviewed in 64]. In this study,

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targeting information and the sequence N-terminal of 2A proved to be important for efficient cleavage when translated by membrane-bound and cytosolic ribosomes respectively (Figure1, Panel B). In addition, expected subcellular localization of the fluorescent marker protein suggested no significant mis-targeting of the 2A-tagged markers. After optimization of 2A cleavage efficiency, mutant forms of the three small GTPases (HACAH1-2A/RABD2a/SAR1/ARF1, Figure1, Panel C) were successfully used to study trafficking of CAH1 through the endomembrane system demonstrating the versatility of 2A in plant systems.

Exchange factors for ARF GTPases (ARF-GEFs) regulate vesicle trafficking in a variety of organisms. In animals and fungi, there are eight ARF-GEF families, but only the apparently ancestral GBF and BIG families are present in plants, suggesting that plant ARF-GEFs have acquired multiple roles in different trafficking pathways [65, 66]. In *Arabidopsis* the ARF-GEFs GNOM-like 1 (GNL1) and its close homologue GNOM jointly regulate the retrograde COPI-mediated traffic from the Golgi to the ER, which is the ancient eukaryotic function of the GBF1 class [67]. Another line of research by Teh and Moore (2007) revealed secretory traffic is resistant to the trafficking inhibitor brefeldin A (BFA), whereas endosomal recycling involves GNOM – GNL1 is a BFA-resistant GBF protein that functions with the BFA-sensitive ARF GEF GNOM [68]. The 20aa 2A peptide from FMDV was used in this study to construct polyproteins that expressed trafficked fluorescent protein markers in fixed stoichiometry in different cellular compartments: N-ST-RFP-2A-GFP-HDEL produces a Golgi-localized RFP (red) and an ER-localized GFP (green), N-secRFP-2A-GFP-HDEL produces an ER-localized GFP and an RFP that is targeted to the vacuole *via* the ER and Golgi.

2.4. The use of 2A multigene expression strategies in plant science — Caveats and proposals

The take-home message from F2A mutagenesis experiments is that the sequence is largely intolerant to amino acid substitution over its entire length [31, 37]. While mutations of conserved amino acids have, in general, more pronounced effects than changes to non-conserved ones [31], variations at most positions within the peptide reduce activity – 2A peptides are optimized to function as a whole [37]. Sequences immediately upstream of 2A are known to be either critical or very important for activity [57, 69-72]. Longer versions of F2A with extra sequences derived from the capsid protein ("1D") – upstream of 2A in the FMDV polyprotein – produce higher levels of cleavage [23, 29, 47]. Specifically, N-terminal extension of 2A by 5aa of 1D improved "cleavage", but extension by 14aa of 1D or longer (21 and 39aa) produced complete "cleavage" and an equal stoichiometry of the up-and downstream translation products [23]. After "fine-tuning" of the F2A sequence we suggest that researchers opt for $F2A_{30}$ (+11aa 1D). This 2A proved to be the most favourable in terms of both length and cleavage efficiency and was unaffected by the sequence of the upstream gene [73,74]. In the case of shorter 2As, *cleavage efficiency* has been improved by insertion of various spacer sequences such as Gly-Ser-Gly or Ser-Gly-Ser-Gly [41, 44, 45, 75-77], the V5 epitope tag (-GKPUPNPLLGLDST-) [78], or a 3xFlag epitope tag [79] ahead of the 2A sequence. If opting for a shorter sequence, users should be aware activity can be affected by the short amino acid tract linking the protein upstream with 2A introduced by the cloning strategy. For example, the $F2A_{20}$ encoded by pGFP-$F2A_{20}$-GUS was highly active [29, 31], whereas the pGFP-$F2A_{20}$-

CherryFP was noticeably lower [73]. The only difference was the short "linker" between GFP and F2A created by the cloning strategy:-SGSRGAC-(pGFPF-2A$_{20}$-GUS; linker derived from *Xba1* and *Sph1* restriction sites) and-RAKRSLE-(pGFPF-2A$_{20}$-CherryFP; linker derived from furin and *Xho1* restriction site) [73]. Taken together, these observations are consistent with our translational model in which 2A activity is a product of its interaction with the exit tunnel of the ribosome which is thought to accommodate a nascent peptide of 30-40 amino acids [80].

When using the 2A system, it should be noted that the 2A oligopeptide remains as a C-terminal extension of the upstream fusion partner and the downstream protein must have an N-terminal proline residue. Although an N-terminal proline confers a long half-life upon a protein [81], it does prevent many N-terminal post-translational modifications that may be essential for activity. If this is the case, proteins that require authentic termini can be introduced as the first polyprotein domain. The need to target proteins to different subcellular locations within plant cells by C-terminal localization signals may be compromised if they contain a 2A-extension. In the case of proteins translocated into the ER, a strategy was adopted to include a furin proteinase cleavage site between the upstream protein and 2A [82,83]. Furin is a subtilisin-like serine endoprotease that cleaves precursors on the C-terminal side of the consensus sequence –Arg-X-Lys/Arg-Arg ↓ (-RX(K/R)R-) in the *trans*-Golgi network (TGN) [84,85]. The furin cleavage sequences ↑ -RKRR-,- ↑ RRRR-, and – ↑ RRKR-consisting of only basic amino acids, which can be efficiently cut by carboxypeptidases (↑), was used to remove 2A peptide-derived amino acids from the upstream antibody heavy chain during protein secretion [83]. Proteins expressed in plants could have their 2A extensions removed by endogenous proteinases acting on similar hybrid linker peptides. In 2004, François and colleagues connected the first nine amino acids (SN ↑ AADEVAT) of the LP4 peptide to the 20aa F2A to generate a hybrid linker peptide, LP4-2A [86]. LP4 is the fourth linker peptide of the naturally occurring polyprotein precursor originating from seed of *Impatiens balsamina* [87]. Cleavage of the polyprotein with plant defensin DmAMP1 from *Dahlia merckii* at its amino-terminus and plant defensin RsAFP2 from *Raphanus sativus* at its carboxy-terminus resulted in the release and targeting of (DmAMP1-SN) and RsAFP2 to different cellular compartments [86]. Recently, 2A and LP4-2A were used to connect the *Bacillus thuringiensis* (Bt) *cry1Ah* gene, which encodes a protein exhibiting strong insecticidal activity, and the *mG$_2$-epsps* gene, which encodes a protein tolerant to glyphosate, the world's most important and widely used herbicide [72]. The expression level of the two genes linked by LP4-2A was higher than those linked by 2A, regardless of the order of the genes within the vector. Furthermore, tobacco plants transformed with the LP4-2A fusion vectors showed better pest resistance and glyphosate tolerance compared to plants transformed with the 2A fusion constructs.

3. 2A in plant biotechnology

3.1. A strategy to improve transgene expression from the *Chlamydomonas* nuclear genome

Micro-algae have the potential to be low-cost bioreactors for recombinant protein (RP) production due to their relatively rapid growth rates, favourable transformation time, ease of

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containment and rapid scalability [88-90]. The availability of a complete genome sequence [91] coupled with the ability to manipulate all three genomes (chloroplast, nuclear and mitochondrial) makes *Chlamydomonas reinhardtii* an attractive species for biotechnologists [90,92]. While transgene expression from the alga's nuclear genome offers several advantages over chloroplast expression, such as post-translational modifications and protein targeting and/or secretion [93], yields of target RPs are often inadequate for industrial purposes. In an attempt to overcome this limitation, Rasala and colleagues constructed a *C.reinhardtii* nuclear expression vector using F2A to fuse GFP or *xylanase 1* (*xyn1*) from *Trichoderma reesi* to the bleomycin/zeocin antibiotic resistance gene *sh-ble* [94]. High-value xylanases have numerous applications in the textile, paper, pulp, food and feed industries [reviewed in 95]. Efficient cleavage of GFP from Ble-2A was observed in the algal cytoplasm, leading to high level GFP expression and built-in resistance to zeocin. Co-expression of Xyn1 with Ble-2A led to the selection of transformants with higher xylanase activity (~100-fold) compared to unfused Xyn1. Subsequently, the ble-2A expression system was used to secrete enzymatically active Xyn1 by insertion of a secretion signal peptide (SP) between F2A and Xyn1 (*ble2A-SP-xyn1*). In a follow-on study, the same strategy was used to express and compare six recombinant fluorescent proteins (FPs: blue mTagBFP, cyan mCerulean, green CrGFP, yellow Venus, orange tdTomato and red mCherry) in *C.reinhardtii* [96]. All FPs were easily detectable in live cells, and the ble-2A-FP polyproteins were efficiently processed to yield unfused FPs. CrGFP was shown to be the least fluorescent due to its low signal-to-noise ratio, while the FPs with longer emission wavelengths (Venus, TdTomato and mCherry) had the highest signal-to-noise ratios. In this study, the ble-2A vector was used to tag an endogenous gene (α-tubulin) with a fluorescent protein tag (mCerulean) that was readily detectable in *Chlamydomonas* using standard live-cell imaging techniques. Taken together, these results suggest this new expression system will become an important tool for both algal biotechnology and basic research.

3.2. Engineering plant metabolomes

Manipulating plant metabolomes ranges from modifications, building extensions or branches onto existing biochemical pathways to very extensive changes-such as the rice C_4 project. Here, the aspiration is to covert rice from a C_3 plant (3-carbon molecule present in the first product of carbon fixation) to a more efficient C_4 plant – eliminating photorespiration. As a major global crop species, rice has been the subject of intensive research. The 'Golden Rice' project was undertaken to address the problem of vitamin A deficiency (VAD), and is discussed in some detail here since it provides an interesting 'vignette' of the progress in transgenesis and plant metabolome engineering. It should be noted, however, that another 'biofortified' crop also designed to reduce VAD is the "Super-banana", originally developed for the Ugandan population, which recently gained approval to begin human trials in the United States. In this case, however, knowledge gained from a cultivar identified in Papua New Guinea, enabled development of super-bananas by genome editing, rather than transgenesis, of a commercial cultivar.

A shortage of dietary vitamin A leads to VAD resulting in impairment in sight, increases in the severity of a range of infectious diseases and is estimated to lead to the premature deaths

of 650-700,000 children under the age of 5 each year: a particular problem in parts of S.E. Asia and Africa. Whilst no rice cultivars produce provitamin A within the endosperm, the precursor geranylgeranyl diphosphate (GGDP) is produced. This precursor could be converted into β-carotene, which functions as provitamin A in humans. To convert GGDP to β-carotene was originally thought to require the activities of phytoene synthase, phytoene desaturase, ζ-carotene desaturase and lycopene β-cyclase, although recent analyses have shown endogenous rice enzymes can substitute for lycopene β-cyclase in the conversion of lycopene to β-carotene. The agrobacterial vector construct pB19hpc encodes the daffodil phytoene desaturase gene (*psy*) driven by the endosperm-specific glutelin (*Gt1*) promoter, and the phytoene desaturase (*crtI*) gene from the bacterium *Erwinia uredovora,* driven by the cauliflower mosaic virus (CaMV) 35*S* promoter, arranged in tandem (Figure 2, Panel A) [97]. Whilst the *psy* gene already encoded an N-terminal transit peptide sequence, the *crtI* sequence was modified to encode the N-terminal transit peptide sequence from the pea Rubisco small subunit – ensuring both proteins were imported into plastids, the site of GGDP biosynthesis. Selectable marker genes (*aphIV* and *nptII*) were also included. To complete the β-carotene biosynthetic pathway, plants were co-transformed with vectors pZPsC and pZLcyH. Vector pZPsC carries *psy* and *crtI* (as pB19hpc), but lacked the *aphIV* marker expression cassette. Vector pZLcyH encodes lycopene β-cyclase (*lcy*) from Narcissus driven by the rice glutelin promoter and the marker *aphIV* gene controlled by the CaMV 35*S* promoter, arranged in tandem. Again, lycopene β-cyclase carried a functional transit peptide to direct plastid import. Whilst this proved to be a successful strategy – it is rare one's experimental results are given a Papal blessing, the use of multiple promoters in such a strategy of transgenesis often leads to 'interference': known as 'transcriptional interference' or 'promoter suppression'-probably caused by competitive binding of transcription factors and/or modification of DNA structure at one site that affects the other site. Such problems may be overcome by the inclusion of 'insulators' which serve to segregate an enhancer and an adjacent promoter into independent domains-'enhancer blocking'.

The first-generation Golden rice *encoded the* daffodil phytoene synthase gene, but this daffodil enzyme proved to be a rate-limiting step in β-carotene biosynthesis, since substitution with *psy* from maize produced a 23-fold increase in total carotenoids (with a preferential accumulation of β-carotene) compared to the original Golden Rice (Figure 2, Panel B) [98]. Again, tandem (rice glutelin) promoters were used. To avoid the problems of promoter interference, a third generation of Golden Rice was developed using two systems both of which produce multiple proteins from a single transcript mRNA. In this strategy, the *crtI* and *psy* genes were linked by either a 2A sequence (creating a single ORF; pPAC construct), or, the two genes were separated by an IRES (pPIC construct; Figure 2, Panel C) [99]. In this strategy phytoene synthase from Capsicum was co-expressed with phytoene desaturase from Pantoea from a single transcription unit in both cases. The rice globulin promoter drove the expression of [Psy-2A-CrtI], or, [Psy-IRES-CrtI]. The endosperm of transgenic PAC rice had a much more intense golden colour than did the PIC rice transformants, demonstrating that 2A was more efficient than an IRES in co-expression of PST and CRTI and hence the synthesis of β-carotene. Indeed, immunoblot analyses of CRTI (the downstream protein in both cases) showed that 2A was nine-fold more effective than an IRES. It is well-known that in the case of a bicistronic mRNAs using an IRES, the gene downstream of the IRES is only translated ~1/10th as efficiently

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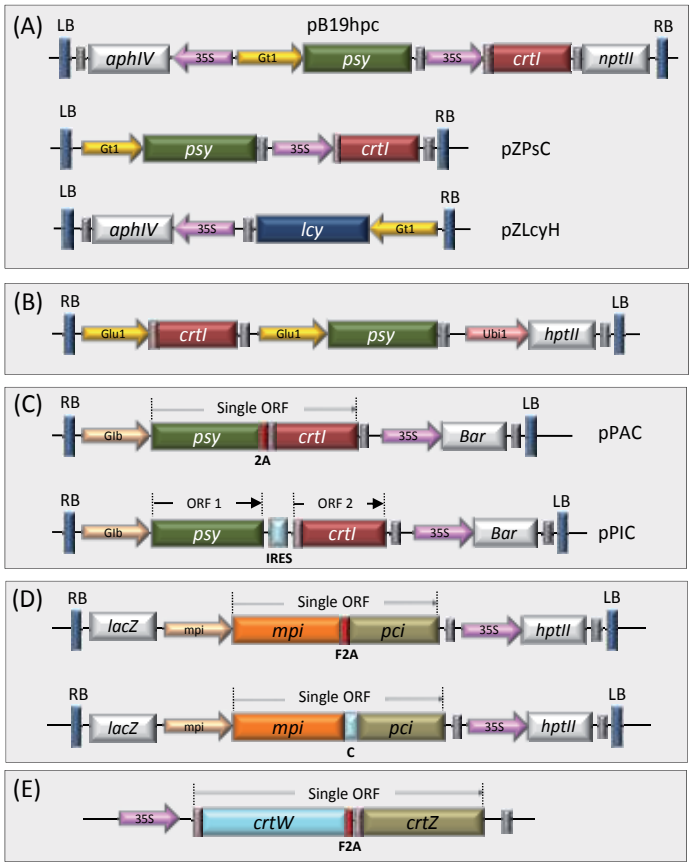

Figure 2. Strategies of plant transgenesis. In all panels, genes / transcription termination / poly(A) addition sequences are shown as boxed areas and promoters as arrows. Panel A: The transcription of the daffodil phytoene desaturase gene (*psy*), *Erwinia* phytoene desaturase (*crtI*) and the Narcissus lycopene β-cyclase (*lcy*) genes are driven either by the endosperm-specific glutelin (Gt1) or CaMV 35S promoters, as indicated. In all cases shown in this figure, sequences encoding the N-terminus of CRT1 were preceded by those encoding functional transit peptides-to ensure import into plastids. The *aphIV* and *nptII* selectable markers used are shown, together with transcription terminators / poly(A) addition signals (light grey boxes) and Agrobacterium vector left and right borders (LB, RB). Panel B: In the second generation Golden Rice, *Erwinia* phytoene desaturase (*crtI*) gene was co-expressed with the maize phytoene desaturase gene (*psy*) gene under the control of tandem rice glutelin promoters (Glu1). The Agrobacterial vector also encoded a selectable marker cassette comprising the maize polyubiquitin (Ubi1) promoter with intron, hygromycin resistance (*hptII*) and nos terminator are shown as grey boxes. Panel C: In the third generation of Golden Rice, Capsicum phytoene synthase (*psy*) and Pantoea carotene desaturase (*crtI*) were co-expressed by linkage into a single ORF *via* a synthetic 2A sequence that was optimized for rice codons (pPAC construct), or, the genes were linked by the insertion of an IRES into the intergenic region (pPIC construct). In both cases, transcription was driven by the rice globulin promoter (GIb). The Agrobacterial constructs also comprised a selectable marker (*bar*) driven by the 35S promoter and were flanked by a 5′-matrix attachment region (Mar) from the chicken lysozyme gene are not shown. Panel D: Sequences encoding the maize proteinase inhibitor (*mpi)* and the potato carboxypeptidase inhibitor (*pci*) were fused into a single ORF. The two proteinase inhibitors were linked using either (i) the processing site of the *Bacillus thuringiensis* Cry1B precursor protein (C) or (ii) the 2A sequence from foot-and-mouth disease virus (F2A). In both cases the wound-inducible *mpi* promoter (arrow) was used to drive the expression of the *mpi-pci* fusion genes. Panel E: Genes encoding the 3,3′-β-hydroxylase (*crtZ*) and 4,4′-β-oxygenase (*crtW*) from marine bacteria (*Paracoccus* spp.) were linked *via* F2A to form a single ORF. Again, both gene sequences were preceded by those encoding functional transit peptides to ensure import into plastids. Selectable markers (e.g. *lacZ* and *hptII*) are shown as grey boxes.

as the gene upstream of the IRES. In comparison to IRESes, the use of 2A and 2A-like sequences has the advantages of producing stoichiometric levels of the translational products and can be used to express multiple (>2) proteins at similar levels.

In light of the developments in the synthesis of β-carotene outlined above, it is interesting to note that whilst higher plants synthesize carotenoids, they do not possess the ability to form ketocarotenoids – potent antioxidants with numerous reported health benefits. Organisms capable of synthesizing ketocarotenoids are rare, although an early report showed that co-expression of the 3,3′-β-hydroxylase (*crtZ*) and 4,4′-β-oxygenase (*crtW*) from marine bacteria (*Paracoccus* spp.), linked *via* 2A (Figure 2, Panel E), lead to the formation of ketolated carotenoids (astaxanthin, canthaxanthin and 4-ketozeaxanthin) from β-carotene and its hydroxylated intermediates by the construction of an astaxanthin pathway [100].

3.3. Improving resistance to abiotic/biotic stresses

Abiotic stresses such as drought, excessive salinity, high and low temperature are critical factors limiting the productivity of agricultural crops. The development of genetically engineered plants with enhanced tolerance presents an important challenge in plant gene technology. A common response of plants to these environmental stresses is the accumulation of sugars and other compatible solutes. Trehalose is a nonreducing disaccharide that functions as a stress protection metabolite in many organisms [reviewed in 101]. In yeast, trehalose-6-phosphate synthase (TPS1) and trehalose-6-phosphate phosphatase (TPS2) enzymes catalyse the conversion of glucose-6-phosphate and uridine diphosphate (UDP)-glucose to trehalose in a two-step pathway [102]. Several efforts have been undertaken to engineer the drought- and salt-tolerance of economically important plants using *TPS1* and/or *TPS2* genes from yeast and bacteria [103-105]. Both *TPS1* and *TPS2* genes of *Zygosaccharomyces rouxii* were co-introduced into potato plants as a ZrTPS2-F2A-ZrTPS1 polyprotein in an attempt to develop stress-tolerant transgenic plants [106]. The resulting plants showed increased tolerance to drought and no visible phenotypic alterations. Glycinebetaine (N,N,N-trimethyl glycine; betaine) is regarded as an extremely effective compatible solute, which is able to restore and maintain the osmotic balance of living cells, in response to high salinity, cold and drought [107]. In plants, betaine is produced by the two-step oxidation of choline *via* the two enzymes choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH). Transferring betaine synthesis genes *CMO* and *BADH* from the halophyte *Suaeda salsa* to *Pichia pastoris* produced "*CMO-F2A-BADH*" recombinant yeasts with higher tolerance to salt, methanol, and high temperature stress [108]. The result indicates that this strategy could be used to improve the tolerance to stress of commercially important crops such as potato, rice, tomato, and tobacco, which do not accumulate betaine.

Plants are substrates for a wide range of pests and pathogens, including fungi, bacteria, viruses, nematodes, insects, and parasitic plants [109]. To defend themselves against pathogen attack, plants produce a battery of antimicrobial peptides (AMPs), secondary metabolites and reactive oxygen species [reviewed in 110-112]. AMPs (such as defensins) are attractive candidates for transgenic applications for several reasons: their diverse antimicrobial activity, low toxicity for non-target cells and low cost in terms of energy and biomass involved in their expression

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[113]. To achieve resistance against a broader range of pathogens in plants, co-expression of transgenes encoding AMPs with different biochemical targets is an attractive approach. In the case of plant defensins DmAMP1 and RsAFP2 (see above), biological activity of the hybrid protein was higher compared to the individual parental proteins [86]. The potato AMPs snakin-1(SN1) and defensin-1 (PTH1) were fused to improve plant protection against phytopathogens [114]. SN1 is active against both bacterial and fungal species, whereas PTH1 shows primarily antifungal activity [115, 116]. Antimicrobial activity of SN1 and PTH1 (linked by the F2A sequence) as a single-fusion protein in *E.coli* systems was better against the majority of tested microorganisms compared with the activity of individual proteins. In a sense, this (finding) is a surprise since F2A does not display any cleavage activity in *E.coli* [54]. Nevertheless, increased antibacterial and antifungal activity was reported in tobacco and potato plants expressing the snakin-defensin hybrid.

The expression of plant proteinase inhibitors is one strategy for increasing resistance against insects. The maize serine proteinase inhibitor (MPI) and the potato carboxypeptidease inhibitor (PCI) were co-expressed in rice using two strategies (Figure 2, Panel D) [117]. The first was to link the two gene sequences into a single ORF *via* a sequence encoding the proteinase processing site of the *B. thuringiensis* Cry1B precursor protein. The rationale here is that the translation (fusion protein) product would be post-translationally cleaved into MPI and PCI by an endogenous rice proteinase. The second strategy was to link the *mpi* and *pci* genes *via* 2A: here, the translation products would be translated as discrete products ([MPI-2A] +PCI). Both co-expression strategies were successful and both types of rice transgene showed increased resistance to the striped stem borer (*Chilo suppressalis*). Whilst both strategies benefit from co-expression using a single transgene, the additional merit of using 2A for similar approaches is that post-translational processing by an endogenous proteinase limits the subcellular targeting of proteins downstream of the Cry1B linker to the cytoplasm or other subcellular sites using post-translational import – not the exocytic pathway which requires the co-translational recognition of signal sequences by signal recognition particle. Since 2A works within the ribosome, proteins downstream of 2A can be modified to comprise N-terminal signal sequences and direct these translation product(s) to the exocytic pathway.

Glucosinolates (GLSs) present in cruciferous plants (e.g. cabbage, broccoli, and oilseed rape) play a defensive role against generalist insects [118] and pathogens [119]. However, crucifer-specialist insect herbivores like the economically important pest diamondback moth (DBM; *Plutella xylostella*) frequently use GLSs to stimulate oviposition [120]. An increase in the global area of brassica crops between 1993 and 2009 coupled with the high fecundity of DBM especially in tropical regions has resulted in development of resistance to many broad-spectrum insecticides used in the field [121]. Recently, genetic engineering has been used to produce non-host GLS-containing plants as a first step towards the creation of "dead-end trap crops". The transfer of the six-step benzylglucosinolate (BGLS) pathway into tobacco plants using only two ORFs, consisting of the first three genes (*GGP1-F2A-CYP83B1-F2A-CYP79A2*) and last three genes (*SOT16-F2A-UGT74B1-F2A-SUR1*), give rise to BGLS-producing plants [122,123]. Importantly, these non-host plants were more attractive for DBM oviposition than wild-type tobacco plants. As larvae are unable to survive, the strategy of engineering ovipo-

sition cues into non-host plants offers an alternative trap crop approach to crop protection. The combination of abiotic and biotic stresses presents an added degree of complexity, as responses at a molecular level are largely controlled by different signalling pathways that can act antagonistically [124]. The pyramiding of several defence genes may therefore provide further opportunities for creating broad-spectrum stress tolerance in agronomically important crop plants [125].

3.4. Cost-effective production of cellulose degrading enzymes for biomass-to-fuel conversion

Dwindling fossil resources and increasing energy demands are driving the development of alternative feedstocks for producing fuels and chemicals. Cellulosic feedstocks such as crop residues, wood products and dedicated crops (e.g. switchgrass, salix) are among leading alternatives because they are sufficiently abundant, low cost and do not compete with food sources. The bioconversion of lignocellulose biomass into fuels involves three major transformations: the production of saccharolytic enzymes (cellulases and hemicellulases), the hydrolysis of carbohydrate components present in pretreated biomass to sugars, and fermentation of sugars to produce fuels such as ethanol and butanol [reviewed in 126, 127]. Unfortunately, the high cost of enzymes is a major barrier in the biomass-to-fuel industry [128]. Observed results indicate *in planta* enzyme expression offers a potential method for low cost large-scale enzyme production [129, 130]. Current indications are subcellular targeting [131, 132] and simultaneous expression of recombinant cellulolytic enzymes [133] are key factors in optimizing their accumulation in transgenic plants. Transgenic expression of 2A-linked cellulase enzymes (β-glucosidase, BglB; xylanase, Xyl11; exoglucanase, E3; endoglucanase, Cel5A) in chloroplast-targeted tobacco plants induced synergistic effects that led to more efficient hydrolysis of lignocellulose materials for bioethanol production [134]. Chloroplast transit peptides, small subunit of Rubisco complex (Rs) and Rubisco activase (Ra) [135], were fused to the N-terminal of the enzyme genes. This study found a synergistic effect between BglB and Cel5A in the (RsBglB-F2A-RaCel5A) lines and between E3 and Cel5A expressed in the (RsE3-F2A-RaCel5A) lines. The enzymes had higher activities which led to enhanced carboxymethyl cellulose (CMC) hdydrolysis into glucose and cellobiose. A similar observation was made by Jung et al., [2010] with chloroplast-derived BglB and Cel5A [136]. Furthermore, supplementing the protein extracts of transgenic (RsBglB-F2A-RaCel5A) with CBH11 exoglucanase increased hydrolysis activity. While the cost or yield may not be the same for all chloroplast-derived enzymes, these are important steps in cellulose bioconversion.

3.5. Recombinant plant viruses as (Co-)expression systems

Plant viruses are both important crop pathogens and very efficient vectors for transient protein over-expression. The majority have comparatively small RNA genomes, encoding a limited number of genes. Modifying such viruses for research or biotechnology purposes is often impeded by limitations imposed by the genome organization. Absolute genome size may be restricted by virion capsid size, open reading frames can overlap each other as well as regulatory sequence elements, and duplication of regulatory sequences to drive expression of

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foreign genes can lead to genomic instability. 2A peptides can be used to overcome some of these limitations.

One major use for plant virus-based over-expression vectors is the production of immunogenic epitopes in plants. Using plants as hosts has the benefits of a eukaryotic expression system with fast growth rates that can be produced on large scales and shares no pathogens with humans or animals. Viral vectors naturally achieve extremely high over-expression levels and can systemically infect whole plants from small, inexpensive inocula and avoiding the requirement to produce transgenic plants. To achieve this, the modified virus has to retain its infectivity and ability to move through the plant. A common approach to overexpress foreign proteins from a plant virus genome is to fuse them to the capsid protein (CP). CPs are often the most highly expressed viral proteins and this ensures efficient overexpression without the need to re-engineer any regulatory sequences in the viral genome. If the epitopes are displayed on the virus particle surface, they are also easy to purify. However, CP fusions are not always tolerated as they can interfere with viral encapsidation and spread. 2A peptides can be used to rescue encapsidation and infectivity of CP-fusion viruses by providing a pool of unfused CP.

The first such use in a modified plant virus was the *Potato virus X* (PVX) 'overcoat' virus, in which a green fluorescent protein is fused to the N-terminus of the CP *via* an FMDV 2A linker [137]. The 2A-mediated 'cleavage' is incomplete so that both free CP and the GFP-CP fusion are produced, enabling formation of fully infectious virus particles which also incorporate the GFP-CP fusion. GFP is exposed at the virion surface, permitting tracking of infection and imaging of virions [137]. A similar 'overcoat' principle was also used to image infections of the related *Plantago asiatica mosaic virus* (PlAMV) [138]. Obviously this overcoat approach also enables the fusion of other peptides or proteins to the PVX CP. This was first demonstrated by expression of a single chain antibody against the herbicide diuron [139]. Approximately 100-250 μg protein/g leaf fresh weight of the antibody was produced, and 'overcoat' virions were easily purified by diuron-based immune-capture.

Subsequently, a number of antigenic epitopes have been expressed using PVX 'overcoat' vectors, including Rotavirus inner capsid protein [140], *Classical swine fever virus* glycoprotein [141], tuberculosis antigen ESAT-6 [142], and a consensus epitope from a *Hepatitis C virus* envelope protein [143]. Between 1-125 μg protein or virus/g leaf fresh weight, or up to 0.5-1% of total soluble protein, were obtained in these studies, and 'overcoat' virions could be purified by centrifugation [141, 143]. In one case, greater amounts of protein were achieved with an alternative expression strategy using a duplicated subgenomic promoter [140], but such constructs tend to be genetically very unstable compared to 2A-based 'overcoat' vectors [144]. PVX virions carrying the *Classical swine fever virus* glycoprotein epitope produced an immunoprotective response in rabbits [141], and antibodies from a mouse immunized with PVX particles displaying the hepatitis C virus R9 epitope reacted with sera from infected patients [143]. For smaller epitopes, direct fusion to the PVX CP was sometimes possible, but for all larger foreign peptides, the 2A-mediated partial separation was required to enable encapsidation of systemically infective virus [143]. Filamentous viruses like PVX are particularly suited for surface presentation of antigens as they have a large number of CP units (~1270/PVX

virion), and 'overcoat' overexpression vectors using 2A have also been developed for the related viruses *Pepino mosaic virus* (PepMV) [144] and PlAMV [145]. So far, plant virus-expressed epitopes have been purified mainly as virion-attached surface peptides, but with 2A linkers, it is also possible to produce them mainly as free proteins. For efficient surface display, an optimal ratio of free and fused CP has to be found that maximizes virus-displayed epitopes whilst still enabling efficient encapsidation. The range of 2A-like sequences with different 'cleavage' activities [31, 37] will be useful in the development of further 'overcoat' vectors.

Expression vectors using 2A peptides have also been developed based on *Cowpea mosaic virus* (CPMV) [146], *Bean pod mottle virus* (BPMV) [147], and *Wheat streak mosaic virus* (WSMV) [148]. In the case of the CPMV and WSMV vectors, 2A linkers were used to overcome the problem that these viruses encode large polyproteins that are processed into functional subunits by viral proteases. Foreign proteins inserted into the polyprotein open reading frame need to be released and 2A sequences provide an alternative to viral protease cleavage sites. In CPMV, using 2A instead of viral cleavage motifs reduced the number of additional amino acids attached to the over-expressed protein, and as in PVX 'overcoat' vectors, some of the foreign protein is displayed on virus particles, which are very stable and easy to purify. In WSMV, use of FMDV 2A or (and also FMDV 1D/2A) sequences resulted in more efficient release of the foreign protein (GFP) than with viral proteinase sites [148]. WSMV vectors enable protein expression in cereal hosts. In the soybean-infecting BPMV vectors, 2A linkers were used both to enable insertion of foreign genes into the viral polyprotein open reading frame, and to facilitate simultaneous co-expression of two different foreign proteins.

4. Food for thought

The first demonstration the 2A was active in plant cells used an artificial polyprotein which comprised two reporter proteins flanking 2A [38]. This co-expression system was soon adopted by plant virologists for use in both rod-shaped and icosahedral virus particles either as high-level expression systems, or, to produce particles 'decorated' with fluorescent proteins, immunogens, single-chain antibodies etc. [137-147]. Here, plants are used simply as 'bioreactors' for production of recombinant proteins / virus particles – the plants are not transgenic. In the case of transgenic plants the first reports of the use of 2A to co-express multiple proteins were as a 'proof-of-principle' or research tools [38, 57], but within a few years plants were being genetically engineered to demonstrate how nutritional properties could be improved [105, 149]. Whilst the use of 2A rapidly expanded in the arenas of animal biotechnology and biomedicine (e.g. monoclonal antibody production, cancer gene therapies, production of pluripotent stem cells: reviewed in [25]), progress in transgenic plants was slower-due to a number of reasons, including the 'trickle-down' effects on plant biotechnology from the EU policies concerning genetically-modified plants. Over the past few years, however, the 2A co-expression system has been used in the development of methods to engineer plant genomes [149], the expression of high-value proteins, the improvement of plant tolerance to biotic and abiotic stresses, the improvement of nutritional properties through metabolome engineering

[*vide supra*] and the expression of plant storage proteins with amino acid content more suited to human nutrition [150]. The drive to improve agricultural productivity through the development of 'dual-use' crops necessitates complex strategies of plant engineering and it seems clear that the use of 2A will continue to expand.

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Chapter 9

Synthetic Biology and Intellectual Property Rights

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Additional information is available at the end of the chapter

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1. Introduction

The pioneering work of Cohen and Boyer in recombinant DNA (deoxyribonucleic acid) technology [1] gave birth to genetic engineering and the biotechnology industry. The related Cohen-Boyer patents [2-4] that protected the technology played a stellar role in the rapid rise of the biotechnology industry [5, 6]. The next landmark was the creation of a bacterial cell controlled by a chemically synthesized genome by Craig Venter and his group in 2010 [7]. More recently, Floyd Romesberg and colleagues in 2014 [8] reported the creation of a semi-synthetic organism with an expanded genetic alphabet that has raised both hope and fear [9]. The new letters in the alphabet are artificially created nucleotides not found in Nature. Along with these breakthroughs, the great promise of CRISPR (clustered regularly interspaced short palindromic repeats), and in particular CRISPR-Cas9 gene editing technology pioneered by Feng Zhang in 2012 [10] as a new way of making precise, targeted changes to the genome of a cell or an organism (see Section 2.3) has set the stage for major advances in synthetic biology, which aims to design and construct new biological parts, novel artificial biological pathways, organisms or devices and systems including the re-design of existing natural biological systems for useful purposes.

Researchers are now focussing on developing tools and methods that would enable them to encode, in artificially created or natural DNA, basic genetic functions in novel combinations by design. The aim is to artificially create biological systems of increasing size, complexity, and tailored functionality. Currently synthesis capabilities far exceed design capabilities in the sense that we know how to build but not yet with clarity what to build [11]. Synthesis capabilities are developing at a pace where DNA synthesis can be automated and the desired DNA produced once the sequence is provided to vendors. This integration of biology and traditional engineering is occurring so rapidly, it appears likely that a couple of decades hence researchers may begin producing synthetic organisms that can produce not only pharmaceutical products but also industrial

products such as bio-fuels on a commercial scale. Possible socio-economic benefits from synthetic biology research is thus enormous, but then so is the possibility of the technology's misuse. The concerns range from bioethical and environmental worries to bio-terrorism, say, by malicious release of genetically engineered viruses targeted at specific ethnic groups. The main concern here is the illegal creation and growth of bio-weapons.

The socio-economic promise of synthetic biology has spurred both public and private investments and made people introspect about its consequences and impact on human society. All players involved in creating and commercialising this knowledge-and-capital intensive emerging technology are obviously deeply interested in knowing how they would gain or lose from the intellectual property (IP) system in place and whether that system needs to be changed, replaced, or abolished from their respective perspective.

DNA as an information carrier gained currency in the 1950s with the discovery of the double-helix structure of cellular DNA by James Watson and Francis Crick in 1953 [12]. Prior to that biologists talked of biological "specificity". In 1953, Watson and Crick noted: "...it therefore seems likely that the precise sequence of the bases is the *code* which carries the genetical *information*..." (Emphasis added) [13]. Now the language of information is pervasive in molecular biology—genes are linear sequences of bases (like letters of an alphabet) that carry information (like words) for the production of proteins (like sentences). The process of going from DNA sequences to proteins we use words like "transcription" and "translation", and we talk of passing genetic "information" from one generation to another. It is rather uncanny that molecular biology can be understood by ignoring chemistry and treating the DNA as a computer program (with enough input data included) in stored memory residing in a computer (the cellular machinery). It is this aspect that bioinformatics exploits. It is analogous to viewing Euclidean geometry not in terms of drawings but in terms of algebra. In our current understanding, DNA is an informational polymer. It is a vast chemical information database that *inter alia* carries the complete set of instructions for making all the proteins a cell will ever need. As Albert Lehninger lyrically put it, understanding the DNA is the study of "the molecular logic of the living state." [14].

The intellectual property (IP) system, as it stands, did not anticipate the convergence of the patenting of information carrying living matter, a knowledge-based global economic system, and the ascendancy of a research-centric and innovative biotechnology industry. Therefore, the IP system is already under great strain because biotechnology related IP has been patched onto an existing patent system in an *ad hoc* manner. For example, in the complex legal maze, intellectual property rights (IPR) related to DNA synthesis, which is at the core of synthetic biology, may be inadvertently infringed by DNA synthesis companies in terms of enforceable trade secret, trademark, copyright or patent laws, simply by constructing DNA sequences for their clients [15].

That the DNA is an information encoded molecule, makes the interpretation of IP laws that much more difficult by judges who are generally ignorant about the deep science that supports biotechnology. Indeed organisms are defined by the information encoded in their genomes, and since the origin of life that information is believed to have been encoded using a two-base-

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pair genetic alphabet (A–T and G–C). Recent research has expanded the alphabet to include several man-made unnatural base pairs (UBPs) which can be efficiently PCR-amplified and transcribed in vitro and whose unique mechanism of replication has been characterized. Clearly, the expansion of an organism's genetic alphabet leads us into unknown scientific territory related to DNA replication, gene expression, unknown proteins, DNA repair, etc. [8]. While the core principles of synthetic biology are common to those of well-practised recombinant DNA techniques, the biggest differences lie in the size, scope, accuracy, and speed of genetic changes that can now be accomplished [16]. Note that genetic modification incorporates DNA from one species into another; genome editing introduces new mutations into an organism's own DNA (similar to what Nature does or we do through selective breeding but on an accelerated time scale).

The critical IP issue in synthetic biology is determining, in an equitable manner, the nature of the IP rights to be allocated, to whom they should be allocated and the context in which they should be allocated for the overall socio-economic benefit of society. This chapter therefore briefly introduces synthetic biology and its relevance to human society, the intellectual property it may generate, equitable modes of protecting the generated intellectual property, and suggests changes to patent laws keeping in mind the changing socio-economic circumstances in which it must operate.

This chapter is written for young researchers and students in synthetic biology for whom a basic understanding of IPR issues related to their subject has assumed great importance.

2. Synthetic biology — Its aims and relevance

Synthetic biology is a revolutionary development in life sciences. It is highly multidisciplinary where molecular biology, physical sciences and engineering merge to design and construct new biological parts, novel artificial biological pathways, organisms or devices and systems including the re-design of existing natural biological systems for useful purposes. We may call it bioengineering. It has already produced tumour-seeking microbes for cancer treatment, photosynthetic systems to produce energy, artificial life, etc. Like engineering, it too aims to produce standardized components and connectors, manufacturing and assembly processes, test vehicles and certification processes, etc. to enable production and marketing of increasingly sophisticated and functional systems on a mass scale. In a sense, "Synthetic biology is the engineering of biology: the synthesis of complex, biologically based (or inspired) systems which display functions that do not exist in nature. This engineering perspective may be applied at all levels of the hierarchy of biological structures – from individual molecules to whole cells, tissues and organisms. In essence, synthetic biology will enable the design of 'biological systems' in a rational and systematic way." [17].

Enormous expectations rest on future advancements in systems biology as it has the potential to radically change the way we approach key technologies, such as medicine and manufacturing. Current efforts have focused on creating highly generic capabilities (the building

blocks) in the form of bio-tools and bio-processes that can be scaled for industrial application. Given the high intellectual calibre of the synthetic biology research community, it appears inevitable that the scientific knowledge they produce and place in the public domain will quickly be translated into industrial applications of high economic value by equally talented industry researchers. This raises obvious concerns about the ownership and control of generated intellectual property that may lead to high commercial value in a twenty-first century economy that truly belongs to the life sciences. The key enabling technology is DNA synthesis. The workspace includes microbes, mammalian cells, plants, etc. Its applications include therapeutics, energy (e.g., fuels), chemicals, agriculture, etc.

The pioneering paper of Watson and Crick [12] that elucidated the double helix structure of cellular DNA has been hailed as the greatest discovery in biology since Darwin's theory of evolution. In their paper, they showed that the structure was made possible by the unique base pairing of nucleotides guanine (G) with thymine (T), and adenine (A) with cytosine (C), each member of a pair belonging to opposing strands. It is this pairing that allows base pairs to be arbitrarily stacked as a double helix. In a famous understatement, they wrote: "It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material." It was the potential for explaining biological function of DNA that led to the widespread acceptance of the double helix model rather than any compelling structural evidence. The helical structure was not rigorously determined by X-ray crystallography until the late 1970s [18]. Whereas cells were regarded as the basic building blocks of living organisms during the nineteenth century, the Watson and Crick paper [12] shifted attention from cells to DNA molecules in the middle of the twentieth century, when geneticists began to seriously explore the molecular structure of genes.

In his 2013, State of the Union message, President Barack Obama said:

> If we want to make the best products, we also have to invest in the best ideas... Every dollar we invested to map the human genome returned $140 to our economy... Today, our scientists are mapping the human brain to unlock the answers to Alzheimer's... Now is not the time to gut these job-creating investments in science and innovation. Now is the time to reach a level of research and development not seen since the height of the Space Race.[1]

On 02 April 2014, President Obama unveiled a bold new research initiative designed to revolutionize our understanding of the human brain.[2] The BRAIN (Brain Research through Advancing Innovative Neurotechnologies) initiative's ultimate aim is to help researchers find new ways to treat, cure, and even prevent brain disorders, such as Alzheimer's disease, epilepsy, and traumatic brain injury. Undoubtedly, synthetic biology will play a signal role in this initiative and much of the needed basic research will happen in the universities.

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1 See http://www.whitehouse.gov/the-press-office/2013/02/12/president-barack-obamas-state-union-address.

2 Fact Sheet: BRAIN Initiative, The White House, 02 April 2013, http://www.whitehouse.gov/the-press-office/2013/04/02/fact-sheet-brain-initiative

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2.1. DNA carries information

DNA is Nature's digital recording medium. The molecular instructions for creating living organisms are encoded in the complex DNA molecule, a portion of which passes from parent to offspring during the reproduction process. Natural DNA is a linear sequence of four types of nucleotides: A, T, G, and C. Each organism's DNA sequence is unique and autobiographical; it determines an organism's unique characteristics, *e.g.*, the colour of a person's eyes, the shape of his nose, his resistance to disease, etc. Other molecules in a biological cell "read" the DNA sequence and set in motion the physical and chemical processes the cell calls for. For example, the vast information carried by the DNA includes the complete set of instructions for making all the proteins a cell will ever need. Over the years biologists have discovered certain tricks for manipulating DNA in a manner similar to manipulating character strings in a text. For example, they can copy DNA fragments using the polymerase chain reaction (PCR) or clone it using a cloning vector; cut DNA using molecular scissors called restriction enzymes; join two complementary DNA strands into a double-stranded molecule in a process called hybridization; and measure the size of DNA fragments without sequencing them using a technique called gel-electrophoresis. The enormous potential of CRISPR genome editing technology lies in its ability to precisely insert DNA into a cell in vivo. For example, CRISPR, allows one to snip out mutated DNA and replace it with the correct sequence. It thus offers possible means of treating many genetic disorders [19].

2.2. The extended DNA alphabet

Since the late 1990s, researchers have discovered that DNA construction can be extended beyond the natural bases (C, G, A, and T) to include man-made ones and artificial DNA constructed. An expanded "DNA alphabet" will obviously allow cramming of more information by way of larger variety of coding patterns for a given number of nucleotides comprising a DNA strand, *e.g.* an extended genetic code and thus enable a wider range of applications from precise molecular probes and nano-machines to useful new life forms. [20, 21].

Watson and Crick [12] showed that natural bases form two base pairs (A-T, G-C) as a result of specific hydrogen bonding patterns. The unnatural base pairs created by Romesberg's group [8] too pair stably and selectively in DNA. These new base pairs draw upon unnatural hydrogen-bonding topologies as well as upon shape complementarity and hydrophobic forces as opposed to only hydrogen bonding in natural pairs and are also synthesized with high fidelity by DNA polymerases. Romesberg *et al* have succeeded in creating DNA strands using the two natural base pairs and a third unnatural base pair of their design with high fidelity [8]. In a sense, researchers may well be anticipating and pre-empting evolutionary events that left to themselves would have taken a few million years to occur.

2.3. CRISPR technology

CRISPR technology is a new way of making precise, targeted changes to the genome of a cell or an organism. CRISPRs are often associated with cas genes that code for proteins related to CRISPRs. By inserting a plasmid containing cas genes and specifically designed CRISPRs, an

organism's genome can be cut at any desired location. Since its invention in 2012, the CRISPR/Cas system has been widely used for gene editing (silencing, enhancing or changing specific genes) in basic research. The importance of the CRISPR/Cas adaptive immune system is that it is a prokaryotic immune system that confers resistance to foreign genetic elements such as plasmids and phages and provides a form of acquired immunity. CRISPR spacers recognize and silence these exogenous genetic elements like RNAi in eukaryotic organisms. It is in building the elaborate system of DNA-cutting proteins and guide RNA sequences that requires extensive engineering to function in eukaryotic cells, and to insert new genes where the targeted host DNA is excised. For a quick introduction to CRISPR technology, see [22].

On 15 April 2014, the USPTO issued the first patent (US8697359, CRISPR-Cas systems and methods for altering expression of gene products) to cover CRISPR-Cas9 gene editing technology to Feng Zhang, the sole inventor, just six months after the patent application was filed on 15 October 2013. The patent is assigned to MIT, and the Broad Institute with Broad managing the patent's licensing. The patent claims a modified version of the CRISPR-Cas9 system that is found naturally in bacteria and which microbes use to defend themselves against viruses. The patent, *inter alia*, claims methods for designing and using CRISPR's molecular components. It is widely expected that Broad will adopt a liberal licensing policy that would make the technology available to scientists for research around the world.

CRISPR is already revolutionizing biomedical research because it provides a very efficient way of recreating disease-related mutations in lab animals and cultured cells. It also holds the promise of treating genetic diseases in humans in unprecedented ways, *e.g.*, by directly correcting mutations on a patient's chromosomes. Mental illnesses too may find similar remedies. Since 2012, CRISPR's use in research has spread like wildfire. The chemistry behind the Cas9 protein is still being explored.

2.4. NGS + CRISPR technologies

The first generation DNA sequencing developed in 1975 by Edward Sanger [23] remained the gold-standard for two and a half decades. It was used in the Human Genome Project that cost $3 billion and 13 years to sequence the human genome and was completed in 2003. In comparison, next-generation sequencing (NGS) use non-Sanger based, high-throughput technologies to sequence millions and billions of DNA strands in parallel, are much faster and cheaper. In fact, an entire genome can be sequenced in a day. And when it is coupled with powerful computational algorithms, say, to answer questions related to mutational spectrum of an organism on a genome-wide scale, we have phenomenal opportunities to understand our biological selves. Targeted sequencing facilitates discovery of disease causing mutations for diagnosis of pathological conditions, and of genes and regulatory elements associated with disease [24, 25]. For trends in DNA sequencing costs, see http://www.genome.gov/sequencingcosts/. (In 2014, it was less than $0.1 per raw mega-base of DNA sequence compared to about $1k in 2004; during 2007-2010, the cost fell sharply.) NGS is not yet ready for clinical use.

For recent advances in CRISPR-Cas9 technology see [26]. In principle, NGS and CRISPR technology together would allow one to change a genome at will to almost anything one wants and even elicit enough detailed information about disease risks, ancestry

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and other traits of a person to determine his identity. Clearly, such advances raise privacy, ethical, legal, and other social issues that are presently barely understood and therefore need careful study. A NIH initiated study in the U.S. [27] notes: "The ongoing evolution of genomic research and health care requires a continuing analysis of the normative underpinnings of beliefs, practices and policies regarding research, health and disease. In addition, as personal genomic information permeates many aspects of society, it has profound implications for how we understand ourselves as individuals and as members of families, communities, and society--and even for how we understand what it means to be human. Long-held beliefs about the continuum between health and disease may be transformed, as will concepts of free will and responsibility."

3. Intellectual property rights — Its aims and relevance

Forms of intellectual property rights (IPR) are copyright, trademark (including service mark and geographical indication), trade secret, and patent. Depending on the type, government granted rights enable owners to select who may access and use their property and to protect it against infringement. Since the protections granted by a government vary from jurisdiction to jurisdiction, the acquisition, registration, or enforcement of IPR must be pursued or obtained separately in each territory of interest. Intellectual properties, in general, are creative ideas and expressions of the human mind that have commercial value. The owner of an intellectual property can generally transfer (with or without consideration), license (or rent), or mortgage it to third parties. Most exclusive rights are nothing more than the right to sue an infringer. Those wishing to use an intellectual property held by another, must license it from the owner. In many jurisdictions the law places limits on the restrictions the licensor can impose on the licensee.

A *license* results if the IP owner transfers less than all of his IP rights. The party receiving the license is called the licensee. If the license is given to only a single person and pursuant to the terms of the license is not permitted to license others, the license is termed an *exclusive license*. If licenses are given to multiple parties or to one person reserving the right to license it to others at a future date, the license is termed a *nonexclusive license*. In the case of an exclusive license, the owner of the intellectual property cannot make, use, or sell the intellectual property unless he has expressly reserved the right to do so in the license agreement. If all the rights are transferred to someone, it is known as an *assignment*. Variations in the terms of a license agreement are virtually limitless, depending upon the needs, desires, and bargaining positions of the parties involved.

3.1. Copyright

Copyright is an exclusive right conferred by a government on the creator of a work (*e.g.*, original literary, dramatic, musical, artistic works in books, recordings, films, videos, etc.) to exclude others from reproducing it, distributing it to the public, performing it in public, or displaying it in public. Copyright law protects the holder's right to decide how and where his

material is used, not just the right to earn profits from the work. Copyright protection comes into effect immediately upon the creation of something that can be protected and is 'fixed' in some way, *e.g.*, on paper, on film, on electronic media (including the Internet), etc. It is not necessary to register a copyright or take any official action to obtain it. However, registration is advisable as it strengthens the owners claim to copyright in litigation. The doctrine of fair use allows non-owners of a copyright work to use such work in a limited way without being accused of infringement.

Things that cannot be copyrighted include abstract ideas, procedures, processes, systems, methods of operations, concepts and principles, regardless of how they are expressed, whether it be by words, illustration, or in some three-dimensional form. Of course, the manner in which they are expressed may be copyrighted, but not the labour that goes into creating a work. Unfortunately, the line between copyrighted material and non-copyrightable ideas, wherever it is drawn, will seem arbitrary to many.

3.2. Trademark and other marks

A mark used in trade—trademark, service mark, certification mark, collective mark, geographical indication—is any sign which can distinguish the goods and services, as appropriate, of one trader from those of another. A mark may be words, logos, pictures, shape of a product or container, or a combination of these. (Certain kinds of marks are not permitted, *e.g.*, marks which are immoral, deceptive, or scandalous, national symbols, national flags, etc.) A trademark serves to identify the origin of goods and creates goodwill for the owner; it signifies that all goods bearing the mark come from or are controlled by a single source and are of specified quality.

3.3. Trade secret

It is any device or information that gives an advantage over competitors who do not know about it or do not use it. Its value lies in its secrecy. Its owner is responsible for protecting it (*e.g.*, through non-disclosure agreements, by restricting access, etc.). Infringement of a trade secret is a type of unfair competition. The subject matter of trade secrets usually includes sales methods, distribution methods, consumer profiles and advertising strategies, lists of suppliers and clients, and manufacturing processes. The Coca Cola recipe is a famous example of a trade secret. What information constitutes a trade secret is case specific. Unfair practices related to trade secrets obviously include industrial or commercial espionage, breach of contract, and breach of confidence.

3.4. Patent

A patent is a limited period monopoly property right granted to an inventor for his invention by a Government subject to prescribed conditions, which include that the invention must be novel, nonobvious to those ordinarily skilled in the art, useful, and fully disclosed. Four types of inventions are eligible for such utility patents: process, machine, manufacture, or composition of matter. They are known as statutory subject matter. In exchange of a patent, the inventor

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describes the secrets related to the invention, publishing them as per law for all to see, absorb, and improve upon but not infringe. This description must be so clear and detailed as to enable a person skilled in the technologies relevant to the said invention to independently reproduce the invention (enablement requirement) without undue extra-solution activity, such as further research, data gathering, etc. on his part. In fact, this description should leave no doubt that the patent applicant was in possession of the claimed invention at the time of filing his application. Patents may be sought on non-trivial improvements over existing inventions. The life of a granted patent is usually 20 years from the filing date of the first valid patent application claiming the invention.

Patent monopoly differs from market monopoly; a patent is a right to exclude, a right to prevent trespassing. In this sense it is similar to, say, the right to keep one's personal properties free from trespassers. A patent grants its owner the right to exclude others from making, using, selling or offering to sell, and importing the claimed invention in the country of grant; it does not confer any right to practice the invention. This is because in practicing the invention, one may well need complementary patents held by others unwilling to license or there may be other laws, rules or regulations that prevent its practice. Patents are issued only to the first inventor (or group of joint inventors) of an invention who files a legally valid patent application; all others are barred, even if they independently created the invention. Consequently, those other inventors must get a license from the first inventor if they wish to practice the invention. Although grant of patents is subject to country-specific constraints, there is universal agreement among nations that patents seeking pre-emptive monopoly of abstract ideas (*e.g.*, mathematical formulas), laws of nature, natural phenomena, and products of nature are ineligible. What else to exclude from patent monopoly is a national prerogative, largely dependent on government policy related to prevailing socio-economic conditions it must manage, and international treaty obligations.

Two recent rulings by the Supreme Court of the United States (SCOTUS) are of importance to the biotechnology industry since it invokes the dictum that "laws of nature, natural phenomena and abstract ideas" are not patentable. In March 2012, the court ruled against Prometheus Laboratories in California observing that it could not patent metabolite levels to guide drug dosing [28]. Then, in June 2013, the court struck down a patent claim by Myriad Genetics of Utah that linked certain DNA sequences to female breast cancer [29]. It held that a naturally occurring DNA segment is a product of nature and its mere isolation does not make it patentable. However, cDNA may be patentable as it is not a naturally occurring substance. Thus to get a gene patent one will have to show that it is significantly different from any natural gene. However, in a diametrically opposite ruling, the Federal Court of Australia in *D'Arcy v Myriad Genetics Inc.* [2014] FCAFC 115 on 05 September 2014 ruled unanimously that isolated DNA and RNA are patentable subject matter under Australian law (Patents Act 1990 (Cth) s 18(1), Statute of Monopolies s 6). To say the least, this makes gene patenting a complex issue if such patents are sought in multiple countries.

Governments grant patents to human inventors only. Post-grant they may be assigned to people or institutions. A patent granted by a government is enforceable only in the territory it governs. One may, however, seek patents for the same invention from multiple countries.

Patent laws of a country do not over-ride its other laws that might regulate the invention's use. Patent laws of a country may take into account moral, cultural, ethical, social, environmental, or scientific concerns of society. Patent rights may be exercised by the patentee, his heirs or assigns. When a patent expires, the related invention becomes the common heritage of mankind.

Limited period patent monopoly may provide an enormous first mover advantage to an entrepreneur, especially if it involves new technology that could lead to a natural monopoly.

3.5. Traditional knowledge

The World Health Organisation (WHO) defines traditional medicine as [30]:

> Traditional medicine is the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness.

This knowledge, much of it undocumented and available only to small groups of people through oral transmission from generation to generation, predates molecular biology by centuries and hence belongs to prior art (public domain). Its importance to synthetic biology is that such knowledge may provide promising directions of research in the hunt for exotic genes.

4. IP outputs of synthetic biology and public concerns

Deciphering the working of a cell, leave alone creating an artificial one, is far more than just listing its constituent parts, *e.g.*, listing its genes. We also need to know how the parts connect and operate together, *e.g.*, how genes and proteins interact to, say, form larger modules and circuits analogous to those in electronic systems. More sophisticated conceptual understanding is needed to advance synthetic biology towards rational construction and redesign of biological circuitry. In addition, development of new computer models, computational algorithms and experimental techniques are needed for exploring gene interactions. Already known techniques, such as chemical modification of proteins and splicing and rearrangement of genetic information in the DNA have matured to a level where they can be used to redesign basic molecular interactions and pathways of living cells. Further, the development of machines and methods for rapid synthesis of DNA with specified sequences has made it possible to build wholly synthetic, highly complex collections of genes and even to synthesize living organisms from the genome up. In fact, biology inspired templates for engineering nanostructures is emerging as a dominant research theme.

Notable contributions in synthetic biology include those from Dae-Kyun Ro, *et al*, Production of the antimalarial drug precursor artemisinic acid in engineered yeast, [Nature, 2006] in therapeutics; Marc Gitzinger, *et al*, Controlling transgene expression in subcutaneous implants using a skin lotion containing the apple metabolite phloretin, [PNAS, 2009] in therapeutics;

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Shota Atsumi, *et al*, Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels, [Nature, 2008] in fuels; Yoon Sung Nam, *et al*, Biologically template photocatalytic nanostructures for sustained light-driven water oxidation, [Nature Nanotechnology, 2010] in solar energy; John E. Dueber, *et al*, Synthetic protein scaffolds provide modular control over metabolic flux, [Nature Biotechnology, 2009] in chemicals; Tae Seok Moon, *et al*, Use of modular, synthetic scaffolds for improved production of glucaric acid in engineered E. coli, [Metabolic Engineering, 2010] in chemicals; Michael B. Elowitz & Stanislas Leibler, A synthetic oscillatory network of transcriptional regulators, [Nature, 2000] in biological computing, programmability; Eileen Fung, *et al*, A synthetic gene-metabolic oscillator, [Nature, 2005] in biological computing, programmability; Marcel Tigges, *et al*, A tuneable synthetic mammalian oscillator, [Nature, 2009] in biological computing, programmability; Tai Danino, A synchronized quorum of genetic clocks, [Nature, 2010] in biological computing, programmability; Daniel G. Gibson, *et al*, Creation of a bacterial cell controlled by a chemically synthesized genome, [Science, 2010], in extending the principles of design and construction to whole organisms; Denis A. Malyshev, *et al*, A semi-synthetic organism with an expanded genetic alphabet, [Nature, 2014], in expanding the genetic code to incorporate unnatural nucleotides and base pairing; Cong, L., *et al*, Multiplex Genome Engineering Using CRISPR/Cas Systems, [Science, 2013], in gene editing.

Research efforts in synthetic biology are largely concentrated in the United States and to a substantially lesser degree in the European Union. Currently no country has the necessary framework for coordinating its research activities, fostering a community of researchers, and creating a forum for the establishment of goals, shared tools, and professional standards. Biological research, more than ever, needs to address ethical and safety concerns of society, especially with respect to synthetic biology if the research community is to gain public trust without raising Frankensteinian fears.

In general, perceived safety and investment risks involved in converting proof-of-concept products and processes developed in laboratories and making them market ready are very high and intimately related to the mode of IP dissemination, *e.g*., open source, patents, IP commons, and private law initiatives. The last is based on contractual agreements that are basically binding among those involved and not on third parties. The open source movement has generally restricted itself to basic research outputs that form the foundation on which subsequent applied research depends. As synthetic biology results move out of research labs and migrate to industry to be integrated into marketable products, altruistic open source initiatives and private profit motive collide. The potential for fierce litigation suddenly arises whose source is the patent system, which has the unenviable task of delicately balancing the need to encourage innovation through grants of limited period monopoly and protect public interest through minimal free-market encroachment.

4.1. The bright side of IP outputs

Due to genetic engineering, modern biotechnology has progressed well beyond simply using natural strains, classic breeding, and strain selection to produce a variety of chemical products. Artemisinin, a critical ingredient in malaria drugs is now pro-

duced from yeast altered through synthetic biology. Rennet, a key processing aid in cheese making, since the 1990s has been made by a microbe altered with insertion of a single bovine gene and is in wide use in the U.S. Algal oil is produced by genetically modifying algae which is now used in making laundry detergent. Synthetic biology techniques are now used to coax bacteria, fungi and other organisms into producing substances they would not otherwise produce. Some of the micro-organisms synthetic biologists create to make ingredients like orange and grapefruit flavourings have passed the muster of the Environmental Protection Agency of the U.S. while the U.S. Food and Drug Administration says the ingredients they produce are "generally recognized as safe". Some companies also produce food-grade vanillin, resveratrol and citrus flavourings from yeast and other microorganisms via synthetic biology. Yet enough misgivings in public perception exist that companies shy away from admitting that some of their products are created or mediated by artificial organisms made possible by synthetic biology [31]. Nevertheless, synthetic biology continues to tackle far more ambitious goals. Here are some examples.

1. *Three-person IVF*. The Human Fertilization and Embryology Authority in the U.K. that regulates the use of human eggs, sperm, and embryos in treatment and research has assessed two types of in vitro fertilisation (IVF) methods: one that involves removing parental nuclei from a fertilized egg and placing them into a donor embryo from a second woman, and another that moves the nucleus from the mother's egg into a donor egg, which then can be fertilized. The aim is to help women with mitochondrial diseases have healthy babies. The report [32] noted that three-person IVF is expected to be ready for use in preventing the birth of children with mitochondrial disease through assisted conception in about two years. Its use on humans in the U.K. will need Parliamentary approval.
2. *Next generation sequencing*. Fourteen year old Joshua lay in a coma for weeks, his brain swelling with fluid due to an unknown cause. With parental approval, doctors ran a test with an experimental new technology that searched the child's cerebrospinal fluid for pieces of DNA that might belong to the pathogen causing his encephalitis. They were able to pinpoint the cause within 48 hours. The child had been infected with an obscure species of bacteria, which the doctors eradicated within days [33]. The technology although years away from clinical use has raised hopes of powerful diagnostic tools for presently undiagnosable diseases becoming available in the future.
3. *Exome sequencing*. In June 2014, researchers in the *Finding of Rare Disease Genes* (FORGE) project reported analysing 264 rare disorders using exome sequencing and identifying the causal mutations to 146 of them and identifying 67 novel genes [34].
4. *Whole-genome sequencing*. A recent paper in Nature [35] has suggested that whole-genome sequencing can diagnose severe intellectual disability in newborns even when standard tests don't. Based on data on 50 patients with severe intellectual disability and their unaffected parents, the genome-wide analysis found 84 novel sequence variations and 8 novel structural variations associated with the disability. Previous gene screens in the same patients had failed to identify disease markers. The results led to a diagnosis of 42 percent of patients studied. Can a synthetic biology remedy be far behind?

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5. *Gene editing technology*. In Nature Biotechnology [36] researchers reported the use of CRISPR-Cas9 to alter the genome of the human malaria parasite Plasmodium falciparum. This parasite has been difficult to manipulate with existing tools. Researchers were able to specifically disrupt chromosomal loci and generate marker-free, single nucleotide substitutions with high efficiency. They were also able to generate a strain of the protozoan resistant to a key malaria treatment.

6. *Solar energy*. An article in Nature [37] reported that in Caltech's Jorgensen's Lab more than 80 researchers are engaged in using inorganic material (silicon, nickel, iron, etc.) to create artificial photosynthesis. Their goal "is to use sunlight to make hydrogen and other fuels much more efficiently than real leaves ever made biomass." Making fuels using power from the Sun, which is effectively inexhaustible but also carbon-free would be a boon. While not synthetic biology, it is inspired by it.

7. *Genome transfer*. Researchers have found that allopolyploidization can also occur by asexual mechanisms. They have shown that "upon grafting—a mechanism of plant–plant interaction that is widespread in nature—entire nuclear genomes can be transferred between plant cells". They have created a new allopolyploid plant species from an herbaceous species and a woody species in the nightshade family. The new species is fertile and produces fertile progeny [38]. Synthetic biology, in conjunction with a potential asexual mechanism of speciation opens up vast new possibilities for the generation of novel allopolyploid crop species.

8. *De-extinction, reanimation*. Recreating extinct species is no longer far-fetched. Synthetic biology not only makes it feasible to revive them but also improve them by boosting their immunity and fertility, their ability to draw nutrition from available food, and to cope with environmental stress. Just as a new vaccine can reduce demand on medical resources, improved species make for better ecological compatibility and balance. Indeed, George Church is currently modifying genes from an Asian elephant to make them more mammoth-like [39].

4.2. The dark side of IP outputs

New technologies come with unknown risks of using and not using it! They have their share of scary stories and apprehensions. Construction of artificial life that goes well beyond traditional recombinant DNA technology, is both ambitious and ominous. But then modern civilization is the result of past risk taking. With older and mature technologies we gradually found ways of muting their dark side by enacting legislation and creating regulatory bodies.

While possible socio-economic benefits from synthetic biology are enormous, so is the possibility of its misuse. The concerns range from bioethical and environmental worries to bio-terrorism, say, by malicious release of genetically engineered viruses targeted at specific population groups. The main concern is the creation and growth of bio-weapons. They can be created surreptitiously, cheaply, on a mass scale, and released in a variety of inexpensive ways into the environment using a variety of delayed triggering mechanisms that would camouflage their presence. Bio-weapons make the lethality of atomic and nuclear weapons passé.

A panel of life sciences experts in 2003 noted [40]:

- "The effects of some of these engineered biological agents could be worse than any disease known to man."
- "The genomic revolution is pushing biotechnology into an explosive growth phase. … [T]he resulting wave front of knowledge will evolve rapidly and be so broad, complex, and widely available to the public that traditional intelligence means for monitoring WMD [weapons of mass destruction] development could prove inadequate to deal with the threat from these advanced biological weapons."

A decade later, these concerns have become more pronounced. The threat spectrum is diverse and elusive and already impossible to comprehensively defend against. The pace, breadth, and volume of the evolving scientific base in synthetic biology and its easy public accessibility makes the controlled development of bio-weapons a hopeless task.

4.3. The regulatory side of IP outputs

Synthetic biology ingredients are rapidly entering consumer products and food [31]. The legitimate concern of various advocacy groups is that synthetic biology is so new that there are as yet no regulations in place for the creation, use, and disposal of new synthetic organisms or even credible risk assessment methods before such organisms are released in the environment [41, 42]. The fear is that premature, wider, large-scale industrial use of synthetic biology ingredients is likely to cause serious harm to biodiversity and farmers. The fact remains that scientists cannot predict, at this nascent stage of synthetic biology, what new forms of life or attempts to 'reprogram' existing organisms, such as yeast and algae, would do to the environment and human life, given that they can now generate millions of new, untested organisms on a mass production scale. The possible effects range from beneficial, benign, to ecological and economic disaster. The core ecological concern is that artificial organisms breed, reproduce, and once released into the environment cannot be recalled. Hence the fear of unintended consequences. Of course, as synthetic biology matures, many equitable solutions are also likely to emerge.

In this 'good-bad' debate, the real concern is the regulation of artificially created living organisms rather than the non-living chemical products (bio-fuels, pharmaceuticals, oils, etc.) they produce. For the latter, reasonable regulatory mechanisms exist and they are continuously evolving. Chemistry is much better understood than the biochemistry of life. Therefore, the demand, as is sometimes made, for labelling ingredients as having come from synthetic biology processes in products has no scientific basis. The chemical properties of an ingredient are independent of the process used in making them.

The regulatory aspect of such synthetic biology products as genetically engineered microbes, plants and animals, promises to be a nightmare. Concerns related to environmental, health, and food safety require specialized regulating agencies. R&D advances in synthetic biology have been so rapid and novel that existing regulatory agencies are either unable to cope or find themselves without the authority to review. The sheer variety and increasing complexity of artificial life, many of which can be generated within a short span, makes their risk assess-

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ment a great challenge. Not only will the regulators need additional funding to meet increased workload and expertise requirements, but also the legal authority to carry out certain tasks not included in current laws. See, *e.g.*, [16]. Most countries currently lack human, financial, and scientific resources to set up effective regulatory agencies or even frame regulatory policies.

Another major concern is the accidental release of artificial organisms in the environment. In some cases, researchers can design organisms with built-in safety features. For example, by designing organisms that can survive and breed only in an artificially created environment, such as by controlling the chemical sources of energy they have access to or by the reassignment of the stop codon. It was recently discovered that in the standard genetic code the stop codon can undergo recoding in nature. Reassignment of the stop codon has been observed in bacteriophages and bacteria indicating that bacteriophages can infect hosts with a different genetic code. This can lead to phage-host antagonism based on code differences. Its implication in synthetic biology is that the stop codon reassignment may be used as a means to engineer organisms to prevent the exchange of genetic information between engineered and naturally occurring species.

Clearly, synthetic biology requires new methods of risk assessment because it involves exotic biological systems based on an alternative biochemical structure, *e.g.*, genetic code based on novel types of nucleotides, or an enlarged number of base pairs. There is also the risk of synthetic biology skills diffusing into wrong hands (*e.g.*, Do-it-yourself biology, amateurs, and bio-hackers) with time as these skills begin to percolate down the education system.

4.4. The societal side of IP outputs

Since artificially created biological systems will often be expected to interact with natural biological systems, including human societies, there are moral and ethical concerns and the need to develop a rational public–science interface to address those concerns [44]. In particular, what should be the relationship between humans and artificially created living organisms and the moral and legal status of the products, *e.g.*, transgenic humans. Indeed, how would we define human life? What would be the legal status of artificial humans, especially if illegally created? What if they formed their own societies, rules of governance and rules of interaction with natural humans? What if there were to occur a sudden spurt of diversification of the human species, engineered or accidental? Could it lead to the collapse of human society as we know it today and the extinction of natural humans?

Precision editing of DNA will eventually enable us to alter not just individual organisms but also ecosystems. It would then be possible to wipe out diseases like malaria by altering Anopheles mosquitoes, which have evolved resistance to anti-malarial drugs and insecticides (a vaccine against malaria has been elusive), by modifying their genome, disabling or hindering their reproductive cycle or building up resistance to parasites through highly heritable genes, and then releasing them throughout the population. However, the accessible nature of the technology, such a "gene drive" could also be used irresponsibly and raise the risks of accidental or even intentional harmful effects [45]. Given the delicate ecological balance needed for human survival, how is responsible behaviour to be integrated with the patent system?

Historically, pioneering technologies have created intense patentability debates [46, 47] that range from conceptual to political. For example, IPR opponents in the past had argued agriculture was not an industry, patents on pharmaceuticals would be unethical, biotechnology is about trying to play God, software and business methods are non-technical, etc. In the 1970s, concerns surfaced about recombinant DNA technology that innocuous microbes could be engineered into human pathogens resistant to then known antibiotics, or enable them to produce toxins, or transform them into cancer causing agents [47]. Fears have since abated. Recombinant DNA technology now dominates research in biology. In synthetic biology, the fears are more in terms of our ability to regulate research and industrial activities so that these activities are carried out safely [16] and the human species preserved.

4.5. The IPR side of IP outputs

In societies that abhor monopoly rights and favour level playing fields of competition, even limited period monopoly creates social tension. Thomas Jefferson (1743–1826), the third President of the United States (1801–1809), the principal author of the Declaration of Independence (1776), a well-known scientist of his time, the initiator of the first U.S. patent system in 1790, and the author of the 1793 Patent Act, had this to say in 1813 in a letter to Isaac McPherson [48]:

> Considering the exclusive right to invention as given not of natural right, but for the benefit of society, I know well the difficulty of drawing a line between the things which are worth to the public the embarrassment of an exclusive patent, and those which are not.

The demarcation debate between openness and limited period monopoly may never end. In synthetic biology this debate is complex because it involves the assimilation of a new technology by society and of inventions that were never anticipated to become part of the patent system. Indeed, some of these future inventions may well be bio-robots and bio-computers with the DNA serving as programmable memory. It would require tremendous legislative efforts to equitably deal with such live inventions. However, one expects that bio-weapons, like atomic weapons, would be kept outside the patent system.

5. Look before leaping to patent

Before filing a patent application, ensure that a thorough prior art search is done and in relation to that prior art, map out all possible obvious extensions to the art that are likely to occur to a person of ordinary skill in synthetic biology (*e.g.*, the average post-doc). If your invention goes beyond the obvious extensions, and fulfils the statutory requirements of novelty, non-obviousness and utility then expeditiously file a patent application for your invention ensuring that you fully describe the invention (including the best mode) therein. File a provisional application if necessary to claim priority over other inventors and follow it up in a timely manner with a non-provisional application. Scrupulously follow patent office protocols. Getting a patent is expensive, so a business analysis before filing is prudent.

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5.1. Prior art search

Prior art or state-of-the-art is all information, available in any form (including social media), in the public domain. It does not include secret information, *e.g.*, trade secrets or confidential communications. Patentability searches of prior art – to decide whether or not an invention is patentable – especially from the point of view of novelty and non-obviousness are routinely performed by patent examiners. Even then, it is usually prudent to pre-emptively carry out a similar search. *Inter alia*, such a search provides valuable information to the lawyer drafting the patent application. First, it helps him define the prior art and the background of the invention so that he can highlight patentable features of the invention. Second, he will be able to strike a balance between framing too broad or too narrow claims for the invention.

Learn the art of prior art search. Automated searches (*e.g.*, Google scholar) are valuable as a lead-in to conducting a specialized manual search or as a follow-up to locate patents or other prior art after a manual search. If affordable, get a professional search done. Note that no search can guarantee that it is complete or completely accurate. More importantly, only the absence, and not the existence, of novelty of your invention can be established.

5.2. PHOSITA

This legal fictional person (or a team) having ordinary skill in the art, called a PHOSITA who is neither a genius nor a layperson, is considered to possess average skills and knowledge in a particular technical field and hence unlikely to ever become an inventor. He thus serves as a reference for determining by comparison whether an invention is obvious or not. If a PHOSITA is deemed capable of coming up with the invention if required, assuming he/she would make the effort to study relevant prior art, then the particular invention is deemed unpatentable. Note that a "person of ordinary skill is also a person of ordinary creativity, not an automaton." [49]. Further, "in many cases a person of ordinary skill will be able to fit the teachings of multiple patents together like pieces of a puzzle." [49].

The skill profile of a PHOSITA is determined on a case-by-case basis, depending on the level and technological features of the invention. Factors used in profiling include the education level of the inventor, type of problems encountered in the art, known prior art solutions, rapidity with which innovations are made in the art, sophistication of the technology, and education level of active workers in the field. Clearly, a PHOSITA's profile changes with time as he continuously imbibes new advances in related technologies. A PHOSITA of today, may have been an expert yesterday! This is clearly true in synthetic biology where the PHOSITA will most likely be a researcher with a PhD.

5.3. Novelty, non-obviousness, utility, written description, claims

Only an invention that can be classified as machine, manufacture, process, or composition of matter and further if it is considered novel with respect to prior art, non-obvious to a PHOSITA, and useful to society at the time the patent application (provisional or non-provisional) is filed is eligible for consideration of a patent grant provided the invention is clearly and fully described. Patent prosecution is the process by which a non-provisional patent application is

defended before the patent office. Prosecution begins with the filing of the non-provisional patent application and ends with the final decision on the application by the patent office.

Obviousness creates a 'patent-free' zone around the prior art related to the invention and prevents trivial advances from being patented. Under the doctrine of equivalents, straying into the patent-free zone of a valid patent amounts to infringing the patent. (See Section 6.2.)

The invention must have a useful effect or a purpose meaningful to society else the invention is not patentable. The invention's utility must be specific to the subject matter claimed, credible to a person skilled in the field, and should not require further research to discover it.

The written technical description of the invention should enable a person skilled in the art to reproduce and use the claimed invention without undue research or experimentation beyond those normally expected from such a person. Because the experimentation may be complex for a particular invention it will not become undue if a person of skill in the art typically engages in such complex experimentation. The inventor must point out how his invention differs from prior art. Finally, the non-provisional application must include one or more claims that distinctly spell out specific aspects of the invention which the inventor claims are his intellectual property in need of legal protection. Omitted aspects that could have been claimed are deemed to have been gifted to mankind. Likewise, disclosing the invention by putting it in public use, testing it in public, describing it in a speech in a technical conference, sale of the invention, disclosing the invention to people without a signed non-disclosure agreement with them, discussing it in the social media, etc. before filing a patent application may be construed as placing the invention in the public domain and hence ineligible for a patent.

It must be clear from the written description that the applicant was in possession of the claimed invention at the time of filing. There is no statutory requirement that the inventor disclose why the invention works or how it was developed. Inventors are expected to write their invention using the language and ideas that are accepted in the field of the invention, say, by a PHOSITA. In some countries, it is a statutory requirement that the inventor set forth the best mode contemplated by him of carrying out his invention.

While the written description must be followed by one or more claims through which the inventor points out and distinctly claims aspects of the invention he believes are his original non-obvious contributions, he should not pre-emptively claim ideas, laws of nature or natural phenomena. Each claim must be so drafted that patent examiners and potential infringers can understand what the claimed subject matter is. Writing claims is a specialized art, and should preferably be drafted by a patent attorney. Claims lie at the heart of infringement litigation and they form the most important part of a patent.

A patent is invalid if its claims, read in light of the invention's description and prosecution history, fail to inform, with reasonable certainty, those skilled in the art about the scope of the invention. However, when the invention is novel and non-obvious, words may not exist to describe it so the law allows words to be invented and defined to describe the invention to fill unintended idea gaps in a language.

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5.4. Business prudence

Economic viability of a patent depends on the following:

Detectability. Once a patent is granted, the idea and implementation details become public. Hence, to enforce your patent, you must be able to detect infringement easily, otherwise keeping the invention a trade secret may be a better option.

Non-avoidability. If viable alternatives to your invention exist or can be developed within reasonable timeframes and costs, then seeking a patent may be unwarranted.

Business value. Acquiring a patent is both time-consuming and expensive. So weigh the potential benefits that may accrue from a patent against potential risks of not seeking a patent.

Technology obsolescence. Track emerging technologies and technology trends to determine if your invention will become obsolete in the near future.

Since biotechnology patents generally underpin business, it is imperative that patent applications are prepared and prosecuted by experienced patent attorneys and that inventors work closely with them to minimize prosecution hurdles and future litigation possibilities.

6. Look before litigating

Infringement occurs when someone unauthorized makes, uses, offers for sale or sells a patented invention within territories where it is protected, or imports into that territory the patented invention during the term of the patent. Infringement and litigation is mainly about the power to regulate the manner in which patented goods and services are traded, not how people use them. Patent disputes seldom throw up clear-cut good guys and bad guys. Each feuding party is likely to honestly believe its actions are reasonable and lawful. Litigation costs are usually very high, so anticipate spending a million or more U.S. dollars. No infringement occurs outside the term of a patent. Patent offices have no jurisdiction over infringement issues, only designated courts have. The relief sought from courts for infringement may be an injunction to prevent further infringement, and award of damages for past infringement. Alleged infringers, if challenged, are quite likely to counter-challenge by questioning the validity of the disputed patent. While the Government that granted the patent may use the patented invention without permission of the patent owner, it must, nevertheless, compensate the owner.

6.1. Obviousness and obvious–to-try

Obviousness and "obvious to try" are not synonyms. The mere fact that something is "obvious to try" in view of prior art does not automatically imply that the invention resulting therefrom is obvious. This is especially true where the number of things one can obviously try are very many (say tens-of-thousands or millions as can happen with respect to chemical molecules) and the search would amount to finding a needle in a haystack. That is, the prior art does not contain any suggestion or teaching that might

suggest how the invention might be accomplished or any basis for reasonable expectation that beneficial results will accrue by proceeding along the lines taken by an inventor. There are, however, situations where "obvious to try" or "worth a try" may be indicative of obviousness. In *KSR* v. *Teleflex* [49], the SCOTUS indicated one:

> When there is a design need or market pressure to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill has good reason to pursue the known options within his or her technical grasp. If this leads to the anticipated success, it is likely the product [is] not of innovation but of ordinary skill and common sense.

6.2. Doctrine of equivalents

The judicially created doctrine of equivalents, universally followed, is a rule of claim interpretation wherein a product or process, although not literally infringing nevertheless infringes if it performs substantially the same function in substantially the same way to obtain the same result as a patented product or process. The doctrine extends patent protection beyond the literal language of the claim.

Literal infringement of a patent, though rare, occurs when the alleged infringing product or process is an obvious near replica. Generally, people try to work around a patented invention by introducing differences and variations they hope will be large enough to beat the doctrine of equivalents. Deciding equivalency is tricky as it must deal with two opposing public policies: (1) the need to provide public notice as to what infringes by requiring clear and distinct claims, and (2) the need to prevent an infringer from avoiding liability by covert means. Of course, one may ask, "What if a device performs substantially the same function in a substantially *different* way to obtain the same result?" This leads us to the reverse doctrine of equivalents (Section 6.3). In determining equivalency, courts may seek expert opinion as to scientific or engineering facts and the decision may well lean on the more believable expert. Note that things that are equivalent for one purpose may not be so for other purposes.

6.3. Reverse doctrine of equivalents

The reverse doctrine of equivalents circumscribes the doctrine of equivalents. The SCOTUS in *Graver Tank* [50] ruled that:

> The wholesome realism of this doctrine [of equivalents] is not always applied in favor of a patentee but is sometimes used against him. Thus, where a device is so far changed in principle from a patented article that it performs the same or similar function in a substantially different way, but nevertheless falls within the literal words of the claim, the doctrine of equivalents may be used to restrict the claim and defeat the patentee's action for infringement. [Citations omitted.]

Thus, where an invention relies on the fundamental concept embodied in a patent but, say, relies on "a significant advance" in technology, the accused device does not infringe by virtue of the reverse doctrine of equivalents. Once a patentee establishes literal infringement, the

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burden is on the alleged infringer to establish non-infringement under the reverse doctrine of equivalents.

6.4. Prosecution history estoppel

Estoppel means a bar preventing one from making an allegation or a denial that contradicts what one has previously claimed as the truth. This can happen, *e.g.*, during patent prosecution, if a claim is rejected by the patent examiner citing prior art and the claim is then amended and narrowed to avoid the prior art. In such a case the patentee is barred from asserting the narrowed claim in a broader sense under the doctrine of equivalents or recapture what was surrendered in the amendment. Thus when prosecution history estoppel applies, only literal infringement may be invoked.

6.5. Research exemption

Generally, use of patented inventions in pure research is exempt from infringement liabilities. That is, if the pursuit is no more than "for amusement, to satisfy idle curiosity, or for strictly philosophical inquiry." [51]. Still caution is warranted as the scope of exemption varies from country to country and whether the research is associated with a commercial goal. If it is, exemption is unlikely. Researchers in synthetic biology need to be very cautious, especially if their research is funded by industry or is likely aimed towards a commercial product. Generally, use of patented inventions in research and tests in preparation for regulatory approval from government bodies is exempt if conducted within a limited period prior to the patent's expiry. This *e.g.*, allows generic manufacturers to prepare generic drugs in advance without infringing relevant patents.

6.6. Method claims

Unlike product claims, process or method claims are generally problematic in litigation. In the United States where patent litigation is rampant, the SCOTUS has often enough reversed the decisions of the Court of Appeals for the Federal Circuit (CAFC) in patent litigation [52]. In a recent case, *Limelight* v. *Akamai* [53] the SCOTUS while unanimously overturning the CAFC's decision, commented, "The Federal Circuit's analysis fundamentally misunderstands what it means to infringe a method patent. A method patent claims a number of steps; under this Court's case law, the patent is not infringed unless all the steps are carried out." It also held that a defendant is not liable for induced infringement if there is no direct infringement. The decision has raised some concerns in the biotech industry since biotech patents often include complicated, multi-step methods. It now appears that the patent system could be gamed by infringers by simply outsourcing part of the process to avoid lawsuits.

6.7. Balancing conflicting requirements

Balancing the requirements for non-obviousness in litigation, with the constraints imposed by the doctrine of equivalents, reverse doctrine of equivalents, and prosecution history estoppel can be tricky because much depends on prior art related to the patent-in-suit, the profile of the

PHOSITA, and exact wordings of the patent's claims. In most cases, the issue involves the expansionary scope of the doctrine of equivalents and whether obvious-to-try is the same as obvious from the perspective of the PHOSITA. Some of the trickiest situations involve the opposing tendencies of the doctrine of equivalents and prosecution history estoppel. Such matters are best left to experienced lawyers.

7. Sundry IP protection issues in biotechnology

In scientific research, openness in sharing foundational research results and tools promptly with the scientific community advances the field more rapidly than otherwise. This requires that synthetic biologists collaboratively create a basic platform where, *e.g.*, standardized biological parts that are safe, ethical, and cost effective are easily accessible to facilitate the development of other inventions needed by society but require an industrial setting, a profit motive, and IPR protection. A shared basic platform will foster less acrimonious market competition. Basic research is curiosity-driven and largely government funded; product development is market-driven and requires huge private funding. The government owns the mint, the private sector does not nor can it crowd-source funds via taxation. IP laws try to bridge this gulf. The task is far from easy as the following two examples indicate.

1. *Galileo seeks IPR*. The Venetian Senate passed the first patent law on March 14, 1474, granting limited duration monopoly for original devices. That same Venice in 1594 granted Galileo a "privilege" (a patent) for 21 years on a machine which he had invented [54] "for raising water and irrigating land with small expense and great convenience," on the condition that it had never before been thought of or made by others. In his petition for the privilege he said, "it not being fit that this invention, which is my own, discovered by me with great labour and expense, be made the common property of everyone" and adding that if he were granted the privilege, "I shall the more attentively apply myself to new inventions for universal benefit." Clearly, even Galileo, the father of modern science, was not willing to divulge his invention only to have it copied for free exploitation by others. Galileo's argument pervades the modern patent system.

2. *The Bayh-Dole Act*. In the late 1970s the U.S. Government realized with shock that while it held title to approximately 28,000 patents (at the time all patents resulting from federal R&D funding at universities were owned by the government), fewer than 5% were licensed to industry for development of commercial products. Literally, results of billions of dollars of federal R&D investment were under-utilized in commerce. The remedy was the Bayh-Dole Act of 1980. It went against prevailing wisdom that patents resulting from tax-payer funded research should belong to taxpayers and availed by industries under non-exclusive licenses. It turned out that without an exclusive license, companies were wary of investing the huge sums required to turn those inventions into marketable products when the resulting products could easily be appropriated by competitors. The business risks were too high. Therefore, under the Act, the government relinquished its ownership rights to future patents arising from federally funded R&D in the universities

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and small businesses and turned them over to the fund recipients. It also permitted them to grant exclusive licenses thereby creating the needed incentives for private firms to invest. Many countries have since enacted Bayh-Dole type Acts. About the Act, *The Economist* (December 14, 2002) wrote:

> Possibly the most inspired piece of legislation to be enacted in America over the past half-century was the Bayh-Dole act of 1980... More than anything, this single policy measure helped reverse America's precipitous slide into industrial irrelevance.

7.1. Limited period monopoly versus dedicated to the public

The *quid pro quo* of the patenting system is that in exchange for government granted limited period monopoly, the inventor must fully disclose the details of the invention so that further innovation and improvement of the invention by others can continue. On the patent's expiry, the invention falls in the public domain and all patent rights are extinguished. Patent law encourages such inventions where without a patent the incentive to invent products and processes useful to society is unlikely to occur rapidly enough. For example, not having patents may mean not having certain drugs and therapies.

Acquiring patents is expensive; fighting litigation even more so. So the key question in framing a patent system is: "Will concentration of monopoly power in a given technology be detrimental to industrial growth in the long run?" The answer depends on the scale and availability of funding. Only those with deep pockets can afford to acquire a sizable patent portfolio. The second question, "Is the patent office ready to handle this technology?" New technologies that come rapidly to the fore can be a nightmare for any patent office because of lack of examiners, inadequate repository of and access to prior art, inadequate case-law from which they can seek guidance, etc. Not every country has the ability or the resources to cope with such a situation. The third question, "How high should the bar be set for grant of patents in terms of novelty and non-obviousness?" Higher the bar, less will be the cost of enforcing patent law since a great many infringement battles can be eliminated and more inventions will populate the public domain. How the answers to the three questions are dynamically balanced will decide how well the patent system serves society. This balancing act is far from easy given that substantial and rapid technological advancement is not possible if based purely on the innovative capabilities of ordinary people. Only extraordinary people are capable of such feats and many of them require the incentive of government granted and protected privileges in order to be productive, *e.g.*, Galileo. Patents promote trade and commerce and avoid the accumulation of trade secrets.

7.2. Patents common

There is a perennial dilemma: How does one encourage innovation without eroding the vitality of the scientific commons? What is the right balance between philanthropy and profit incen-

tive? Should the balancing be driven by free market mechanisms or government intervention? Reforms in the patent system are undoubtedly warranted but what they should be are unclear.

The biotechnology industry, recognizing these dilemmas, has funded certain initiatives in the past with the clear aim of placing the resulting research output in the public domain in the larger interests of both industry *and* society via patents-information commons. These initiatives sought a balance between the intellectual property system that quarantines new knowledge and information and the goal of science to put them in the public domain expeditiously [55]. For example, to mitigate debilitating competition, like-minded companies have collaborated to create and share IP among themselves to enhance the scale, scope and speed of innovation; used cross-licensing, patent pools, and patent exchanges to lower the cost of exchanging IP; embraced open standards to enhance inter-operability and encourage collaboration; and invested in pre-competitive information-commons to boost their downstream product development. Some well-known examples of pre-competitive information-commons are *Merck Gene Index* (1995), *Merck sponsored project to create patent-free transgenic mice* (1997), *SNP Consortium* (1999), *International HapMap Project* (2002), and *The Genographic Project* (2005).

The National Institutes of Health (NIH) in the U.S. too has been active in creating information commons. Since 1996, all human genomic DNA sequence information that it funds is placed in the public domain. In December 1999, it adopted a general statement of "Principles and Guidelines for Sharing of Biomedical Research Resources"[3] that said:

> [T]he use of patents and exclusive licenses is not the only, nor in some cases the most appropriate, means of implementing the [Bayh-Dole] Act. Where the subject invention is useful primarily as a research tool, inappropriate licensing practices are likely to thwart rather than promote utilization, commercialization, and public availability.

In the same spirit, the Guidelines encourage unencumbered transfer of unpatentable research tools to other needy researchers. Of course, in view of the Bayh-Dole Act, the Guidelines could not restrain grantees from filing patent applications.

In August 2014, NIH issued a final policy on genomic data sharing that builds on and replaces its earlier policy issued in 2007 in an effort to promote the sharing of data from genome-wide association studies, and through the creation of the database of Genotypes and Phenotypes (dbGaP), a two-tiered system for distributing data. One tier offers open-access with no restriction and the other provides controlled access that can be used only for research purposes consistent with the original informed consent under which the data were collected. This new policy (available at http://gds.nih.gov/03policy2.html) will go into effect in January 2015. NIH's preference for open access understandably comes from its top leadership which is typically drawn from academia and the basic research community that sanctifies open access. A survey of deals and business models that highlight the more charitable side of the pharmaceutical and biotechnology industry is available at [56].

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3 Available from http://grants.nih.gov/grants/intell-property_64FR72090.pdf.

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8. Universities taste entrepreneurship

8.1. Some history

The Bayh-Dole Act spurred U.S. universities to seek patents and facilitated university-industry partnerships that turned universities into engines of economic growth. However, a Bayh-Dole type Act is unlikely to succeed elsewhere as it requires a system of world-class research universities, brilliant research faculty, a continuous stream of brilliant doctoral students and post-docs, and access to substantial funds to create and maintain research infrastructure. In 1980, the U.S. had all these. Even then, only companies with the wherewithal to convert university generated basic research results into marketable end-products benefited most. So far the most successful example has been the bio-medical sector [55]. For example, in FY 2007, top licensing revenue earners included: New York University (approx. $791.2 million), Columbia University ($135.6 million), The University of California system ($97.6 million), Northwestern University ($85 million), and Wake Forest University ($71.2 million).[4] Most of these earnings came from biomedical discoveries, rather than physical sciences. Even in biomedicine, it was often a block-buster patent that strikingly stood out. For example, New York University's largest licensing income came from an undisclosed portion of its worldwide royalty interest in the monoclonal antibody Remicade; it was $650 million!

Here is another example of IP treasure troves in universities. World-wide the top 10 universities granted U.S. patents in 2012 were: (1) The Regents of University of California (357); (2) Massachusetts Institute of Technology (216); (3) Stanford University (182); (4) Wisconsin Alumni Research Foundation (155); (5) Tsinghua University (149); (6) University of Texas (141); (7) California Institute of Technology (136); (8) National Taiwan University (122); (9) University of Michigan (97); (10) University of Illinois, National Chiao Tung University, and University of Utah Research Foundation (85 each).

To play the IP game on this scale, U.S. universities have had to change dramatically. Since the founding of Harvard University in 1636 when universities provided their students with the requisite classical background and knowledge of leadership and government, the shift to a radically new training-centred curriculum to accommodate mechanical science, agricultural technology, etc. that would complement the new rapidly industrializing economy and the aspirations of the emerging middle class, was remarkable enough. This shift to science-inclusive education helped propel the U.S. economy well into the twentieth century. Post-Bayh-Dole, universities are once again adapting themselves to remain relevant in a global innovation-driven economy, in which researchers are highly mobile, technology obsolescence rates are high, and knowledge acquisition is a continuous requirement. A unique feature of this transition is the birth of the entrepreneurial professor who sets up companies, sometimes taking his graduate students along with him. (Often the same university that does research in science also does research in business management!) Many young professors now routinely

4 Ben Butkus, Biomed Dominates Tech Transfer in US; NYU, Columbia, MassGen Tops in Licensing Income, GenomeWeb, January 28, 2009, http://www.genomeweb.com/biotechtransferweek/biomed-dominates-tech-transfer-us-nyu-columbia-massgen-tops-licensing-income

acquire managerial skills by participating in multimillion dollar R&D projects. To such academics, university-industry collaboration comes easily. Indeed, they expect and get help from their university in spinning-off start-ups to exploit their research. Such 'commercialization' has not eroded basic research, which continues to fascinate top researchers dreaming of Nobel Prizes. In fact, biomedical researchers strive to find clinical applications of their basic research.

8.2. Technology transfer

The technology transfer process between university and industry is complex because it must contend with two fundamentally different and sometimes opposing cultures of dealing with the profit motive. Universities need to ensure that the process does not unduly compromise their educational and research mission. Bayh-Dole type provisions facilitate technology transfer by giving universities the necessary autonomy and IP ownership rights, which provides greater legal certainty and acts as a strong incentive for industries to collaborate with universities. However, the downside is that universities must involve themselves in hitherto unfamiliar activities, such as creating technology transfer offices, and developing interdisciplinary teams with legal, business, scientific, and licensing expertise. For an informative tutorial on technology transfer in U.S. colleges and universities see [57]. *Inter alia* it discusses "the role technology transfer plays in adding value to the academic and research mission of universities and colleges." Of course, remodelling of universities alone is not enough. An entire ecosystem is required that includes the university system, the intellectual property system, immigration laws, technology transfer offices, venture capitalists, and most importantly, opportunities for researchers to remain mobile—getting gifted people to work in a poor country will therefore be an arduous task.

The corner stone of basic research is insight which begins as tacit knowledge held by researchers. The diffusion of tacit knowledge via university-industry collaboration is crucial for technology transfer and commercial success. This means that star scientists—their accessibility, location, motivation to collaborate at the bench-science level with scientists in industry in converting basic scientific knowledge into commercially viable products and processes—will be crucial in determining the pace at which tacit knowledge is diffused [58, 6]. Graduating students too carry considerable tacit knowledge derived from their faculty mentors with them when they join the biotech industry as employees. Donald Kennedy, a former editor-in-chief of the journal *Science* and President Emeritus of Stanford University, once aptly noted, "Technology transfer is the movement of ideas in people." This movement in biotechnology frequently requires the protective cover of patents to ensure adequate return on investment in commercialization. The biotechnology industry's ascendency has meant that universities are no longer not-for-profit ivory towers.

A crucial activity of university technology transfer offices is the marketing of their patent portfolios. An outstanding example of marketing is the Cohen-Boyer patents by Stanford University. It was master-minded by Neils Reimers who had an unusual talent for balancing academic values and industries' needs. And the Bayh-Dole Act which Congress passed on December 12, 1980 some ten days after the first Cohen-Boyer patent was granted, was a

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godsend. Reimers designed a trail-blazing licensing program. By end of 2001, the three Cohen-Boyer patents had made $255 million in licensing revenues from licenses granted to 468 companies. More importantly, 2,442 products were developed from the patented technology that included drugs to mitigate the effects of heart disease, anaemia, cancer, HIV-AIDS, diabetes, etc. Remarkably, the patents never faced litigation. Reimers showed that cutting edge university-centred research, patents, and industry collaboration could be integrated into a formidable system that can propel a country's economic agenda, without the university sacrificing its core values [5].

It now appears that CRISPR-Cas9 technology is the new superstar in biotechnology. Zhang's patent (U.S. patent No. 8,697,359) is the first to cover this technology. While the Cohen-Boyer patents survived their terms without litigation, one hopes that Zhang's patent assigned to Broad Institute will be so blessed. Zhang's patent significantly simplifies gene editing compared to other contemporary techniques, *e.g.*, TALEN and zinc fingers. Since Zhang's method allows one to basically reengineer any organism by modifying its own genome, it immediately opens up the possibility of engineering a variety of applications ranging from better agricultural crops (*e.g.*, drought resistant) to bio fuels to disease detection to personalised medicine (*e.g.*, by correcting the causative mutation), and, of course, of better understanding of gene functioning [26]. So, one expected development is the blooming of patent thickets. The financial stake around the CRISPR-Cas9 technology in the private sector is expected to be enormous and with patent thickets the potential for fierce litigation will be high. A likely development is that if exclusive licences do not create hurdles, companies would try to gather as many patent licences as they can to ensure their freedom to pursue their research and commercial goals. This could be an optimal solution for rapidly developing a plethora of products and processes that will in any case need a large number of players to chip in, much like the electronics industry, where there is space for many players to compete against and collaborate with.

8.3. Litigation

While research universities now see a patent portfolio as a potential source of revenue generation, few are enthusiastic or even prepared to enforce their patents, when infringed, through litigation. In the U.S., universities, by law, must participate as plaintiffs in enforcement lawsuits over their *exclusively licensed* patents regardless of a university's effective ability or enthusiasm to do so [59, 60]. Therefore to preserve licensing freedom, patent application preparation and its prosecution must be strategized to discourage litigation. Clearly, universities must maintain excellent technology transfer offices, whose members are not only "licensing and business development professionals" but who also "handle technologies from inception through research"; "handle conflict of interest issues"; close deals with commercial partners, and "then (God forbid)" participate in litigation to protect IP rights [61].

A few recent high profile cases indicate that the brave may sometimes inherit the earth. For example, in the Carnegie Mellon University (CMU) patent lawsuit against Marvell Technology Group, which allegedly appropriated CMU research for a computer chip used in high-speed drives, the jury awarded the university $1.17 billion in December 2012 [62]. On appeal, Marvell was ordered to pay enhanced penalties of $1.5 billion

for wilful infringement of CMU patents [63]. In another case, Varian Medical Systems, which allegedly infringed on the University of Pittsburgh patents for a respiratory device, a judge awarded $85.8 million. Such cases have made other universities wonder if their technology transfer offices should get more aggressive in protecting patents [62]. Once a patent is infringed the alternatives are litigation or an out-of-court settlement. In litigation, the patent will almost certainly be dissected in terms of the doctrine of equivalents, prosecution history estoppel, the subjectively determined profile of the PHOSITA, applicable prior art relative to the patent, clarity of description of the invention, the breadth and narrowness of claims, etc. Litigation results are often uncertain. In the U.S., *e.g.*, some one-third of district court decisions on claim boundaries are reversed on appeal [64], while a large number of CAFC patent decisions have been reversed by the SCOTUS on appeal [52].

A commercially successful patent attracting litigation is a fair possibility because a patent's validity is not guaranteed. Post-grant a patent may be found invalid because of erroneous evaluation of the invention by the patent examiner during prosecution, or because he was simply blindsided by undetected prior art, etc. In addition, one must be prepared to deal with intentional predatory moves by patent trolls and the calculated overreach of some patent owners in asserting patent claims against non-infringing entities. Their general aim is to either drag the target into expensive litigation or force it into licensing agreements under the threat of litigation, which small and medium enterprises can ill afford.

9. Patent law reforms

Since Galileo (1564-1642), science has dramatically affected society. Industry, transportation, communications and medicine have all undergone such revolutionary changes that most mortals today appear to have semi-divine powers compared to pre-seventeenth century denizens of the world. The common man's focus has shifted from seeking divine favours to diligently acquiring human invented technological gadgets and services. Today,

> Western industrial technology has transformed the world more than any leader, religion, revolution, or war. Nowadays only a handful of people in the most remote corners of the earth survive with their lives unaltered by industrial products. The conquest of the non-Western world by Western industrial technology still proceeds unabated. [65].

Yet some of these technological and scientific advances, such as genetically engineered plants and animals, human cloning, electronic surveillance, the use of robots, and now the possibility of genetically engineered humans raise serious moral and ethical issues, which demand legislative solutions and hence political intervention. If the track record of politicians, say, in handling problems related to climate change is any indication, we can expect synthetic biology related calamities to inundate us before they act. Their inadequate understanding of synthetic biology and the legislative process driven by one-person-one-vote electoral dynamics in a knowledge-driven society where knowledge creators and knowledgeable people constitute a

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miniscule minority creates an anomaly. Intellect-driven legislations require an entirely different legislative process than mass-and-emotion-driven legislations. When laws of Nature and laws of man collide, catastrophe results.

9.1. Challenges

For patents to be an effective tool to promote innovation, they should be scarce and hard to obtain, especially in today's knowledge driven world, where the population of university educated people is far larger than it was several centuries ago when the modern patent system was instituted in England during the rule of Queen Elizabeth (reign: 1558 – 1603). An enormous knowledge gulf separates the PHOSITA of the Elizabethan era and of today. The biggest challenge patent examiners face today is the objective profiling of the modern biotechnology PHOSITA whose profile is prone to rapid changes, sometimes within months.

The second challenge is related to patent seeking researchers whose desire is to seek patents in anticipation that their discoveries will eventually, but during the lifetime of a patent, lead to substantial, if not miraculous, benefits to society that truly touches peoples' lives. The challenge patent examiners face here is, whether or not the applicant is claiming a 'law of Nature', or whether granting a patent will be against the interests of society (*e.g.*, patents on nuclear weapons are banned), such as creating obstacles to further research or advancement of the invention.

The third challenge is fulfilling the need for a new patent system that would minimize litigation. When patent offices are inundated with patent applications in highly competitive cutting-edge technology areas populated with extremely well qualified PHOSITAS, determining overlapping claims among applications is an incredibly demanding task, and therein lies the source of debilitating and fierce future litigations. Current legal systems are visibly deficient in handling such litigations so remedies may lie elsewhere, *e.g.*, in the form of peacemakers among feuding parties.

The fourth challenge is providing adequate scientific research support to the judiciary. It needs a permanent science advisory body to enhance its understanding of the scientific basis on which biotechnology patent claims rest and in creating a plausible PHOSITA profile acceptable to the scientific community on a case-by-case basis. This will substantially simplify and accelerate judicial proceedings in biotechnology patent litigation and lead to greater consistency in judicial decisions. The science advisory body can bring about greater clarity to the vexing question: "When is obvious-to-try the same as obviousness?" in relation to the profiled PHOSITA.

The fifth challenge is integrating introductory IPR courses in science and engineering curricula in universities to bring home to students the paramount economic relevance of their acquired scientific and technical knowledge and skills.

9.2. Questions

The fact that synthetic biology involves the creation of artificial living matter or modification of living matter through human intervention raises important questions related to biosecurity,

biosafety, bioethics, and environmental health and sustainability. Their answers will require consultation with engineers, scientists, attorneys, innovators, teachers, students, policymakers, and ordinary citizens. However, before doing so society must decide how synthetic biology as a scientific discipline is to be handled. That is, establish rules and regulations of ownership, diffusion, and access to the knowledge the discipline generates and accumulates. Concurrently, to further the bio-economy, it must establish global engagement and collaborative models, mentor and nurture young leaders, create next-generation manufacturing facilities, and address standards-related issues. The crucial questions are:

- When do basic research results benefit society most if placed in the public domain as opposed to limited period IP monopoly of those results?
- When it is appropriate for industry to seek private ownership of inventions derived from the results of open innovation?
- When is an open innovation policy in synthetic biology likely to discourage industry from developing commercially viable products and processes?

Johnson [66] notes that synthetic biology needs "public policies and collaborative mechanisms that promote broad and robust pre-competitive openness, sharing, and access" and "strong and robust IPR" to enable "later-stage economic value creation, IPR-enabled commercialization, and market-based investments". They will indeed help in aligning international investments, in framing lab-to-market policies, and in creating global manufacturing and marketing policies to facilitate global commerce.

9.3. Harmonization

There are serious obstacles to globally harmonizing patent laws [67]. Disparate national laws have caused a number of complicated cross-border IP disputes and multiple infringement suits. For example, software and business method patents are permitted only in some countries. Even when patent laws are similar in two countries, their interpretation by the courts may vary widely. Patent laws operate on the principle of territoriality and the needs of individual nations. Thus in a globalized, knowledge-driven economy, technologically advanced nations support strong patent protection to spur innovation, while the less advanced see it as barriers erected to restrict their access to new goods and dilute their welfare programs. Current national patent laws embody premises and concepts that were shaped by the Industrial Revolution; they are not malleable enough for the knowledge and information-driven age that has given rise to such exotic technologies as nano-technology, information technology, biotechnology, and robotics (and in the future, possibly bio-robotics). Today's inventor is frequently university educated or a researcher or a member of a large R&D team rather than an artisan or a technician. There is thus an acute need for harmonization of patent law and its enforcement. The assumption is that a uniform legal system would reduce legal uncertainties, cost of litigation, and barriers to trade. Other potential benefits include liberalized technology transfer and increased foreign direct investment from developed countries to the developing and underdeveloped countries and thus raise living standards globally. Ideally, harmoniza-

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tion would improve the world's capacity to innovate as a whole, which would be greater than the sum from its parts.

The World Intellectual Property Organization (WIPO) has been leading harmonization efforts. It currently administers the Patent Cooperation Treaty (PCT), the Paris Convention, the Patent Law Treaty, the Budapest Treaty, and the Strasbourg Agreement. However, these are not enough. WIPO's Standing Committee on the Law of Patents (SCP), created in 1998 to spur substantive harmonization efforts, has a wide representation of interested parties. So far, their deliberations have resulted in the Patent Law Treaty in 2000. Its modest aim is to harmonize formal procedures, *e.g.*, related to the filing date for a patent application, the form and content of the application, and representation. During 2001-2006, discussions on framing a Substantive Patent Law Treaty generated enough disagreements that they were put on hold in 2006. The SCP has since focused "on building a technical and legal resource base from which to hold informed discussions in order to develop a work program" while larger issues related to exceptions and limitations to patent rights; technology transfer; quality of patents, including opposition systems; confidentiality of communications between patent advisors and their clients; and patents and health hibernate.

Clearly, a bold experiment in universalising IPR governance and rule of law is sorely needed. Present disparities in IP laws and innovation capabilities among nations have created a "creditors and debtors" relationship where creditors appear to impose conditions that would perpetuate their dominance over debtors via institutions such as the WTO. Biotechnology provides strategic socio-economic advantages to creditor nations because of their research universities. This means that flight of capital and talent to countries with top research universities coupled with their liberal immigration policies for researchers can quickly deplete the talent pool of debtor countries. Of course, smart creditors know that helping debtors improve their circumstances makes for better, amicable, long-term, diversified, and more profitable business. Helping debtor countries build world-class universities would be one such example.

However, for synthetic biology, there is more to IP protection than just utility patents. Products arising from DNA synthesis and construction may also qualify as human authored original "literary work and artistic works" and hence eligible for copyright protection. Further, DNA is an information carrying molecule much like a computer program, which too is copyrightable. Likewise synthetic biology motifs can be used as trademarks and tacit knowledge locked up in a researcher's mind and selectively shared may deserve trade secret protection. And, of course, one can seek design patents for novel DNA designs.

10. Conclusions

The genetic uniqueness of each individual implies the existence of numerous undiscovered non-trivial interactions in the human genome that would make linking individual factors to a disease condition highly complex. These interactions, due to inheritability, must account for

family history to correctly interpret genotyping results to provide personalised medical treatment. Indeed, they must go beyond and ask how a given genome copes with a dynamic environment. The magnitude of this task has propelled the convergence of life sciences with other fields, *e.g.*, physics, chemistry, mathematics, computing, engineering, social sciences, etc. in search of new and innovative solutions. A recent NRC study notes [68]:

> The scientific opportunities enabled by convergence—the coming together of insights and approaches from originally distinct fields—will make fundamental contributions in our drive to provide creative solutions to the most difficult problems facing us as a society. This convergence provides power to think beyond usual paradigms and to approach issues informed by many perspectives instead of few.

Synthetic biology makes personalised medicine appear within reach in terms of developing personalized drugs and diagnostics, minimizing adverse drug reactions, and personalising treatments by enabling people to make personalized health decisions. However, to take research results from the lab to the patient's bedside, to the community (translational application) and finally make it accessible to every human on Earth is a colossal endeavour that calls for a very high level of convergence. The time has come for governments to frame policies that would enable the desired convergence. The U.S. government, *e.g.*, is discussing a "modular" policy [69] with public participation. A module, *e.g.*, may include education, basic research, and infrastructure; another that promotes market-oriented innovation through R&D tax credit, intellectual property policies, etc.; and yet another for catalysing breakthroughs in such areas as clean energy, biotechnology, nanotechnology, advanced manufacturing, information technology, and space technologies.

Genomics researchers are the new super-stars of science. A Thomson Reuters report provides a listing of authors who have written multiple highly cited reports and have thereby demonstrated their tremendous influence on ongoing research in their respective fields. Out of the seventeen hottest researchers a dozen belong to genomics [70]. Not surprisingly, the US sits atop the genomics-related patent filings heap, trailed by Europe and Asia, indicating the dominance of the U.S. both in research and its translation. [71]. An enigmatic world order is in the making.

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Over the recent years, biotechnology has become responsible for explaining interactions of biological tools and processes so that many scientists in the life sciences from agronomy to medicine are engaged in biotechnological research. This book contains an overview focusing on the research area of molecular biology, molecular aspects of biotechnology, synthetic biology and agricultural applications in relevant approaches. The book deals with basic issues and some of the recent developments in biotechnological applications. Particular emphasis is devoted to both theoretical and experimental aspect of modern biotechnology. The primary target audience for the book includes students, researchers, biologists, chemists, chemical engineers and professionals who are interested in associated areas. The book is written by international scientists with expertise in chemistry, protein biochemistry, enzymology, molecular biology and genetics, many of which are active in biochemical and biomedical research. We hope that the book will enhance the knowledge of scientists in the complexities of some biotechnological approaches; it will stimulate both professionals and students to dedicate part of their future research in understanding relevant mechanisms and applications.

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