THE SCOPE OF BIOTECHNOLOGY IN THE 21ST CENTURY

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BIOTECHNOLOGY
IN THE 21ST CENTURY

EDITORS
María Concepción Tamayo Ordóñez, PhD
Yahaira de Jesús Tamayo Ordóñez, PhD
Lorenzo Felipe Sánchez Teyer, PhD

COMPILATOR
Francisco Alberto Tamayo Ordoñez, MSc

COORDINATOR
Dr. Atl Víctor Córdova Quiroz, PhD

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Consejo Nacional de Ciencia y Tecnología
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ABOUT THE EDITORS

MARÍA CONCEPCIÓN TAMAYO ORDÓÑEZ, Phd

She studied her bachelor's degree at the Autonomous University of Campeche (2007), her master's degree at the National Polytechnic Institute. National School of Biological Sciences. Helmet of Santo Tomas D.F (2009). The Doctorate in Biological Sciences Biotechnology Option at the Center for Scientific Research of Yucatán A.C (2013). In addition, she has done postdoctoral stays at the National Polytechnic Institute. Genomic Biotechnology Center. Reynosa Tamaulipas (2014) and has worked as Associate Research Professor C at the Yucatán Scientific Research Center (2016-2018).

She is currently a professor-researcher at the UA de C, attached to the Department of Biotechnology of the Faculty of Chemical Sciences. She belongs to the SNI level 1 and to date she has taught more than 15 courses, has participated in 30 congresses (National and international), has published 22 scientific articles, participated in the publication of book chapters. She is a PRODEP profile. He has trained human resources at the undergraduate and graduate level. She is a current evaluator of CONACYT projects and reviewer in international research journals. Is currently a Member of the AGARED (Comprehensive Sustainable Use and Biotechnology of Agaves). founder of INBIOTO (Biotechnological Innovation TO) and belonging to SONABIIN (National Society for Biotechnology and Engineering). Her research interest is the genetic study of isolated microorganisms from different habitats, with characters of interest to produce alternative energies.

YAHAIRA DE JESÚS TAMAYO ORDÓÑEZ, PhD

Academic training in Environmental Biochemical Engineering (Autonomous University of Campeche, UAC), Postgraduate in Biological Sciences: Biotechnology (Yucatan Scientific Research Center, CICY), Postdoctoral Stay-CONACYT (Postgraduate in Food Science and Technology, Autonomous University of Coahuila-UAdeC). Current line of research, study of evolutionary mechanisms and events in the genome of biological systems with biotechnological potential.

She is currently a professor-researcher at the UA de C. She belongs to the SNI level 1 and to date she has taught more than 12 courses, has participated in 37 congresses (National and international), has published 18 scientific articles, participated in the publication of book chapters. He has trained human resources at the undergraduate and graduate level. Co-founder of INBIOTO (Biotechnological Innovation TO) and belonging to SONABIIN (National Society for Biotechnology and Engineering).

LORENZO FELIPE SÁNCHEZ TEYER, PhD

Biochemical Engineer (Technological Institute of Celaya) with a PhD in Plant Sciences and Biotechnology (Scientific Research Center of Yucatan-CICY). Currently a full professor in the Biotechnology Unit of CICY. He belongs to the SNI level 2, has published more than 60 scientific papers and book chapters, mainly related to the development and application of various molecular marker techniques for selection and genetic improvement. It has trained 22 postgraduate students and 10 undergraduate students.

PREFACE

Biotechnology is a broad interdisciplinary branch of biological sciences that consists of any technological application that uses biological systems and living organisms or their derivatives for the creation or modification of products or processes for specific uses. Within these organisms they may or may not be genetically modified. The bases of this interdisciplinary are biology, engineering, physics, chemistry, and biomedicine. Over the years the field of this science has shown relevance in pharmacology, medicine, food science, the treatment of solid, liquid and gaseous waste, industry, livestock and agriculture.

Biotechnology is present day by day in our daily lives, in the foods that we consume are sometimes supplemented with compounds, enzymes and proteins that help improve the taste, texture or general properties of food. Biotechnology has also presented great impact on the generation of raw materials to minimize the use of fossil fuels. Through different crop optimization systems and genetic manipulation, it has been possible to improve the properties of biomass in crops or biological models that are used as raw material to obtain fatty acids and alcohols.

During the year 2020, the COVID-19 pandemic was experienced worldwide, the participation of biotechnologists for the synthesis of new vaccines or establishing methodologies for rapid detection of the virus, was decisive to control the spread of the virus among the world population.

This book describes what has been the biotechnological advance in selected topics related to genetic improvement of crops, enzyme biotechnology, plant tissue culture, metabolites extraction, advances in the generation of biofuels and generation of vaccines.

The goal of this book is to emphasize the importance of biotechnology today. The editors hope that this book is to your liking and helps you in the different disciplines in which they are developed.

CONTRIBUTORS

Ayil-Gutiérrez B.A. CONACYT- Centro de Biotecnología Genómica, Instituto Politécnico Nacional, Biotecnologia Vegetal. Blvd. del Maestro, s/n, Esq. Elías Piña, Reynosa 88710.

Bello-López J.M. División de Investigación, Hospital Juárez de México, Ciudad de México, Mexico.

Betancourt-Urbina G.A. Unidad Académica Multidisciplinaria Reynosa-Aztlán, Universidad Autónoma de Tamaulipas. Calle 16 y Lago de Chapala S/N Col. Aztlán, Reynosa, Tamaulipas, 88740.

Bocanegra-Alonso A. Unidad Académica Multidisciplinaria Reynosa-Aztlan, Universidad Autónoma de Tamaulipas. Calle 16 y Lago de Chapala S/N Col. Aztlan, Reynosa, Tamaulipas, 88740

Canedo-Lopez Y. Facultad de Química, Universidad Autónoma del Carmen. Calle 56, No.4. Esq. Av. Concordia. Colonia: Benito Juárez, CP. 24180. Ciudad del Carmen, Campeche. México.

Cázares-Sánchez E. Tecnológico Nacional de México, campus Instituto Tecnológico de la Zona Maya, carretera Chetumal Escárcega Km. 21.5. C.P. 77960. Ejido Juan Sarabia, Quintana Roo, México.

Córdova-Alvarado A.A. Centro de investigación Científica de Yucatán A.C. Unidad de Bioquímica y Biología Molecular de Plantas. Mérida Yucatán México.

Córdova-Quiroz A.V. Facultad de Química, Universidad Autónoma del Carmen. Calle 56, No.4. Esq. Av. Concordia. Colonia: Benito Juárez, CP. 24180. Ciudad del Carmen, Campeche. México.

Damas-Damas S. Facultad de Química, Universidad Autónoma del Carmen, Calle 56 No. 4 por Av. Concordia, Campus Principal, 24180 Ciudad del Carmen, Campeche.

De la Cruz-Arguijo E.A. Centro de Biotecnología Genómica, Instituto Politécnico Nacional, Blvd. del Maestro, s/n, Esq. Elías Piña, Reynosa 88710.

Diaz-Palafox G. Laboratorio de Ingeniería Genética. Departamento de Biotecnología. Facultad de Ciencias Químicas. Universidad Autónoma de Coahuila. Ing J. Cardenas Valdez S/N, República, 25280 Saltillo, Coah.

Equihua, M. Red de Sustentabilidad y Medio Ambiente, Instituto de Ecología, A.C. Km. Carretera Antigua Xalapa-Coatepec. 351, El Haya, 91073 Xalapa, Veracruz, México.

Estrada-Alfaro N.A. Centro de investigación Científica de Yucatán A.C. Unidad de Biotecnología. Mérida Yucatán México.

Fuentes-Siller E.J. Laboratorio de Ingeniería Genética. Departamento de Biotecnología. [2]Facultad de Ciencias Químicas. Universidad Autónoma de Coahuila. Ing J. Cardenas Valdez S/N, República, 25280 Saltillo, Coah.

García-Castillo M.J. Centro de investigación Científica de Yucatán A.C. Unidad de Biotecnología. Mérida Yucatán México.

Hernández-Delgado S. Centro de Biotecnología Genómica, Instituto Politécnico Nacional, Biotecnologia Vegetal. Blvd. del Maestro, s/n, Esq. Elías Piña, Reynosa 88710.

Interian-Ku V.M. Tecnológico Nacional de México, campus Instituto Tecnológico de la Zona Maya, carretera Chetumal Escárcega Km. 21.5. C.P. 77960. Ejido Juan Sarabia, Quintana Roo, México.

Lira-Mendez K. Centro de Biotecnología Genómica, Instituto Politécnico Nacional, Blvd. del Maestro, s/n, Esq. Elías Piña, Reynosa 88710.

López-López A. Tecnológico Nacional de México, campus Instituto Tecnológico de la Zona Maya, carretera Chetumal Escárcega Km. 21.5. C.P. 77960. Ejido Juan Sarabia, Quintana Roo, México.

López-Mata L. Macroecología, Departamento de Botánica, Colegio de Postgraduados en Ciencias Agrícolas, Km. 36.5 Carretera México-Texcoco. 56230 Texcoco, Méx., México.

Milán-Cárdenas M.C. Facultad de Química, Universidad Autónoma del Carmen. Calle 56, No.4. Esq. Av. Concordia. Colonia: Benito Juárez, CP. 24180. Ciudad del Carmen, Campeche. México.

Montoya-Hernández D.J. Facultad de Química, Universidad Autónoma del Carmen. Calle 56, No.4. Esq. Av. Concordia. Colonia: Benito Juárez, CP. 24180. Ciudad del Carmen, Campeche. México.

Poot-Poot W. Laboratorio de Biotecnología, Facultad de Ingeniería y Ciencias, Universidad Autónoma de Tamaulipas, Centro Universitario, 87120 Cd Victoria, Tamps.

Pulido-Salas M.T. Banco de Germoplasma, Centro de Investigación Científica de Yucatán, Mérida, Yucatán, México. Parque Cientifico Tecnológico de Yucatán km 5 carretera Sierra Papacal a Puerto Chuburná, 97302 Mérida, Yucatán, México.

Ramos-García V.H. Unidad Académica Multidisciplinaria Reynosa-Aztlán, Universidad Autónoma de Tamaulipas. Calle 16 y Lago de Chapala S/N Col. Aztlán, Reynosa Tamaulipas, 88740.

Robles-Heredia J.C. Facultad de Química, Universidad Autónoma del Carmen. Calle 56, No.4. Esq. Av. Concordia. Colonia: Benito Juárez, CP. 24180. Ciudad del Carmen, Campeche. México.

Rodriguez-Zapata L.C. Centro de investigación Científica de Yucatán A.C. Unidad de Biotecnología. Mérida Yucatán México.

Romero-Duran H.P. Facultad de Ciencias Económico Administrativas. Universidad Autónoma del Carmen, Calle 56 No. 4 por Av. Concordia, Campus Principal, 24180 Ciudad del Carmen, Campeche.

Ruiz-Marín A. Facultad de Química, Universidad Autónoma del Carmen. Calle 56, No.4. Esq. Av. Concordia. Colonia: Benito Juárez, CP. 24180. Ciudad del Carmen, Campeche. México.

Salazar-Bravo A. Centro de Biotecnología Genómica, Instituto Politécnico Nacional, Biotecnologia Vegetal. Blvd. del Maestro, s/n, Esq. Elías Piña, Reynosa 88710.

Sanchez-Teyer L.F. Centro de investigación Científica de Yucatán A.C. Unidad de Biotecnología. Mérida Yucatán México.

Tamayo-Ordoñez F.A. Facultad de Química, Universidad Autónoma del Carmen, Calle 56 No. 4 por Av. Concordia, Campus Principal, 24180 Ciudad del Carmen, Campeche.

Tamayo-Ordoñez M.C. Laboratorio de Ingeniería Genética. Departamento de Biotecnología. Facultad de Ciencias Químicas. Universidad Autónoma de Coahuila. Ing J. Cardenas Valdez S/N, República, 25280 Saltillo, Coah.

Tamayo-Ordoñez Y.J. Facultad de Ciencias Químicas. Universidad Autónoma de Coahuila. Ing J. Cardenas Valdez S/N, República, 25280 Saltillo, Coah.

Vital-Ocampo A.G. Facultad de Química, Universidad Autónoma del Carmen. Calle 56, No.4. Esq. Av. Concordia. Colonia: Benito Juárez, CP. 24180. Ciudad del Carmen, Campeche. México.

CHAPTER

ONE

Biotechnology and sustainable use of biodiversity (Biotechnology, ecological integrity and sustainability)

CHAPTER 1

BIOTECHNOLOGY AND SUSTAINABLE USE OF BIODIVERSITY (BIOTECHNOLOGY, ECOLOGICAL INTEGRITY AND SUSTAINABILITY)

Equihua, M.[1], Pulido-Salas M.T.[2] and López-Mata L.[3]

[1]Red de Sustentabilidad y Medio Ambiente, Instituto de Ecología, A.C. Km. Carretera Antigua Xalapa-Coatepec. 351, El Haya, 91073 Xalapa, Veracruz, México +522288421800; equihuam@gmail.com.

[2]Banco de Germoplasma, Centro de Investigación Científica de Yucatán, Mérida, Yucatán, México. Parque Científico Tecnológico de Yucatán km 5 carretera Sierra Papacal a Puerto Chuburná, 97302 Mérida, Yucatán, México. +529999300767; pulidosalas@gmail.com.

[3]Macroecología, Departamento de Botánica, Colegio de Postgraduados en Ciencias Agrícolas, Km. 36.5 Carretera México-Texcoco. 56230 Texcoco, Méx., México. +525959520200; laurolopezmata@gmail.com.

Corresponding author: **pulidosalas@gmail.com**

ABSTRACT

Our purpose in this chapter is to encourage a reflection on biotechnology from an ecological perspective. Biotechnology operates on biodiversity, thus the applications that are conceived, designed, and developed, should be additionally framed in the workings of the biosphere, assuming a "whole system design" stand. Sustainability requires new ways to improve people's access to well-being, so regarding biotechnology we perceive two parallel and interweaved concerns: 1) Biodiversity erosion itself; 2) Deep ecosystem disruption; both damaging the human support systems in the biosphere. Thus, biotechnology should take an active role in protecting biodiversity, its functionality, and the benefits for human life. On one side, new products and commodities can imply new biological interactions between the existing and the novel entities engineered. On the other side, most of modern human actions are affecting ecosystem integrity. To depart from such a harmful track, many young people around the world is opting for technological designs that cares for the "oikos", understood as the ecosystemic "house of human beings", its functionality, and the multiple benefits provided, as enough food, clean air, and good health. Not only based on the understanding the limits to its resilience when using the resources produced by the complex dynamics of biosphere, but also because the costs of

damaging the provisioning capacity of nature entail to human societies. As the international protocol of Nagoya recognizes, it is of the greatest importance to acknowledge the traditional knowledge preserved by indigenous people about natural resources, as well as the knowledge produced by current biotechnologists. Therefore, the core of our analysis is based on two major axis: "the biodiversity" and "the ecosystem." Consequently we address emerging topics frequently not considered, when the main goal should be conducive to sustainable projects aware of the paramount importance of maintaining the "dynamic ecological balance" of planet Earth.

1.1 INTRODUCTION

While we are finishing the writing of this chapter, the whole world is in the middle of a pandemic caused by the virus SARS-CoV-2. Most likely, new trends for scientific research will be focused this particular organism and its variants to cope with the urgent health crisis. Right before this novel entity appeared before us, the trends in biotechnology research was focused on industrial and investment innovations for a stronger economy, the most visible concern was aimed at reducing risks (Fröhling and Hiete, 2020). A philosophical debate has been emerging from some biotechnological research implications, for example biofuels production (Meyer, 2011) or, around, GMOs (Friedrich *et al*., 2019); or how important is to design new varieties to face climate change producing enough food (Kole *et al*., 2015). This debate has also expanded to political and social implications, especially in developing countries as it is the case of Mexico (Aerni, 2002). The debate impacts well on governance and international regulations (Bratlie *et al*., 2019).

The study by scientists of biodiversity has been strengthened and gained in public relevance due to the current environmental crises generated by activities made from a limited anthropocentric posture that has taken biodiversity dynamics for granted and as an economic externality of little interest to support economic output and human development (Niesenbaum, 2019). The study of biodiversity is increasingly transdisciplinary and somewhat reactive. The latter because the situations and subjects it studies and the issues it attempts to solve are analyzed a posteriori The former because it incorporates to biological understanding the theoretical contributions and practices from several scientific and social disciplines like Anthropology, Economics, Statistics, Ethics, Mathematics, Sociology, and Engineering, and the wisdom of people fostering traditional cultivars use and preservation. It is also an inexact discipline because it deals with complexity. In many cases, its predictive capability is relatively low, which leads to the inherent difficulty of establishing basic universal principles, even when using probabilistic approaches and stochastic processes.

This complexity is matched by the compilation by researchers and partnerships of huge databases as well as the Big data flowing

from social networks and new information technologies (Sayers *et al*., 2020). This new context flooded by data (barely structured, massive and many-sided) is challenging us to device approaches (scientific, technological, social, and economic) conducive to the public good, rather than the prevailing somehow frivolous marketing. Can we use such data to help in the design of improved decision making, based on evidence? Can we integrate into such improvement human wellbeing with a sustainable horizon? Even today there is a lack of linking data between human activities and impacts on nature dynamic balance. Our analysis and suggestions here are based on an ecological understanding which seeks to link the health of "the Oikos", understood as the house of human being interwoven (often by weak link, sometimes by strong ones) with the rest of the living forms in the planet. We comprehend the biosphere as a functioning house where live in all its form is literally produced, not just the house of human beings.

1.2 WHY TO TALK ABOUT BIODIVERSITY?

Biodiversity is one of the planetary boundaries critical for Earth's human life support systems considered by Rockström *et al*. (2009) and Steffen *et al*. (2015). The current human pressure poses a severe threat to the biosphere in interaction with other important challenges as climate change and the alteration of other biogeochemical cycles, in particular, the delicate flow of nitrogen of which the public is made little aware. As a response to these concerns, the world's nations have signed agreements to face the challenges implied in these disturbances as the Convention of Biological Diversity (CBD, 2010) that has operated since its onset during the famous Earth Summit held in Rio de Janeiro, Brazil, on June 21, 1992.

Biodiversity, or biological diversity, is often conceived in terms of the variation of plant, animal, and microorganism species, of which 1.75 million are known by science out of the suspected 15 million that exist. The CBD defines biodiversity as the diversity of living forms on the planet Earth, including its genetic support and the natural patterns of ensembles of biological populations that form ecosystems. Biodiversity is also thought as the product of millions of years of the evolution process that took place on Earth through the processes of natural selection acting upon genetic variability, including, the varieties produce under human selection. Due to its nature, biodiversity is produced by a network of kin relationships and the associations of coexistence established between species sharing space and living conditions in ecosystems (Blicharska *et al*., 2019). In sum, biodiversity is the web of life from which humans are also part, and ecosystems are the originary supporting and creation matrix (Cumming *et al*., 2020). Therefore, biological diversity is assessed by the number, and variety of living organisms at all levels of biological organization from genes to species and even to higher taxonomic categories. It includes the diversity of habitats and ecosystems that are contained in the biosphere and, hence, also the ecological processes taking place in them.

Because natural ecosystems are dynamic and are constantly subject to changing conditions, the study of biodiversity requires copping with complex mechanisms for permanent temporal and spatial monitoring and adaptations. This knowledge is needed to design efficient strategies to mitigate and counteract the effects of past, present, and future threats. Also, it is a scientific multidiscipline permeated by values and, therefore, subjective in many cases in which moral or ethical issues need to be encompassed. The urge to address biodiversity in the planet from the academic, social, political, and economic areas represents a reaction to the inherent codependence on which, directly or indirectly, human well-being is sustained, and even the survival on Earth of *Homo sapiens*.

In the 21st century, biodiversity frontier is increasingly challenged by novel entities generated by biotechnology (ancient or modern) and synthetic biology, thus we propose it should be made visible by including a reference to it in the span of planetary boundaries.

1.2.1 THERE ARE NO ECOSYSTEMS WITHOUT INTERACTIONS

Because of the essential role of interactions between the species that make up biodiversity, acting as an enormous set of ensembles, the modern biotechnologists must, in parallel with the day to day tasks, become concerned with understanding the varied ecosystems in which the biological diversity being studied lives, be it forests, wetlands, alpine tundra, lakes, oceans, and even croplands and grazelands. At present, the CBD is committed to achieving the biodiversity targets adopted by party countries in the city of Nagoya, Aichi Prefecture, Japan in October 2010, known as the Aichi Biodiversity Targets as follow:

1. To address the underlying causes of biodiversity loss by mainstreaming biodiversity across government and society.
2. To reduce the direct pressures on biodiversity and promote sustainable use.
3. To improve the status of biodiversity by safeguarding ecosystems, species, and genetic diversity.
4. To enhance the benefits to all from biodiversity and ecosystem services.
5. To enhance implementation through participatory planning, knowledge management and capacity building.

In its broadest sense, biotechnology has formed part of the forces modeling the biodiversity since 10,000 years ago when plants and animals began to be domesticated (Diamond, 2002). A proposal in that context by Larqué (2016) is to rescue and value the techniques developed in prehispanic time in Mesoamerica to write a whole new chapter of innovative research in the biotechnological subdisciplines. Human beings have also influenced the fate of biodiversity by promoting changes in ecosystems on a global scale. Regarding the direct use of species and genomes, the CBD has promoted international agreements resulting in two protocols significant for biotechnology, the 2000 Cartagena Protocol on Biosafety and the 2010 Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization (ABS).

1.2.2 THE UNAVOIDABLE SYSTEMIC APPROACH

Certainly, technological design is implied in the advancement of civilization but it also has had its share of environmental damage, because of which the proposal to adopt a 'total system approach' has gained relevance in order to prevent collateral damage that may be brought by technological innovation (Stasinopoulos *et al.*, 2009; von Weizsacker *et al.*, 2009; Blizzard and Klotz, 2012).The idea is to take into account not only the specific requirements of the technology being developed but to add to the equation the necessary components of the context of their production and use that link them with the health of ecosystems and which determine their articulation with societies.

The call for adopting a prudent total system approach is urgent because of the appearance in the biotechnological scenario of the so-called 'synthetic biology,' operationally defined by the CBD as "a further development and new dimension of modern biotechnology that combines science, technology and engineering to facilitate and accelerate the understanding, design, redesign, manufacture and/or modification of genetic materials, living organisms and biological systems." As can be perceived, the area of application of synthetic biology is ample but, although it may be estimated that the Cartagena Protocol on Biosafety could appropriately cover its safe application, there is no doubt that this Protocol must be adjusted to incorporate the methodologies and specific approaches of this emergent area of biotechnology. It is essential to insist on the need to consider the environmental, socioeconomic, cultural, and ethical dimensions of the possible effects, both positive and negative, of the organisms, components, and products resulting from synthetic biology. Technology is the cultural process that mediates between human desires and the environment, but in the face of the challenges implied in the global human impact (Montagu, 2020), the biocultural dimension of biotechnology must represent a plain reminder of the inescapable anchorage of human societies to the evolutionary dynamics of Earth's socio-systems.

1.3 THE TIME FACTOR IN CHANGES DERIVED FROM NATURAL EVOLUTION OR BIOTECHNOLOGY

Because of the essential role of evolution as a generator of biodiversity, genetic components of species must be studied, in particular at the level of the molecular diversity that is in charge of providing the genes that will be inherited to provide new adaptations.

In this context, a most important factor to be considered is the time required by the evolutionary change following an adaptive processes, which are continuously occurring in the ecosystem. We would be talking of millions of years which is completely different to the few months or years needed to produce changes in an organism through modern biotechnological methods.

By its nature, biological evolution occurs through the coupling of species, or species assemblages, which can be either lax or tight but always defined by the multiple relationships taking place in the ecosystem.

Its mechanism acts in a continual trial and error exercise through which populations of species modify themselves by the propagation of changes among individuals through reproduction. Changes which we call 'adaptations,' 'new organisms,' or 'new varieties'.

Since this process depends on the time needed for its operation, doubts arise regarding the capability of ecosystems and its components as a network of life, to respond at the velocity suitable to match the speed of the transformations introduced by biotechnological processes. The rate of change is, therefore, an essential factor when analyzing the impacts at a short, medium, and long term produced by this novel human capacity to influence the weave of life.

Biotechnological processes acting through mutagenesis produce changes in time intervals tending to zero giving place to new varieties intended for new ecological conditions and satisfy very particular human needs (Van-Montagu, 2020). In contrast, the traditional selection and changes induced by stakeholders, say small farmers in their fields, may need several generations in the case of plants with high variation as maize, but some plant species may need a longer time as in the case of fruit hybrids needing several years.

1.3.1 HUMAN NEEDS AND ECOSYSTEMS AS BENEFITS PROVIDERS

The vital relationship between humans and nature is driven by multiple perspectives and takes place at several levels. If we think of our needs to satisfy our everyday requirements, we will surely appreciate the inevitability of our dependence upon biodiversity. It is enough to start with three essential needs, oxygen produced by green plants and algae, food directly or indirectly produced by the photosynthetic organisms (unique transformers of sun radiation into organic molecules), and preservation of health through drugs obtained from natural sources (plants, animals, and minerals). Surprisingly, the success in domesticating plants and animals has resulted in human beings, narrowing their direct links to nature to the point of dependence on very few domesticated wild species and the promotion of a massive replacement of natural ecosystems by cropland, grazeland, and urban settlements (Diamond, 2002; Maass and Equihua, 2015).

We may possibly never get to know all of the direct benefits that could be derived from biodiversity, but even without that knowledge we are aware that they form part of our way of life (Adom *et al*., 2019). They are essential for the organized and self-regulated functioning of healthy ecosystems, and are beneficial for humanity. According to Bratman *et al*. (2015), there are demonstrable physiological pathways through which the simple contact with nature improves the physical and mental health of people. At this point, it may be worthwhile to ask ourselves if the advancements of molecular biology could help to increase that fraction of species on which human existence depends. Even, if molecular biology could contribute to reduce the loss of natural ecosystems and habitats, which today is the main cause of species extinction (Figure 1.1) and that might be triggering the sixth mass extinction event in the history of the planet. What would be desired is arriving at advancements focused on both parallel goals: to diversify the spectrum of species linked to direct human interests and to reduce the loss of spaces by preserving natural ecosystems.

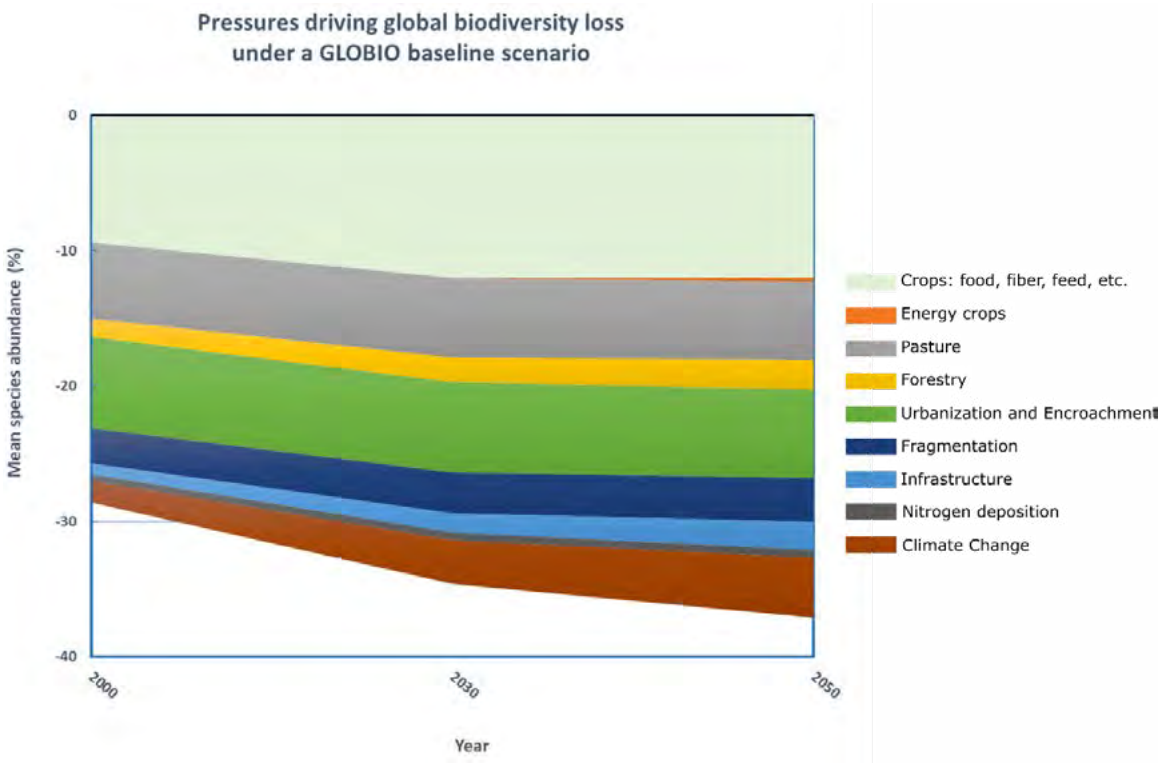

Figure 1.1. *Pressures driving biodiversity loss. Base on the output of the GLOBIO3 model, modification of NEAA (2010). This figure is a modification of NEAA (2010). Source: Netherlands Environmental Assessment Agency (2010).*

With that background, it is clear that three challenges exist for biotechnology involving its future in the context of damaging the integrity of ecosystems and the search for sustainable development when facing the social and environmental dilemma we are experiencing in the 21st century. In consistency with it, we pose three priority challenges as follow:

- Expanding the anthropogenic capabilities for the sustainable use of a larger number of wild species.
- Preserving the genetic diversity of cultivars and their wild relatives *in situ*.
- Maintaining enough spaces with the structural and functional integrity of ecosystems, that is, preserving their capabilities for self-organization (resilience) and natural evolution.

1.3.2 VALUING THE LOSSES

Ecosystems simultaneously offer all the above-mentioned benefits, and many more, and they do it as a consequence of being thermodynamic systems that, through diverse living organisms (plants, animals, fungi, bacteria, etc.), flowing materials and dissipating energy. The vitality itself of the ecosystems relates to their capacity to offer benefits enjoyed by humanity.

Base on the output of the GLOBIO3 model, the trends of change on relative mean species abundance of original species (MSA) depict a clear risk of biodiversity erosion worldwide. The main drivers of this change are included. Clearly agriculture, cattle ranching and urbanization, by promoting landscape conversion are strong drivers of this trend, of course exacerbated by climate change.

Source: Netherlands Environmental Assessment Agency (2010) Rethinking Global Biodiversity Strategies: Exploring structural changes in production and consumption to reduce biodiversity loss.

It is easy to see that the ways in which nature generates value used by humans with a dosage of tension. On one side, there is technology trying to increase the benefits derived from the appropriation of specific components of biodiversity focusing on socio-economic priorities. On the other side, this appropriation of nature has, until now, disturbed the spontaneous thermodynamic processes that determine the dynamics of natural ecosystems and, therefore, their capability to provide the ecosystem services that we require. This alteration is mainly shown as a trend towards homogenization of our surroundings and, thus, to an alarming reduction of biodiversity at all levels of organization that has now reached a global dimension (Borghini, 2019). This effect is observed as specific footprints of human presence, which has gained recognition as a global geological imprint that begins to be called the Anthropocene (Bertolami and Francisco, 2019; Zalasiewicz *et al.*, 2018; Equihua *et al.*, 2015).

The number of species directly linked to human economic cycles is limited. According to FAO data, over 80% of the human diet is based on plants; of which, throughout human history, people have cultivated nearly 7,000 species in 12 centers of origin (See Fig. 1.2), and among those only four (rice, wheat, maize, and potato) contribute 60% of the global caloric intake by people. The same source states that humanity is currently dependent on 38 domestic animal species. Fish provide 20% of the animal protein consumed by about three million people, 10 species of marine fish account for 30% of

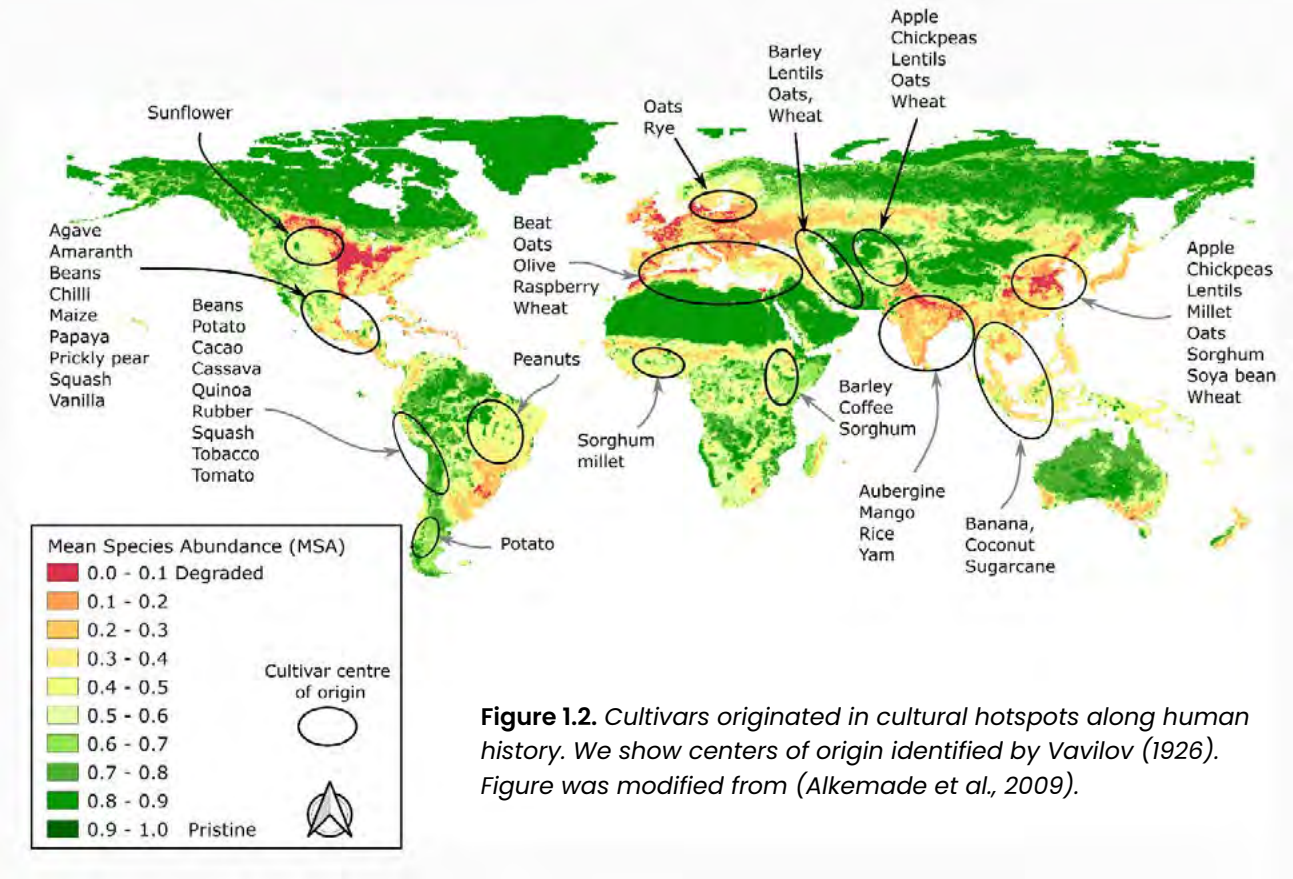

Figure 1.2. *Cultivars originated in cultural hotspots along human history. We show centers of origin identified by Vavilov (1926). Figure was modified from (Alkemade et al., 2009).*

the commercial fisheries, and aquaculture is based on only 10 species. The human food security critically depends on maintaining high genetic diversity in these few species, requires innovative solutions (Higgins, 2020).

Cultivars originated in cultural hotspots along human history. We show centers of origin identified by Nicolai Vavilov in 1926. Currently, these centers of origin seem to correlate with areas where remaining biodiversity, as estimated by the relative mean species abundance of original species (MSA), is highly altered (low remaining MSA index), suggesting both a threat to biodiversity in general and agrobiodiversity in particular.

Table 1.1 *Number of varieties of the main crop plants in the world. Source: FAO.*

200,000	varieties of rice
120,000	varieties of wheat
4,000	varieties of potatoes
35,000	varieties of finger millet
3,000	varieties of coconut

Table 1.2. *Loss of varieties of some important crop plants. Source FAO.*

Country	Loss
Spain	400 melon varieties in 1970, only 12 today
Germany	all apples now grown originated from only 6 varieties
China	90% of rice varieties since 1950
Mexico	80% of corn varieties since 1900
India	90% of rice varieties since 1900
USA	90% of fruit and vegetable varieties since 1900

1.3.3 THE GENETIC MODIFICATIONS

The number of food resources that have not been sufficiently explored or valued is huge. It is something we will discuss further ahead in this chapter, which could suggest that the modifications due to gene insertion or edition are unnecessary or irrelevant. However, since the proposal in 1953 of the structural model of DNA by Watson and Crick (Watson and Crick, 1953), the advancement of molecular biology and its technological tools have taken several paths opening numerous possibilities of intervening in the genetic build of organisms. The many transgenic projects that resulted are the center of debate regarding their applications, scopes, and ethical implications. The debate mainly stems from the potential alteration of the 'human nature' and the assessment of the possible relationships that could be established between the genetically modified organisms for agriculture and the natural environment (Cué and León, 2012).

Certainly, there is a debate strongly loaded with market components from agribusinesses and the manufacturers of the agrochemicals. What is open to debate is not the need to satisfy a scientific curiosity but the answers given by each project to the questions of what, why, for what, for whom, and how; frequently answered under a not holistic but limited perspective.

In agriculture, genetic modification through direct intervention in the genome of native species undoubtedly poses several ecological risks. One such risk is that some genetically modified organisms could cross with other individuals of the same or a phylogenetically closely related species, which could eventually lead to the genetic erosion of the wild populations through hybridization. In addition, if the introduced modification involves resistance to a pesticide, its application would eliminate the native plants lacking such resistance. Thus, according to Johnson (1999), super-varieties could be created that would become competitors of the native varieties with consequences that are until now unpredictable.

Because biotechnology comprises techniques to improve production processes, gene translocation to obtain individuals genetically edited in particular characteristics is one of the most active, less understood, and most questioned of its sub-disciplines (Cumming *et al*., 2020). We can briefly analyze at least one of its justifying arguments: To increase the productivity of the land through improved varieties more resistant against pest or droughts, which, in turn, reduces the need to apply soil-contaminating agrochemicals (Bolívar-Zapata, 2017). This is a plausible justification; however, it could be analyzed from the premise of that being only one side of the coin, which invites us to look at what the other face shows. The CRISPR (clustered regularly interspaced short palindromic repeats) techniques allow modifying the genome of any organism with extraordinary precision, are relatively inexpensive, and are easy to implement, which provides agro-food monopolies an enormous potential to manipulate the genes of plants with commercial importance for feeding human beings. Studies are still lacking to examine, in parallel and with an equal passion, the subject of innocuousness and the potential impacts on human health, which is implicitly involved but often relegated.

Therefore, the analysis must proceed by considering that genetically modified organisms (GMO) have their genetic resources transformed through transgenesis techniques to modify the expression of one of its genes or to add an external gene (Sánchez-Milani, 2000). Also to be taken into consideration is the statement made by Gould and Cohen (2000) about genes not acting alone but multiple interactions occurring between them and that whenever selection for improvement exists, a compensation arises, particularly in animals. The latter authors also point out that, for example, whenever pest-resistant seeds are released, they may give quite good results for some years; however, the pest can always develop resistance or until now overlooked consequences at ecosystem functioning level may appear. That could happen not so much for lack of scientific prevision but rather because of the complex nature of the biological systems involved.

Multiple references in that regard exist for maize. The academic search engine found 15,400 references to two words: transgenic and maize. In Mexico, there are ongoing de-

bates about the recommendations of specialists in maize genetics worried about the possibility of negative effects of transgenes on native maize in its region of origin. The recommendation strongly states that transgenic varieties of maize must not be cultivated until no experimental certainty exists that a genetic invasion of native varieties would not take place (Kato-Yamakake, 2004).

Johnson (1999) suggests another scenario in which, for example, plants that were created to be resistant to herbicides allow application of these agrochemicals to eliminate the crops' pest. However, herbicides application will affect other organisms in the natural environment, possibly impacting food chains through exposure of the insectivores, which the author considers is already happening with birds in Europe and soil beetles. Furthermore, in the case of Mexico, this concerns would require to add bats and reptiles among the species whose populations would be exposed to that threat that may compromise the self-regulatory capability of wild populations and, with that, the ecological balance of ecosystems.

In a review about the debate on GMO effects some authors propose multidisciplinary view for new projects; Zilberman *et al.* (2018), conclude that there is no conclusive evidence that they could be of danger to human health, neither to its claimed that agricultural production can be increased and effects on environment reduced. Authors show a series of studies focused to obtain healthier solutions with more benefit for small farmers who has been struck by climate change. This analysis unfortunately do not refer cases from Mexico, however authors recognize in general, that all biotechnological proposals can be improved by incorporating a good balance between risks and benefits, including social aspects (based on regulations that should be relevant, sensible and not excessive).

As follow in the image (see Fig. 1.3) we provide an image designed with the set of ideas involved in the current debate represented as a word cloud built from the titles and abstracts of scientific papers with the words maize and transgenic in them.

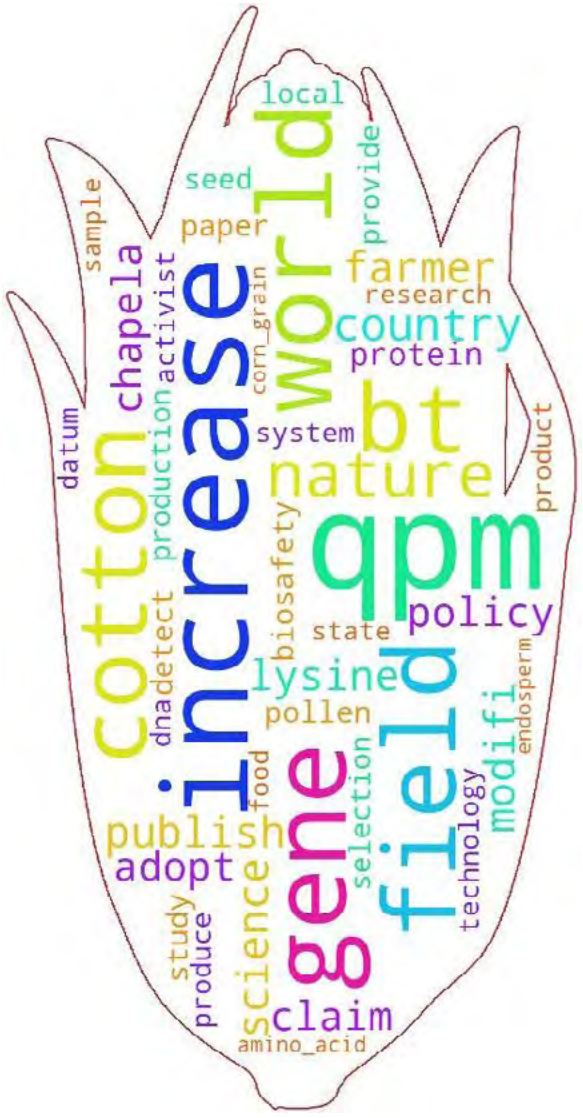

Figure 1.3. Word cloud representing in proportion the number of mentions found by search engines with the words maize and transgenic. Author. M. Equihua.

A search on the world wide web using scientific literature search engines also provides nearly 40,000 references including the words risk and GMO. It would be irresponsible not to take into consideration the results of researchers published in thoroughly scrutinized journals evaluated by highly qualified experts.

The arguments in favor of molecular research for the edition of 'improved' genomes seems to be based on three points:

1. Reduction of the use of agrochemicals because of the use of varieties resistant to pests and diseases.
2. Production of higher yields of food with higher quality (e.g. quality protein maize or QPM) and in less space.
3. Treatment for diseases of broad global distribution.

From the perspective of sustainability, these three arguments have a counterpart in the multispecific production used by small ethnic communities through empirical knowledge, at a local level, and with minimal inputs. The success of these agroecosystems has been noticed not only in Mexico (Toledo-Manzur *et al*., 2013; Herrera-Castro *et al*., 2012; Mariaca, 2012) but also in an ample diversity of systems in different latitudes like, for example, in China (Huai *et al*., 2011), Switzerland, Turkey, and Nepal (Bardsley, 2003), among many other places. A huge amount of information has been generated and recorded in the case of treatment of diseases in rural environments. For instance, in the year 2004, the Atlas of the Plants of Mexican Indigenous Medicine reports 1000 plants throughout Mexico of which only 524 had chemical or pharmacological tests of their therapeutic activity (Argueta and Cano, 1994). In the same way, the tests for 100 plant species used by the Mayan culture in Yucatán (71 of them native) were published by Méndez-González *et al*. (2012). The knowledge of local resources to solve health issues has not yet been enough studied for their responsible use. The management of multiple resources has been relegated not because of a lack of functionality or profitability, neither because of inadequate application for a healthy life, but because it does not count with the necessary verifications to be included in the large production systems. Also, the inclusion of traditional production systems can be foreseen because they are strategies particularized for highly specific conditions that would take part in the conservation of the local biodiversity covering a broad spectrum of plant/animal relationships. Recently in the context of agricultural production, more researchers recognize the role of traditional knowledge that evolve adaptive processes, named as "traditional ecological knowledge" (TEK), and they recommend to combine with scientific knowledge for a newest design of agricultural systems (Alzate *et al*., 2018). For same reasons, it is necessary to maintain and restore the great diversity of local varieties (Ficiciyan, 2018). With same importance we have to include wild relatives through a complete conservation strategy, in order to preserve those valuable genetic resources (Khoury *et al*., 2019a) which involve a high potential value for a sustainable food production in long term (Khoury *et al*., 2019b). All of the above would undoubtedly result in the ecological balance that is essential for a biologically and culturally megadiverse country as is Mexico.

1.3.4 THE ASSESSMENT OF IMPACTS

Another important issue to review, is the scarcity of multidisciplinary and widescope proposals for the objective and regular monitoring, as well as the follow-up assessment of the repercussions on the environment, on human health and on social appropriation.

Alvarez-Morales (1999) mentioned that an important reason for pressure on the environment in developing countries as Mexico, is the presence of opposing groups without a serious discussion and no agreements. And also that some prevalent situations are: the richness in biodiversity with the presence of wild relatives for cultivated plants, which means the possibility for a transgenic derivative; the scarce or lack of legislation for release GMOs (Brown *et al*., 2020), and a limited public education. We think that, to reach agreements, to offer information to the society, and to measure risks and impacts to prevent, we need more scientific research and a huge concentration of data.

The promotion of local, regional, and national agreements would need to be favored, which would, in turn, work to implement the outstanding international agreements (Cartagena Protocol on Biosafety and the Nagoya Protocol on Access and Benefit-sharing).

The recognition of several levels of risk cannot be stressed enough. At any rate, the need remains to design clear procedures allowing evaluation of possible environmental and human health repercussions in an integrated way, such that long term monitoring is enabled and reliable data gathered and disclosed to the public as open data. As an example, the Environmental Impact Quotient (EIQ), which is a method created by the staff of the College of Agriculture and Pest Management of Cornell University, designed to measure environmental impact addressing pesticides with the purpose of improving decision making based on evidence when selecting and using them (Kovach *et al*., 1992).

If an EIQ was regularly assessed, it would produce time series of high worth. Based on the EIQ proposal, finding an index to calculate the future impact of biotechnological projects in the multiple scenarios of Mexico would be pertinent. For that, perhaps the starting point would be to generate the necessary data to know the tolerance to the different pesticides of the components of biodiversity in a location (plants, animals, fungi, and microorganisms), or in the biotechnology case, to document the probability of hybridization of transgenic crops with their wild relatives, or to know the impact these transgenic crops would have on the health of the native fauna. Such an index would result in complementary data to match as a stressor with the resilience capacity of an ecosystem. In any case, the most essential thing is, once again, the time factor, which must be taken into account to the close monitoring of short, medium, and long-term effects that could emerge in each biotic component that could be in touch with the 'new' varieties designed through biotechnological methods. A good example of a study about small farms was conducted in Zambia (Amondo *et al*., 2019). It was based on the adoption of drought-tolerant maize varieties, where authors were valuing the risk and effects through a multivariable assessment including seeds availability, productivity and social data; concluding that flow of relevant information about new technologies can reduce risks and impacts.

Along Humphries and collaborators (1995) reasoning and defining biodiversity as "the number and frequency of ecosystems, species, and genes in a determined assemblage," a biotechnologist that decides to genetically modify a species would need to start with analyzing the natural geographic distribution of that species and assess the genetic representativity of such variety, besides ensuring the number of individuals that represent it according to their *in situ* abundance in each place. Of equal importance would be that, as part of the initial project, the biotechnologist poses a model from the point of view of alteration of ecosystem integrity level for future monitoring at different time periods of the impacts of the research project, at the same time the preservation *in situ* of the species is promoted objectively beyond the possibility of hybridization, that is, of genetic erosion, to happen.

Caswell (2000) analyzed the impact of GMOs concerning regulatory policies and instruments. The author recommends that the analysis of the risks derived from scientific studies and based on international standards should also be subject to political evaluation in each country, to legitimate and minimizing deployment risks. In great measure, this suggestion arises from concern on the influence that an economic assessment designed to satisfy the prevailing mercantile interests in that country may have.

Because of that, an objective measurement would need to be designed for each organism and for each country due to each one of these having particular conditions; she is apparently assuming without stating it that a country has homogeneous environmental conditions. In the case of Mexico, that would require taking into account the environmental, biological, and cultural diversity to design a series of indexes appropriate for each environmental and cultural scenario.

It seems necessary to mention here that there is an unavoidable relationship with the economy in any of the subsectors of the agricultural industry including seed production, agrochemicals, or genomics (Sánchez-Milani, 2000). These corporations contribute important subsidies to innovation research favoring the generation of patents, which in turn will lead to the control of the market. These fundamental relationships are reflected in that, in the year 2000, four agrochemical corporations controlled 100% of the GMO market. Crespi and Marette (2003) also mention a component having an impact on pricing both at the international level as at the level of consumers, which is the legend 'Does contain' or 'Do not contain' in product's labels, because of which the presence of transgenics is not always clearly stated.

These synergic effects of policies and economic dynamics have also been analyzed as part of possible effects on ecosystems. Carpenter *et al*. (2009) mentioned that The Millenium Ecosystems Assessment (2005) has new challenges to assess, project, and manage flows of ecosystem services and the effects on human well-being. These challenges are especially important because attempts to improve some policies and practices have been based on insufficient information about the dynamics of social-ecological systems and the relationships of ecosystem services to human well-being. Often, these attempts are also based on untested assumptions. The new researcher must consider the full ensemble of processes and feedbacks to biophysical and social systems.

It is in this context where the above-mentioned conceptual proposal of planetary boundaries of Rockström *et al*. (2009) can be linked to their relationships with human development, because of which these boundaries must not only be limits but a guide (Steffen *et al*., 2015). The genetically designed or modified individuals produced by mutagenesis would be biological entities within one of the three sections for which objective or complete information about their effects is unavailable (see Fig. 1.4). Therefore, we propose that these biotechnological products would be labeled as 'novel entities' representing a foreseeable risk and requiring strict monitoring to generate the knowledge allowing the assessment of their local and global effects. This idea is reinforced by Paull (2018) who analyze GMOs cases in Australia, looking at the potential to escape and become deleterious invasive entities. According to Rockström *et al*. (2009), planetary boundaries must guide and limit human development for it to be sustainable.

Human development has pushed the biosphere beyond the safe boundaries that we enjoyed over most of the Holocene. We show here the main subject issues that we are recognizing today in the environmental agenda. At the center of the Figure we illustrate the leverage power of the processes involved in the biosphere dynamics. Some of these processes are understood to be dependent on tis history (they show hysteresis), and so, for instance, they could be degraded slowly but recover relatively fast (like the biogeochemical cycles) Dotted arrows suggest week controlling effect or complex and poorly understood today. The illustration was modified from both Mace *et al*. (2014) and Steffen *et al*. (2015).

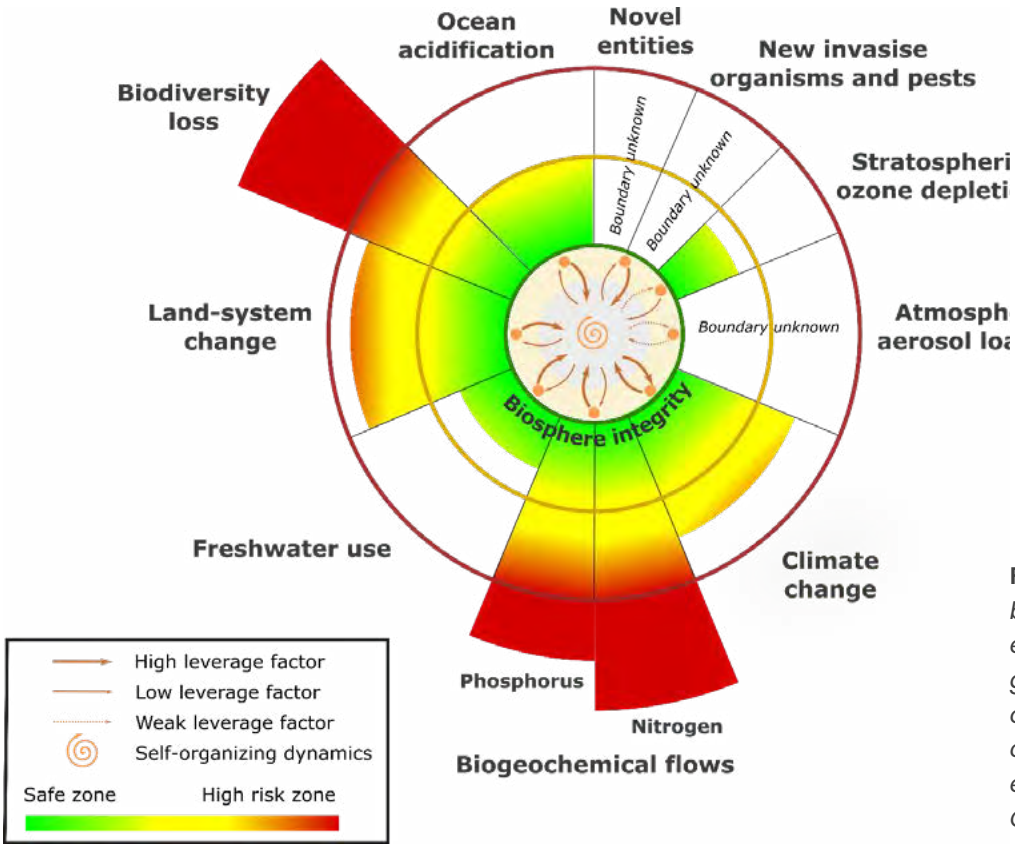

Figure 1.4. *Planetary boundaries of Rockstrom et al. (2009) in which genetically modified organisms can be considered among 'novel entities'. Adapted from: G.M. Mace et al., 2014.*

1.4 BIOTECHNOLOGY FOR THE SUSTAINABLE USE OF BIODIVERSITY

To continue the analysis purpose of this chapter, an evaluation must be made of the relationship between the services provided by a healthy ecosystem –according to their integrity and resilience– and the issues that motivate the search for integrated alternatives.

1.4.1 SUSTAINABILITY SCIENCE, THE FOUNDATION OF SUSTAINABILITY

In the 2005 United Nations report on the Millenium Ecosystem Assessment in which 2000 authors from around the world collaborated (Sarukhán and Whyte, 2005), four areas were determined for the analysis of the ecosystem services provided for the well-being of humans: Conditions and trends, Scenarios, Responses and Sub-global assessments. In Box 2.1 (p. 40) of the document, ecosystem services are classified into four categories: Provisioning (food, fiber, fuel, genetic resources, biochemicals and natural medicines, ornamental, and fresh water); Regulating services (air quality, climate regulation, water regulation, erosion regulation, water purification, disease regulation, pest regulation, pollination, natural hazard regulation); Cultural services (cultural diversity, spiritual values, knowledge systems, educational values, inspiration, aesthetic values, social relations, sense of place, cultural heritage values, recreation and ecotourism); and Supporting services which are those needed to maintain other services (soil formation, Photosynthesis, Primary production, Nutrient cycling and water cycling). This so complete list of benefits received from functioning ecosystem reflects how essential it is to pay attention to the factors involved in the health of the environment as a whole made up of abiotic and biotic components assembled in groups of individuals (populations) and groups of species (communities) functioning in a mutually dependent relationships. This further makes evident what we have already stated above, the importance of reaching the pursued sustainable development by adopting a systemic approach when making decisions, either at the professional, personal, or group level.

Sustainability science, in general terms, seeks to understand the fundamental character of interactions between nature and society and encourage those interactions along sustainable trajectories (Kates, 2000 and Kates *et al*., 2001; Anyshchenko, 2019). In that approach, it seems necessary to incorporate sustainability to the scope of biotechnology, an approach that promotes the analysis of the causes and consequences of our projects at different levels going from the phenomenon of globalization of the economy, throughout the multiple instances that involve manipulating the components and functions of ecosystems, and ending in the genetically modified individuals. Kates (2000) also stated the multiple threats and opportunities in the route towards sustainability that are interactive and cumulative, and that sustainability must be founded on the specific regional conditions, which means that we should be considering the ecological and social attributes.

1.4.2 AGRICULTURE, HEALTH, AND NUTRITION: A TRINOMIAL IN CO-EVOLUTION

Biotechnological research has focused on three sub-disciplines: Agriculture, health, and nutrition. Because of that, to achieve the well-being of *Homo sapiens*, consideration must be made of the fact that the present genetic grounding is the result of multiple adaptations originated to successfully process an assortment of foodstuffs that would provide the necessary nutrients to accomplish a resilient health in specific environmental conditions (latitude, longitude, elevation, climate and surrounding biota).

In the case of agriculture and nutrition, the purpose has been to increase large-scale food production and its quality. In the case of health the objective has been to obtain new drugs to treat broadly spread diseases. However, serious debates exist regarding the ecological, social, and economic impacts involved. The United Nations 2005 Millenium Assessment Report warns on page 37 about the global decline of the genetic diversity of crop plants, which could probably lead to the extinction of wild species due to lack of use or their substitution by modern varieties. The loss in wild populations of unique varieties and subspecies would imply an enormous loss of genes (natural capital) that have until now not been studied and that is absent from cultivated plants (Yang *et al*., 2006; Ford-Lloyd *et al*., 2011).

Otherwise, several studies as those of Dewey, 1989; Burlingame, 2000; Johns and Sthapit, 2004, and Johns and Eyzaguirre, 2006, among others, have shown the direct relationship there is between the dietary diversity using local components and human health, as well as the existing link of the latter to the health of the ecosystems. The latter mentioned study points at the relationship of traditional production systems with health and food self-sufficiency, which is called 'quality foodstuffs for human health,' which has also been supported further by epidemiologic studies. The authors of this paper also highlight the existence of a conceptual communication void between professionals of health and environmentalists, which causes discrepancies in the priorities identified by different sectors of society. Furthermore, the same authors recommend paying attention to the underutilized local varieties and species due to their contribution to dietary diversity and health, which they demonstrate with quantitative data (Johns and Eyzaguirre, 2006). Quoting Johns and Sthapit (2004): "Nutrition offers a nexus for the changes in individual behavior and motivation essential for fundamental shifts in production and consumption patterns. Mutual consideration of biocultural diversity and nutrition can guide policy, research, promotion, and applied action in developing countries".

One of the consequences of obtaining improved seeds through biotechnological processes directly acting upon DNA is the dominance and promotion of monoculture. That means that the list of crop species that have provided food to Earth's human population for millennia has not grown, while use has been made only of the opportunities found in the high molecular variability present in some of these crops to generate a large number of varieties. Through this approach, numerous improvements have been achieved in a reduced number of species, between 12 and a maximum of 25 species making up the core of the global

food system. That species core include rice (*Oryza sativa*), wheat (*Triticum* spp.), barley (*Hordeum vulgare*), and oats (*Avena* spp.) in Asia; maize (*Zea mays*), beans (*Phaseolus* spp.), squash (*Cucurbita* spp.), chilli (*Capsicum* spp.), cacao (*Theobroma cacao*), and amaranth (*Amaranthus* spp.) in Mesoamerica; potato (*Solanum tuberosum*) in South America; and sorghum (*Sorghum bicolor*) and sesame (*Sesamum indicum*) in Africa (Toledo-Ocampo, 1998).

In terms of diversity of plant species food producing, the conventional agrodiversity is poor and inequitable, especially if compared with records obtained from a diversity of regions like southeast Mexico. Peniche-Moreno (2010) accounts for the dietary richness found by the Spaniards that arrived at the Yucatan Peninsula and that subsists until the present, including 25 basic species and varieties cultivated in the Milpa system, 42 fruits and vegetables grown in orchards and homegardens, and 24 species gathered from natural forests. The detailed study by Salazar *et al*., (2016) in Xocén, Yucatán, a village with tight cultural cohesion, recorded 74 food dishes with 100% of the main ingredients were produced into the local traditional system called Milpa. Pulido-Salas *et al*. (2017), in the other three Mayan localities engaged in an accelerated process of transculturation, reported that the main purpose of homegardens is having at hand species for daily uses among which food is predominant with 37 species representing 58.3% of the total. This natural and cultural heritage is subject to protection in the germplasm bank of the Yucatán Scientific Research Center (CICY) since the year 2013, holding a collection of plants representing 29 edible native species to the Yucatan Peninsula (Pulido-Salas and Larqué, 2019). This collection is still growing and some of them under study to determine the nutritional content by interinstitutional collaboration (Sánchez-Contreras *et al*., 2019).

Broadening the alternatives for multiple production beyond food plants, up to 811 plant species have been recorded as part of the Milpa and homegarden traditional systems in southeastern Mexico (Mariaca 2012; p. 29), and 757 plant species have been documented in seven states of south and southeast Mexico (Ordóñez-Díaz *et al*., 2018, p. 395). With this heritage, it is undoubtedly plausible to increase the number of species that can be nutritionally and genetically characterized. This heritage also provides an opportunity to, in the first instance, acknowledge their nutritional and cultural value, furthermore, those species contribute today to human health in association to the genetic pool of the local populations. That knowledge will make it possible to apply techniques that can be called as parsimonious biotechnology, that is, the identification and selection of genes that have a correspondence with market demands, without representing the risk of genetic or ecological future erosion.

1.4.3 NATURAL BIODIVERSITY AS THE RAW MATERIAL OF BIOTECHNOLOGY

Biodiversity is the raw material of biotechnology because of which the levels of complexity involved in the term biodiversity must be considered. The first thing is to recognize the substantial heterogeneity of Mexican landscapes that are a product of the environmental diversity produced by physiographic conditions (climate, hydrography,

geology, etc.). In the second place, it must be assumed that dealing with a biological species implies dealing with populations, understood as a collection of individuals capable of genetic recombination, thus maintaining the evolutionary vigor of species through the production of fertile descendants.

Therefore, it is desirable that biotechnologists incorporate in their research protocols to make a diagnosis of their species of interest. The core diagnosis should include their centers of origin, the climatic zones in which they occur, geographic distribution, types of ecosystems in which they live, abundance, degree of domestication, variability, and uses (experience and tradition). That is, to adopt a comprehensive systemic approach, as we suggested above. With that information, biotechnologists may sustain an interdisciplinary and inter-sector dialogue, therefore favoring the certainty of their applications and innovations that could be adopted by societies, even in a transgenerational way. The interdisciplinary research is required to pose systemic solutions, which implies the collaboration of scientists, social actors, and governmental authorities that formulate well-informed regulations.

It is necessary to take into consideration that biodiversity as a raw material is extensive enough to provide new products through sustainable use that would not require biotechnological modifications. For example, the most recent checklist of the number of vascular plant species native to Mexico is above 23, in 297 families. Of these species, 1039 are ferns and lycophytes, 149 are gymnosperms, and 22,146 are angiosperms. In terms of the number of plant species, Mexico occupies the fourth place at a global level, and has the second place for its high number of endemic species (approximately 50%), only under South Africa (Villaseñor, 2016). That means approximately one half of the Mexican flora is endemic to the country's territory.

The potential in the plant biodiversity of Mexico is overwhelmingly patent because of which its use, management, and sustainable strategies for its conservation involve multiple responsibilities loaded of moral, monetary, cultural, and social values. Ulloa *et al*. (2017) published a checklist of species of the vascular flora of the American continent with 124,992 species in 6,228 genera and 355 families. Their compilation efforts were enormous and their limitations were varied, but the worthy purpose of gathering in one publication the lists by country or region is the product of several decades of permanent work.

Equally important is to recognize that, through ethnobiological and ethnoecological approaches, a large amount of information has been gathered having numerous potential applications. This means that a diversity of proven applications can be found in the natural ecological system or in agroecosystems. For example, from a total of 382 plant species in forest management are in Costa Rica, Chazdon and Coe (1999) reported 20 categories of use of 320 of these species in 81 families, representing 70% of the total species. A good example we have with alive local culture as show a study of lumber that show 101 species in the Mayan zone in southeast Mexico, all having a use known by the Mayan culture for several applications (Trabanino and Pulido-Salas, 2017). Ordóñez-Díaz *et al*. (2018) studied species in homegardens with inherited knowledge of the value for their use, reporting that in sev-

en Mexican states in southeast Mexico three zones are noteworthy for the number of plant species with locally known uses: Oaxaca, with up to 757 useful species; the Yucatan Peninsula with 350; and Veracruz with 338 species. This means there are numerous alternatives to explore agro-diversified production and genetic prospection with parsimonious biotechnology methods; that is, of low impact and without threatening the natural harmony of genomes. As an example, we may mention the use of markers to identify with enough precision the molecular combinations in the genomes of wild relatives of known crop plants which have a certain degree of market interest, to afterward achieve improvements in crops of national and international commercial value.

1.4.4 THE POTENTIAL OF BIODIVERSITY AS RAW MATERIAL

The responsible use of biodiversity requires making basic descriptive studies allowing to appropriately value its potential use under sustainable management. The lack of information is evident, and it hampers the inclusion in markets of commodities. Of the approximately 60,000 known species of trees in the world (BGCI), less than 1% have been studied to assess their potential worth and it is estimated that humanity uses a little over 50,000 plant species with medicinal purposes (Golden *et al*., 2012); in fact, 121 globally used drugs are derived from wild plants and 25% of them are derived from rainforest plants.

In the pharmaceutical industry, it is important to consider that, at present, use is made of the combinatorial chemistry and variants of the computational chemistry approaches to identify and design molecules with biological activity (Liu, *et al*., 2017a), which appears to be a purely synthetic pathway to obtain molecules of biological interest. Also relevant is to take into consideration that some estimates suggest there must be about 10^{60} possible combinations of organic compounds in the universe of interest of combinatorial chemistry (Dobson, 2004). To efficiently explore such universe is a monumental challenge, in fact, that is the same universe that the maybe 15 million forms of living beings and their lineages have been exploring throughout their evolution. In the past 20 years, most pharmaceutical and agrochemical corporations in the world have made use of combinatorial chemistry (Liu *et al*., 2017b). To this day, only two chemical compounds are known that have no apparent relationship to molecules in nature and that were registered for their use as drugs (Newman and Cragg, 2016). Until the present, the optimal exploration strategy seems to be the application of combinatorial chemistry to relatively small molecular libraries (of 100 to 3000 compounds) chosen from known structural patterns from natural products. The use of natural products as inspiration for identifying and designing new molecules, continues occupying an essential and active place in the discovery of new drugs or agrochemicals.

Data like this, have led to the conflict remarked by Gómez-Pompa (2004); when attempts are made to protect biodiversity with the expectation of gaining high profit. Because of that, the answer to the loss of biodiversity is not only incorporating it to the markets but, once more, it requires a systemic and multidisciplinary approach with solid information packages to back it up, especially to define criteria of use and clearly defined limits.

After this succinct review of the factors to direct the efforts of biotechnological studies, apparently until today not considered in a comprehensive way, it is highly recommendable to keep in mind that the aspirations of achieving a sustainable development inevitably implies the modification of the predominant model to reduce inequality and to promote a higher level of cooperation and solidarity among people, as stated in the Nagoya Protocol. To accomplish that, it is necessary to work for the common good at the local, regional, national and international levels.

Biodiversity and the conservation of functional ecosystems are the foundation and cornerstone on which to accomplish the longed-for sustainability. Because of that, we must ask ourselves, whether as researchers, project evaluators, or sponsors: How can the new generations of biotechnologists contribute to achieving the indispensable changes needed to arrive at an equilibrated and resilient relationship between healthy ecosystems and human societies? The possible answers to that question reside in the field of the ethics of each involved professional.

1.4.5 BIOTECHNOLOGY AND THE GLOBALIZATION OF BIODIVERSITY

Globalization, understood as the network of events resulting in the transcontinental translocation of animals and plants to cover needs or costumes, must be taken into account as another factor in decision-making, regarding which species to study and which species are susceptible to be incorporated into biotechnological projects leading as alternatives to obtain agricultural products of high local value and potential for commercialization in national and international markets (Brown and Funk, 2008). Globalization exerts an extraordinary pressure upon the direction followed by the use and management of resources in developing countries (Panayotou, 2000), it affects land productivity, and it forces the adaptation to a strong worldwide economic competition by the adoption of adequate resource management programs and innovative proposals (Wolf, 2004). The impact of globalization on the modification of dietary habits occurs in many areas of the world favoring the erosion of identity, also called transculturation. Thus, the role of biotechnology regarding food products is to characterize and potentialize the benefits of products with local gastronomic interest and that are also a part of cultural identity. That role involve participation of large multinational corporations that produce and commercialize food that adequate their products to local culture and demand (Sasson, 2011). For example, there is at least one case of success in Mexico in which there was some advantage of the benefits for health that natural products offer (Ramírez-Sánchez *et al*., 2017). The current biodiversity is the result of the evolutionary process manifested in all the different life forms and scales of organization (Halffter and Ezcurra, 1992). The invaluable genetic asset of biodiversity are the product of natural selection which, at the same time, provides biodiversity with countless dynamic attributes. However, the intentional translocation of species used for food has increased due to trade agreements between countries and the technological advancements in communication means, either favoring crop plants or mobilizing products because of a demand solely created by commercial interests. As stated by Murphy (2002), these exchanges also generate opportunities for large corporations that market new products. Mexico

has received products of interest with clear benefits but the risk of arrival to the country of invasive species has also been favored, in some cases resulting in the extinction of local species because of hybridization or ecological competition (Davis, 2003). This issue has a higher impact, as an example, when observing that Mexico harbors nearly 50% endemic species representing an irreplaceable natural heritage (Rzedowski, 1991). In the context of our analysis, what matters is to emphasize that biodiversity in Mexico includes an inventory of own natural products that, besides being a food resource for its ethnic groups in rural areas, provides sustenance to the local and migratory fauna including endemic species and frequently holding unambiguous plant-animal relationships that have not yet been studied.

To mention just one example, there is the duo formed by quinoa and amaranth, the former arriving from South America promoted by marketing and that, despite its high price, became established among high-income consumer sectors, which contrasts with amaranth surviving as a curiosity of mexican traditional folklore that is marketed regionally at a lower price than its imported cousin. Both quinoa (*Chenopodium* sp.) and amaranth (*Amaranthus* sp.), belong to the family Amaranthaceae, which reflects their genetic kinship and is manifest in their similar nutritional value. Amaranth is an exceptional nutraceutical food containing between 13 and 18% protein, a value above those of maize (9%) and wheat (10%) (Soriano-García *et al.,* 2018).

Modern biotechnologists searching for ecologically-oriented innovations should be working to improve the productivity and quality of local crops and take advantage of the added advantage of these widely distributed plants having regional wild relatives providing an ample genetic diversity (Espitia-Rangel *et al*, 2010). Another potential harm done by the introduction of crops exotic to Mexico –or of transgenic origin– is the still scarce knowledge about the negative effects of these foreign food plants introduced by humans that could be consumed by the local fauna either mistakenly or forced by hunger causing irreversible evolutionary effects (De León, *et al*., 2019). This exemplifies that it is possible to improve the competitivity at a global scale of numerous local crops that have been displaced even before their agronomic or ecological characterization is made and less so explored through biotechnological methods. Many undervalued (or marginalized) crops exist that were displaced by species introduced to the American continent from the Old World (León, 1992) but that are currently susceptible to originating commercial cultivars after parsimonious breeding, that is, breeding that does not threaten the genetic integrity of the species, interspecific equilibrium, or of the health of ecosystems and people.

1.4.6 THE ROLE OF EMPIRICAL KNOWLEDGE AND 'USE VALUE'

As stressed by Gómez-Pompa (2004), indigenous societies are usually not adequately recognized or rewarded either for their knowledge of biological resources, or for having for generations been the guardians and creators of a large part of the biodiversity we enjoy and now want to protect, study, and commercialize. That is how a trend in opinion has arisen that considers the research of biodiversity as a threat and it is in these circumstances that the Nagoya Protocol was formulated. This warns us of

the fact that taken to the extreme, the urge for understanding nature source of wealth and well-being throughout history becomes hampered; it appears as if to avoid injustice it is preferred not to discover transcendental values in biodiversity. However, it is up to new generations of scholars interested in a component of biodiversity as a raw matter to attain a systemic approach when defining their priorities and objectives.

The concept of 'use value' has been redefined for rural Mexico relative to the services of local biodiversity. In that context, use value is that which has the character of being essential to the economy of households despite having exchange value even not commercial (Ramos y Hernández-Xolocotzi, 1985). In general, the valuing of a commodity implies estimating or establishing a cost in return for a reward, while the value is a measurement that reflects the worth of something at the same time assigning it an exchange value (Barbier *et al*., 1997).

Use value generates local knowledge and this is inherited through oral tradition. As an example, in the case of traditional medicine in Yucatan , Mexico, some associations and local government efforts have been taking place to rescue the knowledge and medicinal plants associated, however looks like it is not enough (Hirose, 2018). More coordinated actions are necessary for registration, given that with that knowledge, to an extent probed through hundreds of experiences using those natural resources, modern science and technology could save time to find new cures for emerging diseases. However, safe ways to deal with the cultural rights transactions involved is yet to be device when moving into a market society.

In that way, once more seen through the process of globalization, the question arises of *how inheritable the gene pack in the families is today?*, which comprises the genes of what is grown and eaten that have conferred the needed adaptation for a locality having its own climate and within a determined combination of food resources.

Among other important issues, biodiversity faces the loss of knowledge about it because of the lack of observance of the use values and their disuse (e.g, production of oxygen and carbon capture), or because of lack of a clear recognition of the benefits obtained from their use and transformation. In a country with abundant resources, these issues are exacerbated, because it has been clearly demonstrated that the value given to them is inversely proportional to their abundance (Dasgupta, 2000). We can then say that when minimizing the presence, or the benefits of a locally abundant species, added to a comparison with the benefits obtained from an introduced species –adopted because of a diversity of causes including marketing strategies, we could be endangering our species stock. In that way, we have also impacted on the intrafamilial heritability of the knowledge derived from the use value of local resources. The precarious valuing favors destructive actions that have stolen space for the local biodiversity due to land use changes (Chapin *et al*., 2000), for example, as can be inferred, with the purpose of establishing extensive monoculture.

The current scenario of increased loss and depletion of biological systems, ecosystems, and their diversity has as one of their causes the impulse given by human soci-

eties to strategies that follow the dominant economic models, leading to turn complex ecosystems into simple productive systems that threaten the stability of life's biophysical processes (Núñez *et al*, 2003).

As Sarukhán (1985) mentioned, ethnobotanical research is a task that must cover all the range of knowledge about plant species from the ethnological inquiry to genetics and the horticultural evaluation of biological materials, including research on the physiology, ecology, cytogenetics, etc. The author thus proposes to recreate and intensify the millennial-long process of evolution under domestication by combining the traditional knowledge of the ethnic groups with all which modern occidental science has to offer.

In such a case, the biotechnological products would not have to become invasive species that replace ecological niches or large areas in naturally resilient ecosystems, nor must they become distractors or intruders into the originary cultures from each region. Instead, they could be complementary to local traditions, satisfying the punctual needs of short, medium, and long term well-being. Biotechnologists would need to start with a series of interviews using sociological and agroecological methodologies with the purpose of designing research projects, therefore achieving their acceptance by social groups. In that way, the originary users and even developers of the resources would have the opportunity to contribute the systemic approach that characterizes ethnic groups, their empirical uses, domestication expectations, and their expression of their concerns about relevant local issues.

1.4.7 THE ADDED VALUE

The relationship of human beings with biodiversity has triggered multiple and highly complex processes of generation of monetary wealth, especially through the appropriation of species variants that, through domestication processes, have turned through millennials into sustenance, health, clothing, and shelter for people both in rural as in urban environments. Less evident is the fact that many other benefits received by humanity from nature have their origin in functional ecosystems. In an attempt to create awareness about these and other benefits, the concept of 'ecosystem services' were developed. With that concept, it is attempted to recognize the varied forms by which ecosystems make human life possible end enjoyable. The most evident of these benefits being the maintenance of equilibrium in the gases which makes air to be breathable, remove toxic components in the water, maintain pollinators of the plants domesticated, or moderate the velocity and force of water running down into basins.

1.5 AN INNOVATIVE BIOTECHNOLOGY-BASED ON MULTIDISCIPLINE

Biotechnology is and endeavor with the purpose of solving complex scientific and social problems and defined as an activity sustained on frontier knowledge generated from a variety of disciplines as molecular biology, biochemical engineering, microbiology, genomics, and immunology, among others. It is an approach that allows an integrated study and manipulation of biological systems including microbes, plants, and animals (Bolívar-Zapata, 2017).

The above-mentioned disciplines comprehend essential multidisciplinary associations. However, these associations are not enough to cover the requirements for multidisciplinarity demanded by a sustainable management of the living organisms which are the object and raw matter of biotechnological studies. Such is the case of the relationships of biotechnology with ecology, ethnobiology, or agroecology, and least so for covering the nutriology, sociology, and psychology of the individuals to be impacted by the results of research.

An example of the need for an integrated vision is given by plant breeding as a sub-discipline of biotechnology, which opens a discussion from the scientific and socio-economic approach of the way in which the involved factors interact. Part of that discussion has to do with how to respond to the need of researchers involved in crossbreeding to clarify what an essential resource is. The argument also extends to the policy for the regulation, distribution, and financing, as well as the debates about agriculture in general and its future in Mexico. However, before the national application of any biotechnological innovation, it will be necessary to observe the recommendations that are strongly supported about adequating the specific public programs and policies for the particular conditions of regions (Villa-Issa, 2008; Anyshchenko, 2019).

The question is how to equilibrate the presence of both perspectives: traditional domestication and biotechnology and at the same time deliver economic benefits to farmers in Latin America? The smallholders are those who will use or not the improved varieties that they initially contributed with but, eventually, their experiences in use, the particularities of their land (climate, soil, microsites, etc.) what will make a crop, a species, a variety, or a cultivar to have a successful adaptation. The International Maize and Wheat Improvement Center (CIMMYT), which has for decades worked in seed improvement, have evolved by integrating the in-situ experience of farmers to implement innovative projects adopting conservation agriculture (Hellin, *et al*., 2019). In that way, CIMMYT has achieved a significant advancement adding both the experience of users (Camacho, *et al*., 2016) and inclusive the creativity of local farmers, the latter of which also plays an essential role to accomplish sustainable management (Pulido-Salas *et al*., 2017).

But how to give equal value to expert farmers and biotechnology experts? How to pay them a similar or equivalent salary? How to make the recognition of farmers not to be sporadic but incorporated into a permanent program, in particular when knowledge is transmitted through kinship and in which is involved a vocation for primary production? The answers to these questions do not de-

pend on the daily work of a biotechnologist but the latter must choose which species (native or endemic) and in which socioeconomic regions to have impact without eroding the local knowledge and traditions which give support to this valuable knowledge.

1.5.1 BIOTECHNOLOGY AS ONE OF MANY ALTERNATIVES

Once the effort is made to preserve the genetic diversity at the species level giving priority to the native species of each particular ecosystem, we may in the future preserve vegetation patches that provide food, refuge, and shelter to the local resident or migratory fauna. In this, natural protected areas play an essential role, but the future ecological balance will depend on the management decisions of microscopic and macroscopic, natural and urban ecosystem that are defined in the present. Thus, the reasons to look for several productive alternatives are the real threat involved in biotechnological proposals implying the release of genetically modified organisms in ecosystems, be these small or large, complex or simple. Each modified genome or organism must be conceived as part of a micro-ecosystem network with possible macro-impacts, and vice versa. If that is not done, maintaining or recovering the equilibrium of something becoming more and more unbalanced would have a very high cost. To plan the future of biodiversity keeping time in mind, requires to make an evaluation of how many elements we add towards disequilibrium and how many to maintain or recover the balance.

Another key factor for equilibrium is to consider that species checklists need to be bivalent, that is, they should include a list of species which span to those species it needs to be fed and reproduce. In the case of plant checklists of uncultivated land, they record the native species that, by definition, are well adapted to each ecosystem (climate, soil, fauna) and that are the base of food chains, in turn, adapted to prevailing environmental factors, but it is also necessary to know the associated species that may facilitate its development. An example is the work by Cancino-Oviedo *et al*., (2020) which include 171 plant species whose propagules are consumed by vertebrates in tropical forests of the Yucatan Peninsula. This author crossed information found for 171 plant species and 44 mammals and bird species some of them endangered. This example demostrate the need of having mixed listings of both flora and fauna, to clarify food chains. This is a vital fact for terrestrial and also marine environments, or intermediate environments, especially when are in contact with human activities. This type of study would need to be replicated to better understand the interdependence between groups of species that drive processes that are essential for the ecological balance.

We still are currently in the stage of proposing a ways to efficiently implement an integrated approach for higher efficiency in resource management that produce commodities for human life. The solutions will demand the harmonization of very diverse methods that, in parallel, also contribute to maintain or recover the environmental resilience and restore biodiversity balance. Also agro-ecology principles and biotechnology will need to be incorporated (Still, 2019).

It is important to acknowledge the concept of “One Health” that was introduced at the

outset of the XXI century. It is relevant because of its emphasis on an idea that has been known for more than a century: human health and animal health are interdependent and bound to the health of the ecosystems (OIE, 2020; WHO, 2017). The concept is now being promoted by the World Organization for Animal Health (OIE) and the World Health Organization (WHO) as well as many other organizations, rendering a framework for a global collaborative strategy to understand risks to human health, connected to both domestic animals and wildlife, framed on a context of ecosystem health, in a whole system approach.

1.5.2 BIOTECHNOLOGICAL ADVANCEMENTS FOR A RESILIENT DEVELOPMENT

In the face of intense technical and economic competition, the new biotechnologists must make a stop and put themselves in the situation of Mexican farmers that for decades have been subject to the offers of governments without achieving, as in prehispanic times, a diversified, multispecific, productive, and resilient agro-ecosystem. This is because it has not been a priority to contemplate the ample diversity of local conditions that make up a reality still present in some tracts of Mexico. These conditions attest the possibilities of a diversified, healthy, and sustainable production, for example, of food. To maintain resilient production systems, as has been sufficiently documented through decades of studies of several ethnic groups in Mexico (Toledo-Manzur *et al*., 2013), must be an important part of innovative studies because of the huge cultural heritage it involves and the agricultural expertise of small farmers settled throughout the country. This heritage of knowledge is defined as an indigenous multiple-use strategy (MUS) that represents a dynamic system of adaptive management (Toledo-Manzur *et al*., 2003).

Another important aspect is recover functionality and natural processes in ecosystem, especially after long disturbing (by extensive agriculture, forestry, hydrologic disruptions or oil spills). Restoration should be designed as a part of innovative projects standing the knowledge about ecological interactions as well as specific restoration tools in order to design strategies with appropiate tailored methods considering local ecological and socioeconomic conditions with the participation of local government, industries, scientists and communities (Jones *et al*., 2018).

Then, at this point, looking towards the future, some questions are pertinent that a modern biotechnologist must ask.

- What are the characteristics of the ecosystem naturally inhabited by the species wanted to be studied?
- How are the societal linkages likely to be affected by the deployment of the specific biotechnology?
- What observation and monitoring methods must be implemented for negative effects on ecosystems, of both microorganisms and macroorganisms as well as people?
- What are the properties and functions naturally performed in the ecosystem by the object of study?
- What species must have priority to design new studies having positive ecological and economic impacts?
- How many individuals must be studied to know a generalization of results

can be made at the end of the study to characterize and know the behavior of a species?
- What scientific disciplines will be impacted by the results of the study?
- What biological groups (from microorganisms to flower plants and mammals) will be impacted by the results of the study?
- Which variables must be considered to complete a multi-temporal index of environmental impact from the insertion of GMOs?

As stated by Hess (1987), the reactions of the public show a lack of trust of biotechnological innovations being innocuous and that these are provided without answers or sufficient safety levels. Unintended complications are suspected due to not enough regulation guidelines within and between involved agencies. Thus, well-structured programs of science popularization through mass information media are needed, which should include legal aspects in a well informed and coordinated way. As Bhatia (2018) suggest, Biotechnology can be broadly defined as "the engineering of organisms for the purpose of human usage", which is a wide scope for the discipline, however do not consider the impacts on biodiversity by and large, meaning the living matrix that contain all other forms of life as the source of the raw materials for biotechnology research in long term.

1.6 CONCLUSION AND REMARKS

The innovative biotechnologists having a vision of ecosystem integrity must keep in mind a concept of species that implies a minimum number of individuals representative of the total genome of a group made up of the sum of localities in which it is naturally distributed and characterized by the possibility of reproduction, and resulting in a fertile offspring conducive to self-regulated and resilient populations. The new projects must promote suitable actions to promote both the conservation of biodiversity and the physical, social, and economic spaces required by the expert producers that generate knowledge with their own hands through the accumulation of everyday experimentation.

It must be kept in mind that the application of biotechnological innovations implies the human appropriation of biodiversity, which inevitably turns biotechnology into a discipline with biocultural repercussions. That is, any biotechnology gives impulse to a specific cultural model with consequences on the surrounding biological lineages. Therefore becoming essential that the projects and proposals involving biotechnological innovations reside within the equations of analysis of human influence. Particularly, regarding the condition imposed on ecosystems when using them, therefore assuming the responsibility of the management of their degree of integrity.

1. Observing integrating concepts and developing an integrated vision must be an essential part of the formation of new generations of 21st-century biotechnologists and beyond, with the purpose of focusing their creativity towards the visualization of innovative proposals that include scholar knowledge in phase with knowledge gained

by experience of use in extensive rural Mexico. The main purpose will continue to be to contribute to improving the quality of life of human beings, which in turn requires including within that wellbeing other forms of life in all ecosystems and as many life forms that inhabit them as possible.

2. Regarding biodiversity, adopting a systemic approach to sustainability, and based on the concepts analyzed in this chapter, we recommend the following principles:
3. Sustainability is a continual process.
4. The physical processes that govern the dynamics of nature are inviolable.
5. Altering an ecosystem increases its fragility.
6. The vision of the world must be systemic and results of research must come from multidiscipline to be sustainable.
7. The living systems require monetary reinvestment for conservation and restoration of health ecosystems around us.
8. There are ecological limits to land use and property rights.
9. Intergenerational equity must be taken into account to maintain the availability of natural resources for humanity.
10. Human health is linked to animal health and ecosystem functionality, as the One health approach suggest by highlighting the human-animal-ecosystem interface.

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CHAPTER

TWO

Genetic improvement of crops of commercial importance and their transcendental impact on man's quality of life

CHAPTER 2

GENETIC IMPROVEMENT OF CROPS OF COMMERCIAL IMPORTANCE AND THEIR TRANSCENDENTAL IMPACT ON MAN'S QUALITY OF LIFE

Tamayo-Ordoñez F.A.[1], Tamayo-Ordoñez Y.J.[2], Rodriguez-Zapata L.C.[3], Cázares-Sánchez E.[4], Lopez-Lopez A.[4], García-Castillo M.J.[3], Fuentes-Siller E.J.[5] and Tamayo-Ordoñez M.C.[5]*

[1]Facultad de Química, Universidad Autónoma del Carmen, Calle 56 No. 4 por Av. Concordia, Campus Principal, 24180 Ciudad del Carmen, Campeche.

[2]Facultad de Ciencias Químicas. Universidad Autónoma de Coahuila. Ing J. Cardenas Valdez S/N, República, 25280 Saltillo, Coah.

[3]Centro de investigación Científica de Yucatán A.C. Unidad de Biotecnología. Mérida Yucatán México.

[4]Tecnológico Nacional de México, campus Instituto Tecnológico de la Zona Maya, carretera Chetumal Escárcega Km. 21.5. C.P. 77960. Ejido Juan Sarabia, Quintana Roo, México.

[5]Laboratorio de Ingeniería Genética. Departamento de Biotecnología. [2]Facultad de Ciencias Químicas. Universidad Autónoma de Coahuila. Ing J. Cardenas Valdez S/N, República, 25280 Saltillo, Coah.

*Corresponding author: **mtamayo@uadec.edu.mx**

ABSTRACT

Because of the extreme conditions derived from climate change, it is possible that future generations may compete for land for planting food crops, water and energy, impacting future food security. Although the genetic improvement of plants has always caused controversy of opinions, it has been documented how genetic engineering has allowed the improvement of plants of economic importance that are currently used in the food area, alternative energy generation and health, among other applications.

In this book chapter, the advantages and the development of molecular tools that have improved plant transformation processes are discussed, minimizing the negative effects of horizontal or vertical gene transfer in other crops, suggesting the viability of using plants. genetically modified, in different fields of research.

2.1 INTRODUCCION

In 2007, it was estimated that at least 100 million people live in absolute poverty and could not access adequate food for a balanced diet (Flood, 2010). This poverty landscape increased with the losses in productivity of economically important cultivars, because at consequences of climate change and exposure to different biotic and abiotic factors (Aslam, 2019; Hansen et al., 2019).

Although biotechnology has made efforts to increase the productivity of agricultural crops and by-products, targeted efforts are still needed to improve yields on food crops and their by-products.

The term genetically modified organisms (GMOs) was defined by the World Health Organization (WHO) as "organisms (i.e. plants, animals or microorganisms), in which genetic material (DNA) has been altered in a way that does not occur naturally through natural mating and/or recombination". Similarly, the United Nations Food and Alignment Organization (FAO) defined GMOs as products that "do not occur naturally through mating and/or natural recombination. The first GM crop was approved in 1994 and since then the number of approved GM crops has increased and been relatively constant over the past two decades and more than 357 genetically modified traits have been approved in various crops, and the most common have been identified in canola, maize, cotton, soybeans and potatoes (Grigore, 2018). GMO crops are present in the human food chain, either directly as food or indirectly as animal feed. Genetically modified cultures have demonstrated resistance to different biotic factors (pathogen resistance, insect resistance and resistance to pathogenic viruses) and abiotic (high temperatures, salinity and drought) (Table 2.1). In addition, the use of plants as a bioreactor for the production of different compounds such as biodegradable plastics, recombinant proteins and drugs, among other molecules of interest, is already a reality (Schillberg *et al.*, 2019; Tripathi and Shrivastava, 2019).

In this chapter we describe what has been the advancement in the genetic engineering of plants that have been used to minimize the negative effects of plant use genetically in different areas of biotechnology.

Table 2.1. *Genetically modified crops of economic importance*

Genetically modified crops	Genetically modified Gene or Enzyme	Biotechnology application	References
Genetic modifications that result in resistance to abiotic and/or biotic stress			
Potato	Oxalate oxidase	Tolerance to salinity stress	Turkish (2015)
Alfalfa	MsRCI2A gene coding of a membrane protein	Tolerance to salinity stress	Long *et al.* (2019)
Potato	Lyzosyme	Use for the control of bacterial pathogens that cause serious diseases in potato.	Rasche (2006)
Potato	Aspartic protease	Resistance to *Botrytis cinerea* in *Arabidopsis thaliana*	Frey (2018)
Tobacco plant	Endo-A-mananase de *Bacillus* sp.	Resistance against fungal pathogens *(Fusarium oxysporum)*	Hoshikawa (2012)
Eggplant	Glucanase	Resistance to fungi in eggplant	Singh (2014)
Te Plant	Endo-1,3-β-D-glucanase	Resistance to blister blight disease	Singh (2018)
Tomato	Co-receptor JAZ2 of coronatine (COR)	Resistance to the causal agent of tomato bacterial speck disease, produces coronatine (COR)	Ortigosa *et al.* (2019)
Strawberry	Mitogen-activated protein kinase gene FaMAPK19	Resistance to *Botrytis cinerea*	Zhang *et al.* (2020)
Genetic modifications that improve the biosynthesis of a product of commercial interest			
Rice	N-acetyltransferase	Biosynthesis of melatonin, which catalyzes the conversion of Serotonin into N-acetylserotonine	Byeon *et al.* (2015)
Flowering tobacco	Melatonin synthese	Melatonin synthesis	Zhang *et al.* (2012)
Soja	O-acetyl serine sulfhydrylase	Accumulation of improved levels of cysteine and Bowman-Birk protease inhibitor in seeds	Kim *et al.* (2012)
Sugar Cane	phosphomanose isomerase	Increased hexokinase activity	Zhang *et al.* (2015)
Goji Berry	Carotenoid isomerase	Conversion of cis-lycopene into lycopene and increment in carotenoid production	Li *et al.* (2015)
Potato	Triosfosfato isomerasa	Triosphosphate isomerase catalyzes the conversion of dihydroxyketone-P and glyceraldehyde 3-P into the glycolytic pathway.	Dorion *et al.* (2012)
Unshu mikan	Isomerase	It catalyzes the isomerization of pro lycopene to lycopene in the carotenoid biosynthetic pathway	Eun *et al.* (2015)
Potato	Triosa-phosphate	Starch biosynthesis	Thorbjørnsen *et al.* (2002)
Corn	Phytase	Improving phosphorus digestion in livestock and reducing the cost of forage production	Xu *et al.* (2018)
Sugar cane	Xylase	Release of pentose and glucose, and its use in the generation of ethanol production	Kim *et al.* (2017)

2.2 THE GENETIC TRANSFORMATION OF PLANTS AS A TOOL FOR THE IMPROVEMENT OF CROPS OF COMMERCIAL IMPORTANCE

FAO reported that global demand for food would increase by 40% by 2030; therefore, the need for GMO crops will also increase, since the advantages offered by the products to the consumer (i.e. the quality of the improved product) and also to the producer (i.e. the lower production costs), are improved compared to non-GM plants (Zilberman *et al*., 2018).

There are currently three generations of genetically modified plants. The first generation refers to those crops that tolerate environmental stresses and increase resistance to diseases caused by pathogens (Long *et al*., 2019; Ortigosa *et al*., 2019); the second generation refers to those crops with improved nutritional content (Moghissi *et al*., 2016; Snell *et al*., 2012) and the third generation refers to those crops with non-food applications, for example, the improved production of pharmaceutically active molecules in plants (Horn *et al*., 2004; Laere *et al*., 2016).

Today, while plant systems are now gaining widespread acceptance as a platform for large-scale production of recombinant proteins, there are still bottlenecks that need to be overcome.

Low commercial uptake. The regulation of the different metabolic processes that are carried out in plants is interconnected, not allowing many times to obtain good yields of specific products, also the quality of the products is affected, not making the large-scale production of the different products feasible (Schillberg *et al*., 2009; Tripathi and Shrivastava, 2019).

Presence of antibiotics as selection markers of transgenic plants. The presence of antibiotic cassettes in transgenic plants has caused controversy due to the impact that the consumption of products with antibiotics can increased tolerance, susceptibility and / or resistance of microorganisms managing to affect human health (Stockwell and Duffy, 2012; Zhu and Wu, 2008).

Horizontal and vertical transfer of genes. The horizontal and vertical transfer of genes, caused by the invasion of genetically modified plants into natural ecosystems, has always been a debate and therefore the transformation via chloroplastic plants has been better accepted compared to nuclear transformation (Day and Goldschmidts-Clermont, 2011; Adem *et al*., 2017).

Since the 1980s, where the first reports of transgenic crops were reported (Bevan, 1984), the introduction of genetically modified plants into natural ecosystems has always been discussed, addressing issues such as the political, ecological and economic consequences of the use of genetically modified plants. Despite this, the genetic modification for tree breeding, has shown advantages compared to conventional: a) genes can be inserted from any organism to another, facilitating the acquisition of a specific phenotype (Sticklen, 2008; Leister, 2019), b) Genetic engineering could allow accelerated reproduction of species that have life cycle is long and takes a long time to enter the reproductive phase (juvenility). Many of the commercially important tree species do not bloom until they are at least 15-20 years old (Hackett, 1985; Hoenicka and Fladung, 2006).

Although there is still much work to be done for every day outweigh the advantages compared to the disadvantages of the use of transgenic plants, progress in the development and implementation of new strategies of genetic engineering has managed to obtain case studies with success that have allowed solving different problems present in the environmental, food and health areas and contribution to the generation of energy sources. Thus, the manipulation of plants has improved the quality of life of man.

2.3 FIRST GENERATION GENETICALLY MODIFIED PLANTS

The first generation of genetically modified plants involves those crops that tolerate being exposed to different biotic and abiotic factors. Stress can be considered as a situation that can disrupt growth and cause a substantial loss in performance.

The responses of plants to these stresses can be transitory to provide plants with the necessary tools to acclimatize and survive, however, they are exposed to multiple stimulations with the possibility of stress, but capable of adapting to their environmental and physiological development. Some environmental stresses can occur during the life of the plant (seasonal changes of the year), but others caused by global climate change can be transient. There are different genetic modifications such as epigenetics (such as DNA methylation) and chromatin remodeling, which have been associated with both biotic and abiotic stresses, including the climate change that they suffer during their lives (Kumar, 2018; Begcy and Dresselhaus, 2018; Ueda and Seki, 2020).

Plants can respond effectively to biotic and abiotic stress environmental conditions and modify their development and physiological conditioning.

2.3.1 ADVANCES IN THE GENERATION OF GENETICALLY MODIFIED PLANTS RESISTANT TO BIOTIC AND ABIOTIC FACTORS

Abiotic stress is the main cause of food shortages that are responsible for an estimated 50% loss in staple crops such as corn, rice, beans, and potatoes (Mishra *et al.*, 2015; Mammadov *et al.*, 2018). One of the main abiotic stress's plants face is extreme temperatures, both high and low. The heat is likely to increase in the future and will affect many countries, including developing countries where hunger is already a problem. Increasing temperature has a significant impact on performance. As part of global warming, heat stress is generally combined with water scarcity, which predictions will become more acute and with more CO_2 and UV radiation, on the other hand, floods, which will be more recurrent in other regions. of the world, also present an important factor that could harm agronomy (Nikolaos Skliris, 2016).

2.3.1.1 Consequences of high temperatures and drought on plants

Drought is one of the factors limiting agricultural production that will become increas-

ingly important due to the expected climate change in the coming decades (Stocker, 2013; Hu *et al*., 2019).

In crops, drought causes reduced germination, negative effects on plant height and obtaining less plant biomass, affecting water content, changes in photosynthetic activity, pigment content and membrane integrity, accumulation of osmoprotectors such as proline, sugars and antioxidants altered expression of genes related to stress (Farooq, 2012; Iqbal *et al*., 2020). In rice, one of the most drought-sensitive species, in which drought-induced yield losses can be as high as 92%. Intermediate drought stress applied to rice seedlings causes symptoms of dehydration-induced oxidative cell damage (Li, 2011). In corn, a study evaluated the response of this crop to repetitive dehydration/rehydration cycles in seedlings. Plants exposed to multiple stress cycles exhibited variable leaf water content compared to simple stress controls. By comparing the transcriptomic responses of corn and *Arabidopsis*, the authors identified not only conserved acclimatization characteristics, but also species-specific patterns of genetic regulation, indicating not only evolutionary conservation but also a divergence in response to drought stress (Ding *et al*., 2014). At the genetic level, in the field of biotechnology, there has already been an advance in the manipulation of genes or enzymes that have had an impact in increasing drought tolerance in agronomically important crops (Table 2.2).

Table 2.2. *Crops of plants GMO of first generation with tolerance of stress abiotic*

Crop (specie)	Gene or enzyme studied	Genetic modification	Observations	References
Drought				
Potato	Trehalose-6-phosphate synthase	The TPS1 gene of Saccharomyces cerevisiae was overexpressed in transgenic potato plants.	The transgenics plants showed retarded growth and aberrant root development and showed significantly increased drought resistance.	Yeo *et al*. (2000)
Tomato	AT12 (encodes a C_2H_2 zinc finger protein)	Integration of ZAT12 gene into nuclear genome	Parameters of relative water content, electrolyte leakage, chlorophyll colour index, H2O2 level and catalase activity suggested that tomato transgenic lines have significantly increased levels of drought tolerance.	Mishra *et al*. (2012)
Wheat	Inositol polyphosphate kinase	Heterologous expression of *ThIPK2* gene of *Thellungiella halophila* into *Triticum aestivum* L.	Transgenic plants showed higher seed germination rates, better developed root systems, a higher relative water content (RWC) and total soluble sugar content, and less cell membrane damage under drought stress conditions.	Zhang *et al*. (2020)

Crop (specie)	Gene or enzyme studied	Genetic modification	Observations	References
Apple	MdERF38 an ERF transcription factor	Expression of MdERF38 in transgenic lines of apple	It was show that MdERF38 interacted with MdMYB1, a positive modulator of anthocyanin biosynthesis, and facilitated the binding of MdMYB1 to its target genes. Therefore, MdERF38 promoted anthocyanin biosynthesis in response to drought stress.	An *et al.* (2020)
Banana	aquaporin gene MaPIP2-7	Overexpression of MaPIP2-7 in banana	Overexpression of MaPIP2-7 confers tolerance to drought, cold, and salt stresses by maintaining osmotic balance, reducing membrane injury, and improving ABA levels.	Xu *et al.* (2020)
Salinity				
Potato	Myo-inositol-1-phosphate synthase (MIPS)	MIPS overexpression in sweet potato under field conditions.	Inositol,phosphatidic acid (PA), Ca2+, BA, K+, proline and trehalose content was increased, in the transgenic plants under salt and drought stresses.	Zhai *et al.* (2016)
Tomato	codA gene encoding choline oxidase	Expression heterologous of coda gene of *Arthrobacter globiformis* into *Lycopersicon* esculentum	The codA-transgenic plants showed higher tolerance to salt stress during seed germination and subsequent growth of young seedlings than wild-type plants.	Goel *et al.* (2011)
Wheat	Fructokinase-like protein2 (FLN2)	Stress tolerance against salinity was evaluated in the knockout line of FLN2 by CRISPR / Cas9 method.	The mutant line presented an inadequate supply of the assimilate required to support growth, not allowing its adaptation against salinity compared to the wild strain.	Chen *et al.* (2020)
Alfalfa	Salt tolerance gene rstB	rstB gene was introduced into alfalfa genome by Agrobacterium-mediated transformation.	*rstB* transgene enhanced calcium accumulation, which could serve as a mechanism of the improved salt tolerance.	Zhang and Wang (2015)
Corn	DREB1A/CBF3 Gene	Transformation of maize (*Zea mays* L.) with DREB1A/CBF3 Gene *Arabidopsis*.	Transgenic plants showed tolerance to cold, drought, and salinity compared with wild-type plants.	Al-Abed et al. (2007)
Cold				
Grapew-ine	calcium-dependent protein kinase (VaCPK20)	Overexpressing of VaCPK20 gene in callus cell lines of Vitis amurensis.	Transgenic grape cell cultures overexpressing the VaCPK20 gene showed higher resistance to cold stress.	Dubrovina *et al.* (2015)

Crop (specie)	Gene or enzyme studied	Genetic modification	Observations	References
Rice	Sucrose:sucrose 1-fructosyltransferase (wft2) and Sucrose: fructan 6-fructosyltransferase (wft1)	wft2 and wft1, were introduced into rice plants.	Transgenic rice seedlings with wft2 accumulated significantly higher concentrations of oligo- and polysaccharidesthan non-transgenic rice seedlings, and exhibited enhanced chilling tolerance.	Kawakami *et al.* (2008)
Maize	Choline dehydrogenase (*bet*A)	betA gene from Escherichia coli was transferred into elite maize inbred DH4866 via Agrobacterium-mediated transformation.	The chilling tolerance, expressed in cell membrane damage, degree of chilling injury, survival rate, and photosynthesis was enhanced in plants transgenic.	Quan *et al.* (2004)
Potato	Choline oxidase (CodA)	Transformation of tomato (cv. Moneymaker) with a chloroplast-targeted codA gene of Arthrobacter globiformis.	GB-accumulating plants are more tolerant of chilling stress than their wild-type counterparts.	Park *et al.* (2004)
Tobacco	Antifreeze proteins (AFPs)	Transformation of tobacco (Nicotiana tabacum) with carrot (Daucus carota) AFP gene.	That the expression of *DcAFP* driven by *Prd29A* promoter could significantly enhance the cold tolerance of transgenic tobacco and had fewer effects on plant growth.	Wang *et al.* (2017)

For its part, the increase in temperatures is one of the main predictions of climate change models that are likely to have a profound impact on food security, since it damages plant growth, affects plant reproduction and, therefore, the yield of crops (Schauberger *et al.*, 2017). Changes in temperature have always been in conjunction with the current global drought.

Plant responses to heat vary with the degree and duration of stress and the type of plant. Heat is now a major concern for crop production and approaches to maintaining high yields of crop plants under stress from high temperatures. Plants have a series of adaptations or acclimatization mechanisms to cope with high temperature situations. They have been described as employing ion transporters, proteins, osmoprotectors, antioxidants, and other factors involved in signaling cascades that are activated to compensate for the biochemical and physiological disturbances induced by high-temperature stress (Hasanuzzaman *et al.*, 2013). In wheat it was demonstrated that the thermal shock mediating by high temperatures after the subsequent injection of iron and cadmium salts in the leaf segments, helped to tolerate the stress by temperature (Zandalinas *et al.*, 2018). In rice, short-term heat pretreatment was shown to reduce cadmium-induced chlorosis in seedlings. These studies suggest that heat shock-induced accumulation of antioxidant compounds plays a prominent role in protecting against subsequent cadmium exposure (Chou *et al.*, 2012). Currently, by manipulating genes or

enzymes related to ion transporters, osmoprotectors, and antioxidants and other factors involved in signaling cascades, progress has been made in the development of crops of economic importance that tolerate high temperatures (Table 2.2).

2.3.1.2 Tolerance to osmotic stress by salinity in crops

High salinity is one of the most damaging factors for agricultural production, both in natural saline soils and in irrigated land with a high level of evaporation or insufficient water management. Abiotic stress induced by salt alters plant growth by reducing water absorption, stoma closure, and decreased photosynthetic activity (Mane *et al*., 2011; El-Moneim *et al*., 2020). In turn, ionic stress caused by specific salts absorbed at higher than optimal concentrations influence the homeostasis of essential ions, metabolic activity and the integrity of plasma membranes. The study of plants grown at low concentrations of salt salinity can increase the tolerance of model plants and different crop species to saline stress, improving the physiological and growth parameters related to plant vigor and aptitude (Rasool, 2012). For example, the response of *Arabidopsis* against osmotic stress and salinity, showed that plants grown in low concentration of NaCl accumulate less sodium in their shoots, have a higher biomass and better survival compared to control plants. In addition, the plants that showed greater tolerance to salinity also acquired tolerance to drought. It is important to mention that for biotechnology applications, plants did not exhibit effects on growth, which suggests that the memory of plants against salinity stress does not have a negative impact on their growth (Sani *et al*., 2013).

2.3.1.3 Stress from cold

Low temperature is also one of the main factors that influences the geographical location where plants can grow. This abiotic factor has been responsible for losses in crop yield. Exposure to low temperatures causes various phenotypic symptoms, such as poor germination rate, reduced organ expansion, wilting, and inhibition of reproductive development (Van Buer, 2016). For example, barley plants subjected to high salt stress show impaired growth, measured by root lengthening. However, this response can be prevented by acute heat shock priming. The beneficial effect of heat shock priming was observed in the protection against damage mediated by cold stress in tomato (*Lycopersicon esculentum*). Harvested tomato fruits exposed to non-freezing cold conditions show signs of cold damage, that is, loss of aroma, leakage of electrolytes, lack of ripening and oxidative stress (Biswas, 2016).

The memory of plants in response to cold stress has been studied in several cold-sensitive agronomic species. Exposure to moderate temperatures before cold alleviates the negative effects induced by cold on plant growth and development. For example, in rice, a cold pretreatment prevents the absorption of cold water at the roots, the wilting of the leaves and the whitening of the color (Alicja Sobkowiak, 2016).

2.3.1.4 Ultraviolet radiation (UV-B) and exposure to chemical agents

UV-B is one of the types of ultraviolet light and a natural component of solar radiation. Increased UV-B intensities is harmful to plants due to their sessile lifestyle and mandatory requirements sunlight are needed to

be carried out processes such as photosynthesis. UV-B stress can be divided into low and high doses, and short-term (acute, seconds to hours) or long-term (chronic, hours to days) exposure. High dose UV-B radiation from plants causes serious detrimental effects and even leads to programmed cell death. For its part, exposing plants to low doses of UV-B causes effective activation of defense mechanisms and acclimatization to UV stress (Hideg *et al*., 2013).

Some plant species that have shown a beneficial effect after long-term UV-B exposure include crops such as wheat, corn, cabbage, rape, beans, and soybeans (TT and Puthur, 2017). The seeds of these crops irradiated with UV-B have been shown to have higher germination, higher growth rate, higher pigment content and greater tolerance to other stresses, such as tolerance to salinity and pathogens. For example, a higher germination rate was observed as a result of UV-B treatment for corn; higher pigment content for cabbage, beets (Beta vulgaris), beans, soybeans, and rice; and increase in the biomass of buckwheat. In addition, an increase in the chlorophyll or carotenoid content was reported for UV-treated rice seedlings and pumpkin (*Momordica charantia*) (Tsunekawa, 2010).

Furthermore, instead of applying a moderate abiotic stress pretreatment, memory signaling against stress in plants can also be induced by treatment with chemical compounds. Such chemicals can be synthetic or naturally occurring and include, i.e. amino acids, hormones, nutrients, pesticides, reactive oxygen-nitrogen-sulfur species (RONSS) (Savvides *et al*., 2016). Pretreatment of *Arabidopsis* seedlings with the non-protein amino acid β-aminobutyric acid (BABA) by 1 day, either treatment with high salt content or drought, showed better tolerance to subsequent stress: lower wilting rate and loss of water. Interestingly, BABA is also a commonly used agent that improves systemic acquired resistance (SAR) for pathogen protection, indicating that the compound triggers the activation of a common pathway for biotic and abiotic stress. *Arabidopsis* plants pretreated with melatonin showed better growth after cold stress, manifested in increased fresh weight, root length, and shoot length (Bajwa *et al*., 2014). Melatonin increased cold-inducible gene expression at different time points during stress (van Buer, 2016).

The exogenous application of chemical compounds in crops has been used frequently for seed treatment, because seeds can be treated more easily and at a lower cost than adult plants. It has been described that chemical pretreatment of seeds can increase the germination rate and percentage. Furthermore, it can have a long-term beneficial effect by improving seedling vigor, especially during growth under stress conditions (Lutts *et al*., 2016).

2.3.2 RESPONSE OF PLANTS TO BIOTIC STRESS

Plants are constantly exposed to pathogens and have developed defense mechanisms that allow them to survive against these pathogens (Teixeira *et al*., 2019; Ganusova *et al*., 2019). The first of these comprises pre-existing defense mechanisms, formed by structural characteristics of the cell wall and the presence of some compound with antimicrobial activity. On the other hand, also in plants are the inducible defense

mechanisms formed by the de novo synthesis of antimicrobial compounds, in response to the presence of any pathogen.

In plants, the basal defense mechanism can be activated by some inducers, called PAMPs (pathogen-associated molecular patterns), such as flagellin from bacteria, liposaccharides, and chitins from some fungi (Hayafune *et al.*, 2014; Macho and Zipfel, 2015). The presence of non-pathogenic microorganisms also triggers the primary defense response, which is why they are also known as MAMPs (microbe-associated molecular patterns) (Ipcho *et al.*, 2016; Ranf *et al.*, 2017). Subsequently, in plants, there is the recognition of effector proteins by proteins of the plant itself. *Avr* factors are recognized by R proteins, products of the host R genes, called effector-triggered immunity (ETI) (Hatsugai *et al.*, 2017). ETI is generally associated with programmed cell death at the site of infection to prevent it from spreading. This mechanism is known as a hypersensitive response (HR) (Laflamme *et al.*, 2020).

Certain pathogens activate the hypersensitive response in plants, which limits the growth of the pathogen at the site of infection. The HR response is characterized by the presence of brown and black spots formed by dead cells at the site of infection. In this type of response, a physical barrier is created to prevent the proliferation and spread of pathogens (Lu *et al.*, 2015). This type of response is associated with high levels of plant growth hormone salicylic acid (SA), a regulator with a key role in the mechanisms of response and resistance to pathogens, as well as the synthesis of proteins related to pathogenesis and is due to This is associated with the establishment of systematic acquired resistance (SAR) (Kulye *et al.*, 2012).

For its part, the SAR response is a type of secondary response that allows the uninfected parts of the plant to be resistant to attack by pathogens. This response is triggered by an increase in SA concentration, which occurs during the HR response, the increase in this plant growth regulator is taken as a signal by the plant to induce the expression of genes related to R proteins, throughout the plant (Kulye *et al.*, 2012). This type of response induces the synthesis of genes related to pathogenesis (PR) (Choi *et al.*, 2015). In SAR, the NPR1 protein is the one that censuses the SA signal. SA interacts with transcription factors TGA, acting as a transcriptional coactivator of the PR genes (Loake *et al.*, 2007; Fu *et al.*, 2012).

2.3.2.1 The R genes and their biotechnological application against biotic stress

To date, many genes have been isolated in the models of *Arabidopsis thaliana* sp. and *Oryza sativa* sp. In *Arabidopsis thaliana* at least approximately 150 genes encoding NBS-LRR proteins have been identified (Mondragón-Palomino *et al.*, 2002) and in Oryza (Rice) at least 400 sequences with high homology to these genes have been reported (Liu *et al.*, 2017). Likewise, the divergence of these NBS-LRR families has been documented in many crops of commercial interest such as rice (Ma *et al.*, 2015), *Vitis vinifera* (Goyal *et al.*, 2020), *Brassica napus* (Alamery *et al.*, 2018) and mango (Lei *et al.*, 2014), among others.

There is evidence of a relationship in the increased expression of genes that encode NSB-LRR proteins in response to the presence of pathogens. The level of response appears to be related to the degree of resistance or susceptibility of the plant to the pathogen

(Shimizu *et al*., 2014). Variation in the expression or transcript levels of these NBS-LRR genes has been described in many economically important crops such as sunflower and squash under different infection conditions.

Given the importance of these genes and their participation in the plant's response to pathogens, the isolation of R genes creates new possibilities to incorporate them into the genomes of plant susceptible to pathogens. Also, from these it is possible to create selection tools assisted by molecular markers that allow identifying crops resistant to pests and diseases, as has been shown in wheat, chili and rice (Ma *et al*., 2019; Naresh *et al*., 2018; Kim and Reinke, 2019).

In conclusion, with advances in genetic engineering, a variety of genes and enzymes have been explored, involved in different metabolic pathways, and that their genetic modification has increased tolerance to different abiotic and biotic factors in several crops of economic importance (Table 2.1 and Table 2.2), this has allowed impact in the area of food and alternate energy generation.

2.3.2.2 Genetic engineering and improvement of traits of economic importance in plants of commercial interest

Genetic manipulation of plants has been more accepted in ornamental plants, timber plants and crops with the application of biomass generation for bioenergy. It is estimated that at least 50 ornamental plants have been transformed, the main species including rose (*Rosa hybrida*), chrysanthemum (*Chrysanthemum morifolium*), petunia (*Petunia hybrida*), and carnation (Dianthus caryophyllus) (Azadi *et al*., 2016; Kishi-Kaboshi *et al*., 2018; Boutigny *et al*., 2020; Noman *et al*., 2017).

Through genetic engineering of plants, it has been shown that characteristics such as the color of the fruits or flowers can be modified. Several ornamental plants, including carnation, rose and gerbera have been engineered for modified flower color. Some of the biosynthetic pathways that have been manipulated for these purposes is the pathway to produce anthocyanins or carotonoids (Nakatsuka *et al*., 2010; Nishihara and Nakatsuka, 2010). Some reported genes that have been manipulated are the expression of dihydroflavonal-4 reductase (dfr) gene from maize in a petunia line (Meyer *et al*., 1987). Chalcone synthase (Chs) gene that causes the generation of pink flowers of pink, white and variegated in petunias, chrysanthemum, gerbera and roses (Fukusaki *et al*., 2004; Van der Krol *et al*., 1998). Pigmented flowers have been obtained by genome editing with the CRISPR/Cas9 system in torenia (Nishihara *et al*., 2018).

Another characteristic that has also been genetically manipulated is the size and quality of the fruit. Fruit ripening has been modified by altering the activity of cell wall enzymes such as polygalacturonases. Calgene Inc., USA (1994) developed the first commercialized transgenic plant, a long shelf life tomato by the suppression of polygalacturonase (PG) gene by antisense strategy (Smith *et al*., 1988). The Flavr Savr Tomatoes varietes have improved flavor and total soluble solids (TSS), in addition to the enhanced shelf-life. Also, tomato varieties with increased shelf life were developed through antisense RNA inhibition of ACC synthase or ACC oxidase, two ethylene precursors. Delayed leaf senescence has been achieved in tobacco

and petunia by manipulation of cytokinin synthesis (Clark *et al*., 2003), and Park et al. (2005) demonstrated that fruit from tomato plants expressing Arabidopsis thaliana H+/ cation exchanger (CAX) gene have more calcium (Ca2+) and prolonged shelf life when compared to controls.

Crop maturity indicated by the percentage of ripening fruits on the vine was delayed in a CaMV35S-ySpdSyn genotype, with fruits accumulating higher levels of the antioxidant lycopene. Notably, whole-plant senescence in the transgenic plants was also delayed compared with wild-type plants.

2.4 SCOPES OF THE SECOND AND THIRD GENERATION OF TRANSGENIC PLANTS.

Today the population faces food insecurity and in poor countries such as sub-Saharan Africa and South Asia. The world population is expected to increase to 9-10 billion in the next 30 to 40 years (Stewart and McLean, 2005), triggering global food insecurity and the establishment of agronomic crops with improved nutritional content could be an alternative to the food shortage in these countries.

The second generation of genetically modified plants includes those plants where the organoleptic characteristics of the fruit, nutritional content and longer shelf life are improved. One of the first transgenic plants with improved nutritional content was known as "Golden Rice", this cereal contains moderate levels of beta-carotene, and initially the goal was for the population with vitamin A deficiency to be the consumers of Golden Rice (Ye *et al*., 2000; Paine *et al*., 2005). In India it has been estimated that Golden Rice could save up to 40,000 lives per year including poor in danger of losing eyesight and life (Potrykus *et al*., 2012).

Another scope of the second-generation GMOs is obtaining a soybean with high iron contents. Researchers transformed the storage protein gene ferritin into rice under the control of an endosperm-specific promoter, obtaining transgenic rice plants with grain that contain three times more iron than the control (Goto *et al*. 1999; Drakakaki *et al*., 2005). The consumption of these cereals was initially proposed in a population with anemia, and it has been shown that healthy people can consume this cereal without presenting negative effects on the absorption of this mineral (Lönnerdal *et al*., 2006). Due to the positive impact of this transgenic, genetic modifications have been made that increase the iron content in crops such as corn (Kanobe *et al*., 2013) rice (Paul *et al*., 2014), and banana (Kumar *et al*., 2011) (Table 2. 3).

Additionally, advances in genetic engineering have shown that they can increase Vitamin content (Pérez-Massot *et al*., 2013), such as Vitamin A in corn (Zhu *et al*., 2008; Naqvi *et al*., 2009) and potato (Lopez *et al*., 2008); vitamin C in Corn (Naqvi *et al*., 2009) and tomato (Bulley *et al*., 2012) and folic acid in rice and tomato (Storozhenko *et al*., 2007).

Table 2.3. *Second and third generation transgenics plants with food, biopharmaceutical and bioenergy application.*

Specie	Enzyme or modified gene	Characteristics of the transgenic	References
Second generation of transgenic plants			
Rice	Soybean *ferritin* gene (*SoyferH2*); Barley nicotianamine synthase gene (*HvNAS1*); nicotianamine aminotransferase genes (*HvNAAT-A* and *-B*); mugineic acid synthase gene (*IDS3*) were transformed into rice.	Transgenic plant showed increased the seed iron level without causing iron sensitivity under iron-limited conditions.	Masuda *et al.* (2013)
Banana	Soybean ferritin cDNA was transformed in banana.	Transgenic plants showed 6.32-fold increase in iron accumulation and a 4.58-fold increase in the zinc levels.	Kumar *et al.* (2011)
Soybean	Barley NA synthase 1 (*HvNAS1*) gene was transformed into soybean.	The NA (Nicotianamine) content of transgenic soybean seeds was up to four-fold greater than that of non-transgenic (NT) soybean seeds.	Nozoye *et al.* (2014)
Wheat	Soybean (Glycine max) ferritin gene was transformed into Wheat.	Compared to the control, transgenic plants have increased iron content of 4.93 to 64.03 %.	Xiaoyan *et al.* (2012)
Rice	Soybean ferritin gene was transformed into rice.	Transgenic plants showed higher iron and zinc levels in transgenic rice grains.	Vasconcelos *et al.* (2003)
Soya bean and grass pea	Expression of an oxalate-degrading enzyme was transformed into soya and grass pea.	Plants transgenic showed the reduction in OA (oxalic acid) level in soya bean (up to 73%) and grass pea (up to 75%) seeds. The reduced OA content was interrelated with the associated increase in seeds micronutrients such as calcium, iron and zinc.	Kumar *et al.* (2016)
Pineapple	Soybean (Glycine max) ferritin gene was transformed into pineapple.	Plants transgenic showed 5.03-fold increase in iron and 2.44-fold increase in zinc accumulation in the leaves .	Mhatre *et al.* (2011)
Maize	Maize was transformed with a single endosperm-specific functional expression/silencing transgene, which encodes a bacterial feedback insensitive DHDPS with an LKR/ SDHRNAi sequence.	Plant transgenic of maize, showed a significant elevation in the seed Lys level.	Frizzi *et al.* (2008)
Maize	Expressing gene encoding HORDOTHIONINE12 or theBARLEY HIGH LYSINE8 (BHL8) protein and a bacterial DHDPS into maize.	Plants transgenic showed an elevation of total Lys to over 0.7% of seed dry weight, compared to around 0.2% in wild-type maize.	Jung and Carl, (2000)

Specie	Enzyme or modified gene	Characteristics of the transgenic	References
Rice	Rice lysine-rich histone proteins, RLRH1 and RLRH2, were over-expressed in rice seeds to achieve lysine biofortification.	The lysine content in the transgenic rice seeds was enhanced by up to 35 %.	Wong *et al.* (2015)
Canola	Aspartokinase (AK) and dihydrodipicolinic acid synthase (DHDPS) was transformed into canola.	Expression of *Corynebacterium* DHDPS resulted in more than a 100-fold increase in the accumulation of free lysine in the seeds of canola.	Falco *et al.* (1995)
Rice	*TKTKK1* and *TKTKK2* were expressed under the control of 35S promoter and were independently introduced into the rice genome.	Transgenic rice seeds significantly increased lysine, threonine by 33.87% and 21.21%, respectively.	Jiang *et al.* (2016)
Triticum	Ornithine amino transferase (TaOAT) was overexpressed in Triticum.	Transgenic plants overexpressing TaOAT showed enhanced tolerance to drought stress and increasing proline accumulation.	Anwar *et al.* (2020)
Third generation of transgenic plants			
Carrot	Production of a recombinant human GCD in a carrot cell suspension culture.	Treatment of this recombinant GCD protein showed no adverse reactions or clinical findings, indicating the potential safety of prGCD.	Shaaltiel *et al.* (2007)
Carrot	Production of interferon alpha-2b by nuclear expression under the CaMV35S constitutive promoter.	Carrot showed highest level of recombinant human interferon alpha-2b accumulation with highest level of plant protein extract antiviral activity (up to 12.8 x 10 (3) IU/mg TSP).	Luchakivskaia *et al.* (2012)
Rice	Development of transgenic rice expressing the capsid precursor polypeptide (P1) gene of foot-and-mouth disease virus (FMDV), under the control of a dual cauliflower mosaic virus (CaMV 35S) promoter.	After intraperitoneal immunization of mice with crude protein extracts from transgenic rice plants, FMDV-specific neutralizing antibodies were detected.	Wang *et al.* (2012)
Rice	The VP2 cDNA of IBDV strain ZJ2000 was cloned downstream of the Gt1 promoter of the rice glutelin GluA-2 gene in the binary expression vector, pCambia1301-Gt1.	Specific pathogen-free chickens orally vaccinated with transgenic rice seeds expressing VP2 protein produced neutralizing antibodies against IBDV and were protected when challenged with a highly virulent IBDV strain, BC6/85.	Wu *et al.* 2007
Potato	Transgenic potato expressing GP5 protein of PRRSV (Porcine reproductive and respiratory syndrome virus) was produced by Agrobacterium-mediated transformation.	Mice immunized with transgenic potato extracts generated both serum and gut mucosal-specific antibodies.	Chen and Liu (2011)

Specie	Enzyme or modified gene	Characteristics of the transgenic	References
Tomato	Development of tomato plants transgenic with the *preS2-S* Gene.	The fruit of which accumulate the PreS2-S anti-gen protein of HBV (hepatitis B virus). The results indicate that the candidate vaccine obtained from tomatoes transgenic for the preS2S gene showed a high immunogenicity and a distinct induction of both mucosal and systemic immune responses in vaccinated mice.	Salyaev *et al.* 2012
Corn	The generation of transgenic corn containing the S protein of TGEV (transmissible gastroenteritis virus).	The oral plant-based TGEV vaccine, when administered to previously sensitized gilts, can boost neutralizing antibody levels in the animals' serum, colostrum and milk.	Lamphear *et al.* (2004)
Banana	Embryogenic cells of banana were transformed with the *ORF 5* gene of PRRSV (Porcine reproductive and respiratory syndrome virus) envelope glycoprotein (GP 5).	A vaccination-dependent gradational increase in the elicitation of serum and saliva anti-PRRSV IgG and IgA was observed in pigs.	Chan *et al.* (2013)
Artemisia carvifolia Buch	Genetic transformation of A. *carvifolia* was carried out with *Agrobacterium tumefaciens* GV3101 harboring the rol B and rol C genes.	Artemisinin content increased 3-7-fold in transgenics bearing the rol B gene, and 2.3-6-fold in those with the rol C gene. A similar pattern was observed for artemisinin analogues.	Dilshad *et al.* (2015)
Spike lavender	Transgenic plants overexpressing the linalool synthase (LIS) gene from *Clarkia breweri*.	In the youngest leaves of transgenic plants linalool increase up to a 1000%.	Mendoza-Poudereux *et al.* (2014)
Rice	Expression of multiple genes encoding enzymes involved in flavonoid synthesis in rice (PAL and CHS, genes encoding flavonol synthase/ flavanone- 3-hydroxylase, isoflavone synthase, and flavone synthases).	The target flavonoids of naringenin, kaempferol, genistein, and apigenin were highly accumulated in transgenic rice.	Ogo *et al.* (2013)
Spearmint	We generated transgenic plants in which MsYABBY5 was either overexpressed or silenced using RNA interference (RNAi). We generated transgenic plants in which MsYABBY5 was either overexpressed or silenced using RNA interference (RNAi). Regulation of MsYABBY5 by overexpressed or silenced using RNA interference (RNAi) into Spearmint	Analysis of the transgenic lines showed that the reduced expression of MsYABBY5 led to increased levels of terpenes and that overexpression decreased terpene levels.	Wang *et al.* (2016)

Specie	Enzyme or modified gene	Characteristics of the transgenic	References
Cotton	Transgenic cotton was development by suppress expression of the endogenous cottonseed †-12 desaturase (*fad2*) of a mutant allele of a rapeseed *fad2* gene.	Increased seed oleic acid content ranged from 21 to 30% (by weight) of total fatty acid content in transformants.	Chen *et al.* (2014)
Rice	Transgenic rice was obtained by constructed a chimeric gene consisting of a maize *Ubi1-P-int* and a soybean *GmFAD3* cDNA, and by *Agrobacterium*-mediated transformation.	The α-linolenic acid content of the seeds increased dramatically up to tenfold that of the control.	Anai *et al.* (2003)
Brassica	A zero erucic acid (C22:1) line of *Brassica juncea* (VH486), was modified for its fatty acid composition in the seed oil with antisense constructs using the sequence of *fad2* gene of *B. rapa*.	Homozygous lines proved to present 73% of C18:1 and 8 to 9% each of C18:2 and C18:3 (α-linolenic acid) fractions in comparison to ca. 53% C18:1, 24% C18:2 and 16% C18:3 in the parental line VH486.	Sivaraman *et al.* (2004)
Rice	Transgenic rice plants over- and under-expressing pyruvate decarboxylase (employing Ospdc1 gene from rice) as well as over-expressing alcohol dehydrogenase (employing Ghadh2 gene from cotton) proteins.	Pdc and Adh over-expressing rice transgenics at early seedling stage under unstressed control growth conditions showed slight, consistent advantage in root vigour as compared to that of wild-type seedlings.	Agarwal *et al.* (2007)
Cotton	Cotton (*Gossypium hirsutum* L.) was transformed with constructs for the over-expression of two enzymes involved in ethanol fermentation, alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC).	Under conditions of O2 deficiency, excised roots from these transgenic plants showed up to 80% increase in ethanol evolution compared to untransformed roots.	Ellis *et al.* (2000)
Switchgrass	Down-regulation of the switchgrass caffeic acid O-methyltransferase gene in transgenic Switchgrass.	Transgenic plants decreases lignin content modestly, reduces the syringyl:guaiacyl lignin monomer ratio, improves forage quality, and, most importantly, increases the ethanol yield by up to 38% using conventional biomass fermentation processes.	Fu *et al.* (2011)

On the other hand, in nature at least 20 amino acids have been described and of these there are essential amino acids, which the human body does not have the ability to synthesize, so it is important to acquire it from different food sources. In order to develop modified transgenic plants with amino acid composition (Ufaz and Galili, 2008; Chen *et al.*, 2010), studies of genetic modifications were started including important cultivars such as corn, rice and potato (Table 2.3).

Maize is the most agronomically studied crop in reference to the increase in lys content (Frizzi *et al*., 2008). Reported studies indicate that that a combination of the traits of DHDPS expression, LKR/SDH suppression, and expression of genetically engineered high-Lys proteins, all in an endosperm-specific manner, shows high levels of lysine production in this culture. The progress made to date to biofortify cereals with some amino acids such as lysine, methionine, threonine, tryptophan, isoleucine, and lysine has shown that genetic engineering of crops could be an important way for biofortification of amino acids in agronomic crops for human or animal consumption (Yang *et al*., 2016; Galili and Amir, 2013) (Table 2.3).

The use of crops with improved nutritional content has caused controversy due to the constant debate regarding human consumption, and the possible negative effects that the consumption of these products may have on health and the environment in the long term. Furthermore, due to the slowness of policies and regulations in the establishment of second-generation transgenic crops for field application, the generation of plants with non-food applications (third generation of transgenic plants) was as an alternative to impact the bioenergy and pharmaceutical area, for example, the improved production of pharmaceutically active molecules in plants.

Currently there is an emphasis on proposing plants as bioreactors to enhance recombinant biopharmaceutical production (Buyel *et al*., 2012; Ma *et al*., 2005). Among the advantages that have been highlighted in third generation cultures for the production of active molecules is that these systems are low cost, low risk of contamination, easy scale-up, stability, presence of metabolites, and ability to produce N-glycosylated proteins, among others (Park and Wi, 2016; Buyel *et al*., 2017; Owczarek *et al*., 2019).

The presence of pathogens that infect human hosts is a risk that will always be present in the world population, therefore the technologies that allow the generation of vaccines is a priority that is in progress. Some transgenic plants have been used to produce vaccines include strawberry, rice, tobacco, tomato, banana and corn (Park and Wi, 2016) (Table 2.3). In carrot, it has been documented that it is possible to produce human recombinant b-glucocerebrosidase for the treatment of treatment of Gaucher's disease, anti-TNF (PRX-106) for treatment of autoimmune disorders, interferon alpha-2b for viral diseases and LTB protein for vaccine against cholera and diarrhea, caused by *Escherichia coli*.

Other plants used to produce vaccines are safflower and tobacco (Table 2.3). The vaccine for the Influenza AH5N1 virus and a monoclonal antibody cocktail (ZMApp) against the Ebola virus, are being produced in tobacco plants. For the treatment of diabetes, the production of insulin is done in safflower (*Carthamus tinctorius*) (Owczarek *et al*., 2019).

Plants have also been positioned as bioreactors for the production of active molecules that are widely used in the pharmaceutical industry (Table 2.3) (Nabavi *et al*., 2020; Oksman-Caldentey *et al*., 2004) For example, *Artemisia annua* has been genetically modified to obtain higher levels of the medicine used against malaria (Dilshad *et al*., 2015). Also crops such as rice have been used as a platform to produce Flavonoids (Ogo *et al*.,

2013) and spearmint has been used to produce terpenes (Wang *et al*., 2016).

In the bioenergy area, plants have proven to be good producers of fatty acids, genetic modification of cotton plants, soybean and Brassica have helped to obtain better yields and quality of fatty acids (Chen *et al*., 2014; Anai *et al*., 2003; Sivaraman *et al*., 2004) that can be used for biodiesel generation.

Also, the generation of greater biomass, or improvement of recalcitrant in plants to obtain alcohol by fermentation has been reported in maize, rice, switchgrass, corn and sorghum (Vermerris *et al*., 2007; Choudhary *et al*., 2020; Ellis *et al*., 2000; Fu *et al*., 2011). In crops, such as cotton, the fermentative metabolic pathway has been genetically manipulated by overexpressing the enzymes involved in ethanol fermentation, such as alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC) demonstrating that transgenic plants showed up to 80% increase in ethanol evolution compared to control (Ellis *et al*., 2000).

2.5 DEVELOPMENT OF RECOMBINANT DNA TECHNOLOGIES TO GOAL TO MINIMIZE THE NEGATIVE EFFECTS OF THE USE OF GMOS

Since the generation of the first plant transgenics and their use with different biotechnological applications, there has been debate about the negative consequences that these can have on the environment and health. Some points of debate have been:

a) The presence of antibiotics as selection markers of transgenic plants,

b) Gene manipulation and genome dysregulation in transgenic plants caused by nuclear transformation.

c) Horizontal and vertical transfer of genes in non-target crops.

In order to minimize the effects of the presence of antibiotics in the transgenes, cloning vectors have been optimized that allow to eliminate the presence of antibiotics once the gene of interest has been inserted into the genome. Strategies are currently being developed that involve the use of CRISPR-Cas9 to eliminate cassettes of antibiotics such as Methicillim (Wu *et al*., 2018). There are also antibiotic elimination systems in transgenes based on the Cre / lox system and their efficient use has been reported in transgenic tobacco plants, barley, rice and Brassica (Éva *et al*., 2018; Moore and Srivastava, 2006; Wang *et al*., 2020). Additionally, gene editing techniques like the use of zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALEN), and the clustered regulatory interspaced short palindromic repeats (CRISPR) associated nuclease (Cas) have allowed site-directed modifications in the genes of interest (Tamayo-Ordoñez *et al*., 2016). These techniques have been used to induce mutations in crops of commercial interest such as tobacco, maize and wheat (Shukla *et al*., 2009; Upadhyay *et al*., 2013).

Regarding the controversy that has caused the transfer of genes from transgenics to other non-target crops, we can mention that the chloroplastid transformation of plants

minimized horizontal and vertical gene transfer in protected crops (Daniell *et al*., 2002). The chloroplastid transformation of plants has had more acceptance because it has advantages such as; higher production of recombinant proteins and the transfer of modified genetic material is only through the mother (Adem *et al*., 2017).

Genetic transformations directed at chloroplast have impacted resistance to herbicides, insects, diseases and droughts, and to produce biopharmaceuticals, in crops such as tobacco (Chen *et al*., 2014), lettuce (Gorantala *et al*., 2014) and Barley (Rotasperti *et al*., 2020). Furthermore, the successful engineering of tomato chromoplasts for high-level transgenic expression in fruits, together with hyperexpression of vaccine antigens, and the use of selectable markers free of plant-derived antibiotics, bode well for administration. oral edible vaccines and biopharmaceuticals that are currently out of reach of those who need them most.

2.6 CONCLUSIONS AND REMARKS

The generation of first, second and third generation transgenic plants has helped to solve problems related to crop tolerance to different biotic and abiotic stresses, improvement in fruit quality and minimization of the life cycles of some crops. The nutritional value of some crops has also been improved and has impacted the production of bioactive molecules of interest for their non-pharmaceutical use.

According to the advances made in transgenic plants, we can mention that due to the controversy generated in the use of transgenics, to date the first and third generation are more accepted since they do not refer to human consumption. However, the development of all generations of transgenics is of equal importance, and the generation of these transgenic plants has had an impact in the biopharmaceutical, bioenergy, food and agriculture. Stressing the importance of continuing with studies aimed at minimizing the risks of the use of these transgenic plants in health and the environment.

Advances in recombinant DNA technologies have developed strategies that day by day minimize the adverse effects of transgenics on human health and the environment. At present, GM of plants is rapidly becoming a viable strategy for crop breeding.

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CHAPTER

THREE

The scope of biocatalysis in enzyme biotechnology

CHAPTER 3

THE SCOPE OF BIOCATALYSIS IN ENZYME BIOTECHNOLOGY

De la Cruz-Arguijo E.A.[1], Lira-Mendez K.[1], Tamayo-Ordoñez F.A.[2], Interián-Ku V.M.[3], Córdova-Quiroz A.V.[2] and Tamayo-Ordoñez M.C.[4] and Tamayo-Ordoñez Y.J.[5]*

[1]Centro de Biotecnología Genómica, Instituto Politécnico Nacional, Blvd. del Maestro, s/n, Esq. Elías Piña, Reynosa 88710.

[2]Facultad de Química, Dependencia Académica de Ciencias Química y Petrolera, Universidad Autónoma del Carmen, Carmen, México.

[3]Instituto Tecnológico de la Zona Maya, carretera Chetumal Escárcega Km. 21.5. C.P. 77960. Ejido Juan Sarabia, Quintana Roo, México.

[4]Laboratorio de Ingeniería Genética. Departamento de Biotecnología. Facultad de Ciencias Químicas. Universidad Autónoma de Coahuila. Ing J. Cardenas Valdez S/N, República, 25280 Saltillo, Coah.

[5]Facultad de Ciencias Químicas. Universidad Autónoma de Coahuila. Ing J. Cardenas Valdez S/N, República, 25280 Saltillo, Coah.

*Corresponding author: **yahatamayo@hotmail.es**

ABSTRACT

For centuries, people have used enzymes produced by several model organisms –like microorganisms, plants, and animals– with the goal of obtaining beneficial biotechnological products. These products are obtained through the functions of the enzymes of the used organisms because of their biocatalizing activity and, at present, they are broadly applied in the industry for production of food, beverages, drugs, animal feed, personal hygiene, products, pulp and paper, diagnosis, and therapy. Through the use of several biotechnological tools like genetic engineering and biocatalysis, overproduction of enzymes from microbes, plants, and animals has been achieved, and the yields of the products of interest have been improved, resulting in the continued increase of the use of enzymes in a diversity of biotechnological processes.

This chapter describes the impact and future perspectives of biocatalysis in enzyme production for various biotechnological applications and its consequences in the paper, food, and distilled beverage industries. Also described is the impact of biocatalysis in the degradation of plastic wastes and the use of agroindustrial wastes.

3.1 INTRODUCTION

The enzyme industry as we now know it, is the product of a fast development of biotechnology, especially in the last four decades. Since antiquity, the enzymes present in nature have been used in food production. The enzymes – naturally occurring substances involved in all biochemical processes– break down complex molecules into smaller units like carbohydrates into sugars. Due to enzymes' specificity, there is a corresponding enzyme for each substrate. Although most enzymes are produced from plants, fungi, bacteria, and yeasts, the microbial sources of enzymes have advantages over their animal or plant counterparts, 50% of which are derived from fungi and yeasts, 35% from bacteria, the remaining 15% being of plant or animal origin. Currently, microorganisms have been and will continue to be one of the largest and most useful sources of enzymes (Deckers *et al*., 2020; Adrio and Demain, 2014; Garg *et al*., 2016).

The production of ever more complex fine chemicals for life sciences (health care, agrochemicals, nutraceuticals) requires selective synthetic biotechnological tools, which complement purely organic chemical synthesis with a rewarding economic and ecological impact (Blamey *et al*., 2017). Enzymes are important due to their many useful properties. Their development, to a great extent, has been possible due to the availability of microbial sources. Microorganisms are of much attention because they can be produced economically and are amenable to genetic improvement. Microbial enzymes have replaced many plant and animal enzymes. They have found application in many industries including foods, beverages, pharmaceuticals, detergents, textiles, leather, chemicals, biofuels, animal feed, personal care, pulp and paper, diagnostics, and therapy. New molecular methods, including genomics and metagenomics, are being employed for the discovery of new enzymes from microbes (Sanchez and Demain, 2017). Catalysts of an enzymatic nature have been used by humans for thousands of years, in their benefit, for example, in fermentation as a means of producing and preserving food products such as cheese, beer, vinegar, and wine, ignoring that the resulting transformations were due to the functions of specific enzymes (Reetz, 2013; Salli *et al*., 2019; Wallace and Balskus, 2014; Jemli *et al*., 2016). In past years, the biocatalysis was based on the use of whole cells in which the bacteria or yeasts were used to carry out chemical processes. Enzymes are proteins that are part of the cells of all living things, because they are able to accelerate the speed of chemical reactions, they are considered biological catalysts and are essential for the cell to be metabolically active, without them, many of the chemical reactions within the cell would be very slow, so much so that they would not be compatible with life (Voet *et al*., 2013) Examples of enzymatic catalysis are fermentation as a mean for producing and preserving foodstuffs (Reetz, 2013). Until a little more than 100 years, these catalyzers began to be used with specific academic purposes, these proteins are classified according to the reactions they catalyze into: oxydo reductases (accelerate oxide-reduction reactions), transferases (transfer chemical groups between molecules), hydrolases (break or synthesize covalent bonds of molecules), lyases (they break bonds forming in turn double bonds), isomerases (catalyze a spatial rearrangement of chemical groups in the molecule without modifying its chemical composition)

and ligases (promote covalent union of two molecules coupled with the breakage of a pyrophosphate bond as a source of Energy). Almost a decade after discovering molecular chirality in 1848, Louis Pasteur changed the direction of the investigation and began investigating fermentations, lactic and alcoholic, and it was not until 1860, that he demonstrated a similar enantioselectivity in the metabolism of tartaric acid by *Penicillium glaucum* This discovery began the process that ultimately established the fundamental importance of molecular chirality in biology (Gal, 2013). Later, scientists like Emil Fischer, Albert Eschenmoser, Friedrich Lichtenthaler and Linus Pauling carried out investigations that led to the origin of biocatalysis, but it was Eduard Buchner who in 1897 overcame the paradigm of the need to use living cells to be able to transport catalyzed processes that open the path to biocatalysis as we know it now and open the possibilities to countless academic and industrial processes in which cell extracts or partially purified enzymes were used for biocatalytic purposes. Today, with increasing emphasis on biologically mediated chemical reactions, beyond the use of stoichiometric reagents, catalysis offers significant benefits, largely thanks to advances in biotechnology, the situation has changed dramatically in recent years. Two decades, building on advances in high-throughput DNA sequencing, more than 20,000 bacterial and fungal genomes have been sequenced, with data available in the public domain (Reetz, 2013; Bolivar and Nidetzky, 2016; Sheldon and Woodley, 2018).

Thus, the term biocatalysis was established in the present century as the use of enzymes in chemical synthesis and makes reference to the use of cells or their isolated enzymes to catalyze reactions or transformations leading to the obtention of compounds of interest for the satisfaction of numerous human needs (Truppo, 2017; Hughes and Lewis, 2018). An example of this is that pharmaceutical enzymes are classified into four main groups, enzymes in replacement therapy, in cancer treatment, for fibrinolysis, and enzymes that are used topically for various treatments. In general they play a critical role in the treatment of common and rare diseases. The enzymes from microorganisms can help to perform changes in the steps of drug synthesis, to increase the diversity of structures until now accessible to the traditional production methods. Enzymes are ubiquitous in all living beings because they are molecules essential for their functioning. One of the most outstanding properties of enzymes is their high specificity because of which they have an ample application in a number of industries. This capability of adaptation has made the microorganisms to be the primary source of industrially applied enzymes. However, most of these (more than 50%) are of microbial origin since, compared to enzymes derived from animal and plant sources, they are more stable and cover a greater variety of catalytic activities. Furthermore, microorganisms can grow rapidly in economic means, thus achieving high yields and representing an always available source, since their growth is not affected by seasonal fluctuations (Borrelli and Trono, 2015). In nature, the products of synthesis like carbohydrates, lipids, amino acids, and biologically active products –like neurotransmitters and hormones– are obtained and act in an enantiomeric way. Enantiomeric forms must be highly specific because they take part in biological processes and their information, which is controlled by messengers that selectively interact with the specific enzyme sites, receptors, transporting molecules, etc., and whose interaction de-

pends on the chemical complementarity of these quiral agents. The evaluation of new enzymes, as well as their improvement, are one of the most important challenges in biotechnology (Salli *et al*., 2019; Yari *et al*., 2017). As for human diseases caused by bacterial infections resistant to multiple antibacterial agents, it has become a serious concern in recent years. The continued emergence of antimicrobial resistant bacteria is increasing the need to reduce the use of medically important antibiotics. A number of approaches are being analyzed and optimized including the development of promising antibiotic alternatives to control bacterial virulence through interruption of quorum detection, use of synthetic polymers and nanoparticles, exploitation of recombinant enzymes / proteins (such as glucose oxidases, alkaline phosphatases and proteases), and the use of phytochemicals. The development of new agents to control the pathogenicity of a disease requires a deep understanding of the virulence factors that occur in the progression of the infection. The long process of conceptualization and a detailed understanding of these factors, as well as the necessary research resources, are considered important causes in their study (Hassan *et al*., 2018) The development of recombinant DNA technology has had a major effect on production levels of enzymes and represent a way to overproduce industrially important microbial, plant, and animal enzymes. It has been estimated that between 50–60% of the world enzyme market is supplied by recombinant enzymes. In addition, directed evolution techniques have allowed design of enzyme specificities and better performance (Sanchez and Demain, 2017). Biotechnology has become a major contributor to gross domestic product in many countries, and this contribution also translates into enzyme applications for different markets, products, biotech startups, international research programs and consortia (Blamey *et al*., 2017).

The importance of producing enzymes with medical applications, without a doubt is the most studied application, is related to the production of antibiotics and other bioactive molecules. Since the 1980s, various lipases and phospholipases have been produced commercially. The porcine and human pancreas were the first used for its production, but today, the main commercially available sources are yeasts, fungi and bacteria (Borrelli and Trono, 2015).

During the 1950's, most focused on redox processes and especially on the selective hydroxylation of steroids, a process whose selectivity is difficult to accomplish using conventional chemical methods (Truppo, 2017). The enzymes used for these processes can be wild, recombinant, or genetically modified to improve their activity or specificity. Compared to conventional chemical catalysts, the use of enzymes offers several advantages such as: (a) specifically directing the course of the reaction towards a predetermined product, thus reducing the risk of side reactions; (b) they work in mild conditions of temperature and pH; (c) avoid the production of toxic end products. Also, if it is immobilized on multiple matrices and supports, they can be reused multiple times, thus reducing the cost and improving the performance of the biocatalyst. In general, the use of enzymes on an industrial scale guarantees energy savings and avoids contamination, which promises ecologically and economically sustainable alternative strategies for industrial transformation (Borrelli and Trono, 2015) The use of recombinant proteins for different industrial applications

has gained increasing importance. In the past years, bacterial hosts have been used in more economic ways, for example, studies were made to synthesize recombinant penicillin G acylase (PGA). One or more enzymes that carry out the required synthetic steps can be present in the complete cells of a microorganism and act simultaneously without interference. The involved microorganism might be in full growth, stationary, or immobilized state. Alternatively, the enzymes may be free, in solution, within a membrane reactor, suspended, or cross-linked (Tripathi and Shirivasava, 2019). The enzyme based catalysis complies with the evermore demanding, highly selective, safe, and sustainable industrial processes. Unlike their chemiocatalyzer counterparts, the biocatalyzers have a very large tridimensional structure that provides multiple contact sites with the substrate of interest, which allows an exquisite level of selectivity. Also, biocatalysis offers economic and environmental advantages over chemiocatalytic methods. The enzymes are produced from renewable, low cost resources and are themselves biodegradable (Wallace and Balskus, 2014; Jemli *et al*., 2016; Truppo, 2017). Enzymes have currently been described in which biocatalysis has increased their yield (Table 3.1).

Nearly 100 types of enzymes are used industrially, most (75%) being hydrolytic enzymes used to depolymerize high molecular weight natural substrates like proteins, starches, pectins, and others. Some of the main areas of industrial application of microbial enzymes are for production of detergents, fuels, human and animal food products, textiles, paper, fats, oils, organic pharmaceutical synthesis products, leather, and personal hygiene products, in clinical analyses, and for fermentation (Salli *et al*., 2019; Ghaffari-Moghaddam *et al*., 2014).

Table 3.1. *Industrial production by biocatalysis of enzymes obtained from microorganisms.*

Species	Enzyme	Concentration	References
Bacillus flexus XJU-1	Alkaline amylase	37.72 U/mL,	Niyonzima and More (2014)
Bacillus licheniformis ER15	γ-glutamil transpeptidasa (GGT)	2 552 U/L	Bindal *et al*. (2018)
Catenovolum agarivorans MNH15	Dextranase	1 U/mL	Lai *et al*. (2019)
Cgaetinekka raogugera	β-glucosidase	33.6 U/mL	Mu-Rong *et al*. (2019)
Coriolopsis gallica	Lactase, manganese peroxidase, lignin peroxidase	9.4 U/mL 0.31 U/mL 0.45 U/mL	Elisashvili *et al*. (2017)
E. coli BL21(DE3) PLysS with asBL/pET-PICh plasmid	Recombinant insulin variant Recombinant antithrombosis insulin (rAT-INS)	150 mg/L	Jing *et al*. (2018)
Lactobacillus acidophilus BCRC 10695	B-glucosidase	3.91 U/mL	Liu *et al*. (2018)

Species	Enzyme	Concentration	References
Paenibacillus macerans	Cyclodextrin glucanotransferase (CGTase)	45.2 U/L	Yang *et al.* (2017)
Rhizomucor miehei CAU432	B-1,3-1,4-glucanase	20 025 U/g	Yang *et al.* (2015)
Saccharomyces cerevisiae	Heterologe α-galactosidasa	19 U/mL	Alvarez *et al.*, (2018)
Streptomyces brollosae NEAE-115	L-asparaginase	162.11 U/mL	El-Ahmady *et al.* (2019)
Streptomyces griseoflavus PTCC1130	Protease	14035 U/L	Hosseini *et al.* (2016)
Streptomyces sp. Al-Dhabi-49	Protease and lipase	139 2U/mL-253U/mL	Al-Dhabi *et al.* (2020)
Trichoderma asperellum PQ34	Chitinases	22U/mL	Hoang *et al.* (2020)
Trichoderma reesei NCIM 1186	Cellulase	3055.65 U/L	Jampala *et al.* (2017)

3.2 BIOCATALYSIS IN THE BIOTECHNOLOGICAL INDUSTRY

Due to their biocatalyzing capabilities, enzymes are amply used in industry (Patel *et al.*, 2016). During the 1980s and 1990s, enzyme engineering based on structural information allowed the extension of their substrate ranges, allowing the synthesis of unusual intermediates, directed evolution has thus made remarkable progresses in industrial applications of biocatalysis (Choi *et al.*, 2015) (Table 3.2).

Table 3.2. *Biotechnological applications of enzymes obtained from different microorganisms.*

Microorganism	Enzyme	Application	References
Filamentous fungi			
Aspergillus awamori	Glucoamylase	Increase 125% the sorghum ran	Makanjuola *et al.* (2019)
Aspergillus niger	Lipase	Provides stability and conditioning to baking doughs.	Singh and Mehta, (2016)
Aspergillus niger	catalase	Cheese processing	Singh and Mehta, (2016)
Aspergillus niger 113	Phytase	Food industry	Joshi and Satyanarayana, (2015)
Aspergillus niger CH4	Endo-pectinase	Breaks the α (1,4)-glycosidic bonds of pectin, either randomly or at the ends. Used in fruit juice and wine industries	Tapre and Jain, (2014)

Microorganism	Enzyme	Application	References
Aspergillus niger CH4	Exo-pectinase	Breaks the α(1,4)-glycosidic bond at the ends. Used in fruit juice and wine industries	Tapre and Jain, (2014)
Aspergillus sp.	Acid proteinase	Milk coagulation	Singh and Mehta, (2016)
Aspergillus terreus M11	Endoglucanases	In composting	Juturu and Wu, (2014)
Chaetomella raphigera	B-glucosidase D2-BGL	F;ifeedback inhibition of exo-glucanases and endoglucanases	Mu-Rong *et al.*, 2019
Geotrichum marinum	Extracellular lipase	Conversion to glycerol and fatty acids, and transesterification reactions	Bonugli-Santos *et al.*, (2015)
Mucor sp.	Amylase	Breaks covalent bonds by inserting a molecule of water and used as additive in the food industry	Bonugli-Santos *et al.*, (2015)
Trichoderma asperellum PQ34	Chitinase	Degradation of cell walls of phytopathogenic fungi	Hoang-Loc *et al.*, (2020)
Trichoderma reesei	Cellulase	Facilitate filtration and clarification processes, in olive oil extraction, and in sy milk production	Novy *et al.*, (2019)
Yeasts			
Candida antarctica	Lipase B	Act specifically on water insoluble esters and used in the detergent production industry	Joshi and Satyanarayana, (2015)
Geotrichum candidum *Pichia guilliermondii*	Inulinase	Hydrolyze inulin into practically pure fructose, amply used in the food industry as dietetic sweetener	Castillo and Chamy, (2013)
Kluyveromyces marxianus	B-D-fructofuranosidase	Breaks covalent bonds	Bali *et al.* (2015)
Kluyveromyces marxianus	Fructosyltransferase (FTase)	Catalyzes transference of the fructosyl fraction of a saccharose molecule by other, producing FOS of higher size	Bali *et al.* (2015)
Kluyveromyces fragilis, Kluyveromyces lactis y Kluyveromyces marxianus	β-galactosidases	In milk lactose and serum hydrolysis	Beltrán and Acosta, (2014)
Saccharomyces cerevisiae	Invertase	Hydrolyzes saccharose into its monomers: glucose and fructose	Gurung *et al.*, (2013)
Saccharomyces cerevisiae; Saccharomyces pastorianus	Acetyl transferases I and II (AATasa I and II)	Responsible for the formation of acetate esters making up the compounds significantly involved in final sensorial quality of ales	Loviso and Libkind, (2018)
Saccharomyces fragilis	Lactase	Hydrolyze lactose into its monosaccharides (galactose and glucose) and used in dairy products	Gurung *et al.*, (2013)

Microorganism	Enzyme	Application	References
Yarrowia lipolytica	Lipase	Conversion to glycerol, fatty acids, and transesterification reactions	Looser *et al.* (2015)
Bacteria			
Arabidopsis thaliana	β-amylase	Breaks covalent bonds by inserting a molecule of water and used as additive in the food industry	Santiago *et al.*, (2016)
Bacillus clausii, B. cereus, B. licheniformis, B. sphaericus, B. subtilis, B. stearothermophilus, B. mojavensis, B. megaterium, B. brevis, B. anthracis, B. thuringiensis	Proteases	Participate in the efficient modification of food proteins to increase their nutritional value, solubility, digestibility, flavor, palatability, and minimizes allergenic compounds	Singh *et al.*, (2016)
Bacillus stearothermophilus	Maltogenic amylase	Converts maltose into isomaltooligosaccharides acting as acceptor molecules through α-(1-6) glucosides. Used in starch and sugar industries	Moral *et al.*, (2015)
Bacillus subtilis JA18	Endo-b-glucanase	Processing of fruit juice and beer	Joshi and Satyanarayana, (2015)
Beauveria bassiana	Proteses	Develops consistency and flavor and aids in beer clarification	Souza *et al.*, (2015)
Catenovulun agarivorans NH15	Dextranase	Removing dental plaque	Lai *et al.*, (2019)
Escherichia coli	Hydrolase	Proteolytic enzymes that aid digestion of proteins in foodstuffs	Meireles *et al.*, (2016)
Escherichia coli,	Lactase (β-galactosidase)	Reduces milk and its serum products	Singh *et al.*, (2016)
Micrococcus luteus, Brevibacterium antiquum, Staphylococcus equorum subsp. equorum	Lipases	Catalyze fat and oil hydrolysis to glycerol and fatty acids	Ozturkoglu-Budak *et al.*, (2016)
Pseudomonas aeruginosa	Lyase	Degrade polysaccharides + antibiotics	Meireles *et al.*, (2016)
Streptomyces brollosae NEAE-115	L-asparaginase	Enzyme of high therapeutic value due to its use in certain types of cancer therapy mainly in acute lymphoblastic leuk	El-Ahmady *et al.*, (2019)
Streptomyces sp.	Transglutaminase	Crosslinking of proteins in dairy products	Singh *et al.*, (2016)
Streptomyces sp. Al-Dhabi-49	Lipase and protease	Proteolytic enzymes	Al-Dhabi *et al.* (2020)

3.2.1 PAPER INDUSTRY

With an increased awareness of sustainability issues, enzymes have been increasingly applied in the paper and pulp industries to counteract negative environmental effects. The main components of wood are cellulose, lignin, and xylan. In paper production, lignin gives pulp a dark color and it must be removed to obtain lustrous quality paper, this is achieved by the addition of large quantities of chlorine and alkaline chemical products in a process called whitening. Alternatively, laccase enzymes that degrade lignin can be applied in whitening. Xylanase is another enzyme that can be used for whitening paper because it easily degrades xylan and facilitates lignin removal. The use of enzymes reduces the processing time, energy consumption, and the amount of chemical substances required during processing. In the processing of recycled paper and cardboard, the pulp contains ink that must be removed before the paper can be reused again. The conventional deinking process requires large quantities of chemicals like NaOH, $NaHSO_3$, and H_2O_2; however, the use in the process of cellulase enzymes softens the recycled pulp and facilitates the release of ink (Jegannathan and Nielsen, 2013; Singh *et al*., 2016).

3.2.2 FOOD INDUSTRY

The food industry is one of the economic activities where enzymes are used for an ample variety of applications. Such applications are based in three basic aspects: (i) controlling food quality (the presence or absence of some enzymatic activities has a large impact on the quality control of the final product); (ii) modifying the properties of some food additives and of foodstuffs themselves (to modify chemical and rheological properties of food as, for example, through the use of enzymes like amylases, lipases, pectinases, etc.); and (iii) to be used as food additives (enzymes with direct application in the food industry). These biomolecules are efficiently involved in the improvement of the properties of foodstuffs and food components like flavor, scent, color, texture, appearance, and nutritional value. The deep understanding of the role played by enzymes in the industrial manufacturing of food ingredients has improved the basic processes to provide better, safer, and higher quality commodities for the market. Throughout the world, the microbial enzymes are efficiently used in bakery, the largest market of their application for improving the stability of the dough, the softness and structure of the crumb, and the useful life of products (Table 3.2). The increased use of microbial enzymes in cheese processing is large responsible of the use of enzymes in the dairy products industry, which is the second largest industry for their application, followed by the beverages industry (Patel *et al*., (2016), Singh and Mehta, 2016).

3.2.2.1 Use In The Cheese Industry Of Lactic Acid Bacteria (LAB)

The lactic acid bacteria (LAB) have different types of fermentation, the homofermentative LAB have lactic acid as the only final product (*Lactococcus, Streptococcus, Pediococcus, Vagococcus*, and some *Lactobacillus*) using the Embden-Meyerhof glycolytic pathway (Axelsson, 1998) and the heterofermentative LAB in which the most abundant final product is lactic acid but it is accompanied by lower proportions of acetate, ethanol, and carbon dioxide, converting hexoses to pentoses by the 6-phosphogluconate/phosphoketolase

pathway (*Leuconostoc, Oenococcus, Weissella, Carnobacterium, Lactosphaera*, and some *Lactobacillus*; (Carr *et al*., 2002).

The genera of LAB found in nature are *Aerococcus, Alloiococcus, Carnobacterium, Dolosigranulum, Enterococcus, Globicatella, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus*, and *Weisella* (Axelson, 1998; Carr *et al*., 2002). Their use in the food industry is varied because they fermentate foodstuffs like milk, meat, and vegetables to obtain yogurt, cheese, pickled food, and for the production of wine and beer; also, they are also probiotic that improve human and animal health. These LAB absorb substances toxic to humans for which they aid in proper food preservation (Jadan-Piedra *et al*., 2017). Some enzyme coding genes having ample industrial application have been identified in some LAB (Table 3.3).

Table 3.3. *Enzymes identified in lactic acid bacteria (LAB) and overexpressed in different hosts*

Enzyme	Gen	Source microorganism	Host	Vector	References
4,6-α-glucanotransferase	*GtfB*	*Streptococcus thermophilus NCC408*	*E. coli* BL21-CodonPlus-(DE3)	pET-28b(+)	Li *et al*. 2018)
Amylases	*Amy2*	*Lactobacillus plantarum* WCFS1	*E. coli* BL21(DE3)	pURI3-Cter	PlazaVinuesa *et al*. (2019)
Arginine deiminase	*ADI*	*Enterococcus faecalis*	*E. coli* DH5 α y BL21 (DE3)	pET28a(+)	Cai *et al*. (2018)
Chitinase		*Bacillus licheniformis* DSM8785	*Pichia pastoris* KM71H	pPICZαA	Menghiu *et al*. (2018)
Endo-1.4-β-glucanases	*Egl*	*Bacillus halodurans* C-125	*E. coli* BL21-CodonPlus-(DE3)	pET-EGl	Zeeshan *et al*. (2018)
Enoate reductases	*ER*	*Bacillus coagulans*	*Saccharomyces cerevisiae*	pADP1	Raj *et al*. (2018)
Glycoside hydrolase	*GST*	*Lactobacillus ginsenosidimutans* EMML 304	*E. coli* BL21 (DE3)	e pGEX4T-1	d Zubair-Siddiqi *et al*. (2020)
Halophylic proteases	*PRW1*	*Halobacterium salinarum*	*Bacillus subtilis*	HProPRW1	Promchai *et al*. (2018)
Phosphomannomutase (ManB)	*manB*	*Escherichia coli K12*	*Lactococcus lactis* subsp. *cremoris* NZ9000	pNZmanB	Li *et al*. (2018)
Transglutaminase	*TGase*	*Bacillus amyloliquefaciens*	*E. coli*	pBAD/3C/bTGase	Silva-Duarte *et al*. (2020)
Xylanase	*xyn2*	*Trichoderma reesei*	*Bacillus subtilis*	pDR111	Amorim *et al*. (2018)
β-glucanase	*Bgluc*	*Bacillus methylotrophicus*	*E. coli* BL21 (D3)	pET-28a-Bgl	Ma *et al*. (2020)

Enzyme	Gen	Source microorganism	Host	Vector	References
β-glucosidase	*bgy2*	*Lactobacillus brevis*	*E. coli* BL21(DE3)	pMAL-c5X	Zhong *et al.* (2016)
β-glucosidase	*BteqBgluc*	*Bacillus tequelensis* BD69	*Pichia pastoris* GS115	pPIC-Bteqβgluc	Raza *et al.* (2020)
γ-glutamyl transpeptidase	*GGT*	*Bacillus licheniformis*	*E. coli* BL21 (DE3)	pET51b-sblg	Bindal *et al.* (2018)

The main LAB used for yogurt production are *Lactobacillus bulgaricus, L. casei, L. acidophilus*, and *Streptococcus thermophilus*, which provide flavor, a soft delicate taste, aid in curdling, and improve the digestibility. *Streptococcus lactis, S. cremoris, S. lactis* ssp. *diacetylactis*, and *Leuconostoc cremoris* are used for the production of sour cream, which promote its characteristic flavor with small amounts of acetaldehyde and large quantities of diacetyl. The LAB transform some materials in the fermentation that richens the nutritional value because they produce vitamins, reduce antinutrients, or increase the availability of nutrients. The antimicrobial properties of the LAB have been broadly studied; however, an antifungal property has been recently discovered because of which they are considered of interest in food production due to fungi being a large issue in foodstuffs for the large economic losses they generate. Russo et al. (2017) tested 88 strains of *Lactobacillus plantarum* isolated from different foodstuffs, demonstrating their antifungal activity against different fungal species like *Aspergillus niger, A. flavus*, *Penicillium roqueforti*, *P. expansum*, *P. chrysogenum*, *Cladosporium* spp., and *Fusarium culmorum*, and it was shown that *L. plantarum* inhibits *P. chrysogenum*, *P. expansum*, and *F. culmorum* in 75%, which makes these bacteria a good antifungal agent.

Another application of LAB is as antioxidants, antithrombotic, antihypertensive, hypocholremic, or have immunomodulatory and probiotic effects. In the U.S., fermented vegetables as pickled cucumbers are normally consumed. Commercially, fresh cucumbers are immersed in brine (0.6-1.7 M NaCl) and subjected to fermentation by naturally occurring LAB until having < 0.05% sugar. LAB like *Lactobacillus plantarum, L. brevis, Enterococcus faecalis, Leuconostoc mesenteroides*, and *Pediococcus cerevisiae* (Fideler *et al.*, 2019) have been used to produce exopolysaccharides (EPS) and eliminate the number of additives added to this foodstuff. Juvonen et al. (2015) studied 37 LAB strains in carrot paste to obtain EPS (dextran, levan, and β-glucan), five of these producing the largest amounts of EPS in the presence of MRS medium with 1% glucose + 1% saccharose (*Lactobacillus lactis* E-032298, *L. mesenteroides* E-093126 and E91461T, *Leuconostoc citreum* E-093497, and *Weissella confusa* E-90392), suggesting these microorganisms can be used in vegetables to replace hydrocolidal preservatives is texturizers.

The LAB have also been used in fermentation of meat products. Meat fermentations might contain biogenic amines (BA) due to the activity of the cells themselves, of the microorganisms, or accumulated during storage (histamine, tyramine, cadaverine, putrescine, tryptamine), these BA are formed by the elimination of the carboxyl group (-COOH) from amino acids forming lower molecular weight peptides with biological activity that

produce poisoning symptoms (headache, reddening of skin, etc.). Pasini et al. (2008) evaluated the effect of two LAB (*Lactobacillus sakei* and *Pediococcus pentosaceus*) on the BA content of sausages, finding mostly putrescine and tyramide when using *P. pentosaceus* and concluding that in order to improve the safety and characteristics of traditionally fermented sausages the appropriate LAB must be selected.

According to the above said, the more extensive study of some enzymes present in LAB and their application for biocatalysis could become a short-term goal in food biotechnology.

3.2.2.2 Bakery

The use of enzymes for food production and processing has a long tradition. Enzymes have become essential in bread making because they improve the quality of dough and baked goods by allowing you to improve its flexibility, stability, volume, and crumb structure. Its manufacture involves the use of microbial enzymes such as amylase, alone or in combination with other enzymes, it is added to the flour to retain moisture more efficiently to increase softness, freshness and shelf life, in addition, lipase and xylanase (EC 3.2.1.8) are used for dough stability and conditioning, while glucose oxidase and lipoxygenase are added to improve dough strength and whiteness. Transglutaminase (EC 2.3.2.13) is used in this industry to improve the quality of flour, the quantity and texture of bread and cooked pasta. Lipases are also used to improve the flavor content of bakery products by liberating short-chain fatty acids through esterification and to prolong the shelf life of the bakery products (Patel *et al*., 2016; Singh *et al*., 2016).

The use of enzymes in the bread industry is mainly due to the enzymatic deficiencies of wheat and flour that leads to a low availability of fermentable free sugars for the yeasts. The content of amylases on flour depends on wheat growth and harvesting conditions. In humid climates, the trend is towards a high activity of α-amylase due to seed germination, while in dry climates the level of α-amylase will be low because of scarce germination. One way of incrementing the sugar content in dough is to add exogenous microbial enzymes like α-amylase and β-amylase (E.C. 3.2.1.2) that preferentially hydrolyze the α-(1-4) glycosidic bond of amylose and amylopectin, thus improving the quality of bread. The thermoresistant amylases, generally produced by bacteria, may remain stable after baking and continue to hydrolyze starch, which results in a soft and sticky bread. Otherwise, the fungal amylases are usually thermolabile and are inactivated during baking. The trend in the bakery industry is the use of novel flours, enzymes, or the design of bakery products for celiacs together with efforts to replace the use of traditional additives that are harmful to health as potassium bromate. Globally, studies are reported of the use of new raw materials different from wheat to manufacture bread and the use of new additives like enzymes, prebiotic, probiotic character products, or vitamins and minerals. The search for natural additives that do not generate residuals in foodstuffs has led to the use of enzymes in their design. In the case of bakery, studies have been made of the use of different enzymes and their effect on bread's properties, examples of this being the effect of xylanase from *Chaetomium* sp. on the volume of steamed bread, different combinations of enzymes like α-amylase, xylanase, protease, transglutaminase, glycosidase, and laccase to improve the rheo-

logical properties of bread doughs and their effect on the useful life of bread, and to determine the physical sensorial and fermentative properties of breads formulated with several types of enzymes like xylanase, amylase, lipase, and glycosidase, transglutaminase, and laccase (Desai *et al*., 2018).

3.2.2.3 Production Of Alcoholic Beverages

The alcohol that is characteristic of fermented beverages is the result of the metabolism of yeasts that transforms into ethanol the sugars present in fruits, grains, honey, and other carbon sources (Figure 3.1). The first produced alcoholic beverages were the result of spontaneous fermentations by the microorganisms (fungi, yeasts, and bacteria) present in the surface of fruits and grains, inside the containers used for the fermentation, or unintentionally introduced by humans.

In the production of distilled beverages, the spontaneous fermentation of musts involves a sequential substitution of different yeast species. Initially, when the alcohol content is low, apiculate yeasts – mostly in the genera *Hanseniaspora/Kloeckera*, *Candida, Rhodotorula, Kluyveromyces*, and *Pichia* (*Hansenula*)– are predominant. These low alcohol producing are used for the commercial production of several alcoholic beverages and can produce high concentrations of acids and volatile compounds. The yeast metabolism is not only responsible for the production of alcohol but also of several hundreds of compounds acting as flavoring, as solvents that favor the extraction of the aromatic compounds present in grapes, the production of many different aromatic metabolites (alcohols, esters. aldehydes, sulfur containing volatile compounds, etc.), or the release of enzymes capable of transforming aromatically neutral compounds of grape –called precursors– into aromatic compounds that give fermented beverages their characteristic scent and flavor. The presence and concentration of metabolites formed during the fermentation, either desired or not, will depend on the contribution of certain yeast strains or species (Segura-Garcia *et al*., 2015; Varela, 2016). Considering brewing in its most simplistic form, it surely represents humanity's oldest biotechnology. It is a complex mixture of components, made from raw materials including water, yeast (the most widely used yeast is Saccharomyces cerevisiae), malt and hops, and contains a wide range of different chemical components that can react and interact in all the stages of the manufacturing process, which are essential to determine what is the flavor and aroma. The volatile fraction can be composed of more than 800 different compounds, but some of them may be directly involved in producing a taste sensation when the product is consumed, these compounds belong to various chemical classes, including higher alcohols, esters, fatty acids, carbonyl compounds, sulfur compounds, furan compounds, monoterpenols, C13 noriso-

Figure 3.1. *Products obtained derived from the fermentation process using yeasts. A) Obtaining bread and derivatives, B) obtaining beer, C) obtaining wine.* ***Sacharomyces*** *spp. strains are used in production, the most frequent being* ***S. cerevisiae****.*

prenoids, and volatile phenols (5–8) (Olaniran *et al*., 2017). Of all of them, esters are of great importance because they represent a large group of flavor active compounds that confer a fruity-flowery aroma, can have very low flavor thresholdsand a major impact on the overall flavor, the major esters can be subdivided into acetate esters and medium-chain fatty acid ethyl esters, the first group comprises acetate esters such as ethyl acetate (fruity, solvent-like), isoamyl acetate (banana) and phe-nylethyl acetate (roses, honey, apple). Ethyl acetate represents approximately one third of all esters in beers, the second group of esters includes, among others, ethylcaproate, and ethyl caprylate (both apple-like) (Blanco *et al*., 2016; Loviso and Libkind, 2018). The different aroma and flavor generated by one type of yeast compared to another in direct link with the esters produced may be due to inter-specific differences in genomic terms, in the mechanisms of regulation of the expression of the genes responsible for the synthesis of said esters and in the activity of the participating enzymes (Loviso and Libkind, 2018; Abe *et al*., 2019).

There is a wide range of phenolic compounds derived from grapes in wines, the environmental conditions of light and temperature, characteristics of the different growing areas, directly determine the photosynthetic activity of plants and the enzymes in the phenylpropanoid pathway responsible for the biosynthesis of these compounds. Commonly, the authors divide them into subgroups and classify them according to chemical structure. This is in relation to the higher molecular weight compounds that are heterogeneous, structurally complex, difficult to separate from each other, but very important in terms of their sensory contributions to wines. Most pigments are derived from anthocyanins extracted from the skin of red grapes, although other phenolic compounds can mediate both extraction and stabilization, as well as convert or engage in subsequent reactions to form new colored compounds. Uncoloured phenolics can also react to form various pigments during winemaking and preserving. There are two main factors that affect the intensity and color of simple anthocyanin species, which are the pH and the concentration of sulfur dioxide (Harrison, R. 2018). Some yeast strains may have an acidifying effect on must due to deviation of their metabolic pathway during alcoholic fermentation, which have effects on wine color, or also the yeast influences the color of the wines by modifying the concentration of anthocyanins by adsorption in their cells, or favoring their Aroma is one of the most important quality factors of wine and one of the key determinants of consumer acceptance, it is a complex sensory characteristic that is determined by more than 1,300 volatile compounds, which include alcohols, esters, acids, aldehydes, isoprenoids, lactones and ketones, with a wide concentration range. The differences in the aromatic profile of the wines are determined by changes in the type, proportion and concentration of these volatile compounds (Fariña *et al*., 2015).

Microbiologically, this quality is influenced by the genera and species of yeasts present in the winemaking process that are capable of withstanding the conditions; in grapes and must, apiculated yeast *Hanseniaspora uvarum* (and its anamorphic form *Kloeckera apiculata*) predominate, however, species of the genera *Candida*, *Cryptococcus*, *Hansenula, Kluyveromyces*, *Metschnikowia*, *Pichia* and *Rhodotorula* are present. Today, it has been verified that many strains that are not Saccharomyces can be positive for

the development of anthocyanins and wine aromas at different stages of fermentation. Furthermore, oenological tannins are widely used in the wine industry to improve the color profile and complexity of the aroma, obtaining products with a more "natural" or traditional profile and image and more similar to those derived from spontaneous fermentations (Chen *et al*., 2018).

In addition, the use of these non-Saccharomyces opens up the possibilities for wine improvement, both in terms of aroma, acidity, glycerol, mannoproteins, color and alcoholic strength, the proportion of non-saccharomyces yeasts at the beginning of fermentation can stimulate chemical and sensory changes, which is due in part to the secretion of esterases, glucosidases, lipases, β-glucosidases, proteases, cellulases and other enzymes. that interact with the substrates present in the medium, improving some fermentation stages (Ciani *et al*., 2016, The identification, isolation, overexpression, and use in biocatalysis of these enzymes could in the next decade become a tool to improve the production of distilled beverages.

3.2.2.4 Functional Food

It was in 1984 when the concept of functional food emerged in Japan, publishing the regulation for "Foods for specified health use" (FOSHU). This term was intended to define those processed foods which contained ingredients that played a specific role in the physiological functions of the human body. And it was in the 90s when the concept of "functional foods" was introduced in Europe. A functional food is any food or ingredient naturally present or intentionally added to a food, which in addition to its own nutritional action, contains additional components that promote health (Rodríguez-Tadeo *et al*., 2017) Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host although probiotics can be administered indifferent regulatory categories of products, probiotic foods include yogurt, cheese, juices, and cereal bars among others, the most common being yogurt (Glanville *et al*., 2015) While prebiotics are defined as "a non digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" was first introduced by Gibson and Roberfroid in 1995, and its greatest effect is in the function and metabolism, such as increase in the expression or change in the composition of short-chain fatty acids, increased faecal weight, a mild decrease in luminal colon pH, a decrease in nitrogenous end-products and reductive enzymes, an increased expression of the binding proteins or active carriers associated with mineral absorption, and immune system regulation, Prebiotics reach the colon without being digested because of their chemical nature. A part of the material is not digested by pancreatic and small-bowel enzymes in the human gut and therefore, reaches the large bowel. The whole length of human gut is occupied by microorganisms with population numbers and species distribution characteristics of specific region of guts, In the gut the relatively more stable colonies are in large intestines than smaller intestines because the transit time in small intestines is faster (4-6 h) than large intestines (48-70 h) in adults.

Prebiotic compounds are short-chain carbohydrates including some fructooligosaccharides, polydextroses, and some oligosaccharides present in soybean and

oats. They occur in edible plants like onion, garlic, banana, asparagus, and artichoke. Of all food ingredients, the non-digestible carbohydrates (oligo and polysaccharides) are the most important candidates for being considered as prebiotics and are easily obtained through direct extraction from aqueous solutions or after chemical or enzymatic treatment. For the latter method, several kinds of enzymes can be used as, among others, ß-fructofuranosidases to obtain fructooligosaccharides (FOS), β-galactosidases to obtain galactooligosaccharides (GOS), and transglucosidases to obtain isomaltooligosaccharides (IMOS). The selection of bacterial, fungi or yeast strains able to efficiently ferment short chains-carbohydrates with prebiotic properties, is a subject of permanent interest, the ability to ferment short chain oligosaccharides is a key property for any bacterial or yeast strain to provide desirable clinical effects (Younis *et al*., 2015; Trujillo *et al*., 2015).

3.3 THE PLASTIC WASTES INDUSTRY

Synthetic polymers of polyesters are omnipresent in our daily life and are responsible for the formidable advancement of new material generation for their use in the building, textile, packaging, and medical device industries. In that context, it was discovered that cutinases play an essential role in the hydrolysis of the wastes of these polymers. Cutinases are enzymes that naturally hydrolyze biopolymers and should therefore be capable of hydrolyzing an ample variety of synthetic polyesters. The cutinases from the microorganisms *Fusarium solani f.* sp. *pisi, Pseudozyma jejuensis* sp. *nov., Aspergillus oryzae, Thermobifida fusca*, and *Aspergillus nidulans* have been reported to hydrolyze plastics by breaking the ester linkage of cutin, thus releasing the monomers. The esterolytic activity of cutinases has been amply explored in vitro because they show hydrolytic activity of an ample variety of esters including hydrolysis of synthetic soluble esters to insoluble long-chain triglycerides. Also, in non aqueous or low water activity media, cutinases can catalyze synthesis reactions like the esterification and transesterification of different substrates. These hydrolytic and synthesis reactions have a potential application in the food industry, in the dairy industry for hydrolyzing milk fat, in the synthesis of structural triglycerides, polymers, and surfactants, in the synthesis of personal hygiene products, in the degradation of insecticides, toxic substances, and synthetic polymers, and in the production of biodiesel, among other applications (Peña-Montes *et al*., 2018). The growing concern to satisfy the increase in the consumption of polymers, human health and the safety of the environment, has led to the use of microbial enzymes for the synthesis of biodegradable polymers. Cutinases that were discovered more than 40 years ago, were tested in a series of technological applications, and have been proposed as biocatalytic tools for the polymer sector. These enzymes are widely distributed in animals, plants and microorganisms, they have caught the attention of researchers because they do not require cofactors, they are quite stable, catalyze both hydrolysis and synthetic reactions, and are even active inorganic solvents. Biopolymers

are environmentally friendly materials, as they are synthesized from renewable carbon sources through biological processes, degrade biologically after use, and return to the natural environment such as CO2 and biomass. The cutinases of the microorganisms *Fusarium solani, Pseudozyma jejuensis, Aspergillus oryzae, Thermobifida fusca* and *Aspergillus nidulans* hydrolyze plastics by breaking the ester bond of cutin, thus releasing the monomers. The sterolitic activity of cutinases has been extensively explored in vitro because they show the hydrolytic activity of a wide variety of esters, including the hydrolysis of synthetic soluble esters to long-chain insoluble triglycerides. In vitro enzyme catalyzed polymer synthesis is an environmentally safe process that has several advantages over conventional chemical methods. Biopolymers such as polyesters, polycarbonates, and polyphosphates are used in various biomedical applications, for example, orthopedic devices, tissue engineering, adhesion barriers, control of drug delivery, etc. (Singh *et al*., 2016; Kumari *et al*., 2016).

3.4 USE OF AGROINDUSTRIAL WASTES

Nowadays fossil fuels are still the world's main energy sources; however, their environmental impact, and their increasing cost has lead scientists to investigate more environmentally friendly, in addition, due to political unrest in the world, diversification of energy sources is needed. The Convention on Climate Change of the EU of 2005 defines biomass as a non-fossilised and biodegradable material that originated from animals, plants and microorganisms. Biomass could originate from energy crops or from residues of agricultural and agro-industrial activities. when it is produced by photosynthetic organisms they fix light, water and carbon dioxide. For example: land and marine plants, or photo-synthetic microorganisms known as cyanobacteria and microalgae. In these organisms, solar energy is stored in chemical bonds and can be released through processes such as combustion, digestion, decomposition, or by hydrolysis and fermentation with liquid or gaseous fuels (Alvares *et al*., 2014).

The originating from energy crops are often criticised as non-sustainable, since they are considered the main responsible for the depletion of soil nutrients and for competing with food crops, so it is well accepted that agriculture can contribute to the increase of renewable energy production, reduce fossil fuel dependency and production of pollutants (Volpe *et al*., 2014) As for biofuels, they are manufactured from plant resources, such as firewood, forest residues, charcoal, vegetable oils, and agricultural waste such as straw, bagasse, and other solids. They are widely used in food cooking, water heating, electricity generation in steam turbines, industrial heat production and electricity. Biomass also produces other types of biofuels, which are obtained through biotechnological processes, from biomass, and are converted to alcohol (bioethanol from sugars), biogas (biomethane from organic waste) and biodiesel (from waste fats and oils). These biofuels can be used in diesel or gasoline engines, cars, buses, cargo trucks, and

ships, or to produce electricity and heat in generators and mechanical work from their use in industrial engines (Alvares *et al.*, 2014) Regarding lignocellulosic wastes, they must be pretended by physical (thermal), chemical or enzymatic methods before beginning the process of enzymatic or microbial fermentation (biocatalysis). Organisms like fungi are adept at extracting sugar from lignocellulosic materials, and fungi that grow on wood are primarily sought because their production of enzymes capable of breaking down lignin to extract the sugars they feed on (Kumar and Chandra, 2020).

3.5 CONCLUSIONS AND REMARKS

The term biocatalysis was applied to the use of biomolecules to catalyze reactions or transformations that render compounds of interest for satisfying numerous human needs (Schmid *et al.*, 2001; Bornscheuer *et al.*, 2012; Tapre and Jain, 2014; Patel *et al.*, 2016; Loviso and Libkind, 2018). Currently, biocatalysis is applied in numerous industries producing drugs and other chemical products, food, or biofuels (Kim *et al.*, 2016). The sustainable manufacturing of commodities for human consumption is one of the main purposes of biocatalysis and it involves many future challenges and opportunities (Choi *et al.*, 2015).

It is irrefutable that biocatalysis has attracted the attention of the scientific community due to the significant advancements that have taken place in recent years in areas like genetic engineering, microbiology, and synthetic biology. Without the recombinant DNA technology, mutagenesis, and directed evolution, it would have been impossible to achieve the expression of massive amounts of enzymes or the obtention of new biocatalysts having an enhanced activity, an improved stability, and a higher specificity.

But despite the relevance of the research made and the developments achieved through biocatalysis, the production by biotechnological methods of a large number of currently used chemical products is yet limited and it is important to realize that this represents several challenges: (i) The improvement of the products' properties that are usually not optimal at the industrial level, (ii) the improvement of the techniques that increase the stability of enzymes, (iii) the inhibition of the substrate and the product, and (iv) to increase the activity of enzymes and even their specificity for the substrate.

Nowadays, work is made along three different axis to improve the biocatalytic processes:

1. The partial replacement of the catalytic process through combined strategies in which the biocatalyzers (enzymes or whole cells) may replace chemical catalysts along the synthetic pathways.
2. To continue developing the biocatalysts by means of emergent technologies of evolutionary or directed engineering that may possibilitate new and shorter synthesis pathways, and
3. To develop a more efficient and sustainable process in which biocatalysts remain active and stable throughout their useful life.

It is important to state that the first step in the development of a biocatalytic process is the identification of a product and the purpose of the reaction. Next, the system must be characterized based on the constraints of the reaction and, if necessary, of the process, or of both. Once the conditions are adjusted, the biocatalyst is applied in the process of bioconversion and the product is obtained and recovered.

As mentioned above, the proper selection of the biocatalyst is essential. This process can be an issue due to two causes, one related to the characteristics of the process and, in that case, if the process might be completed in one, two, or a combination of separate steps, it is preferable to configure the process using enzymes. Contrariwise, if specific cofactors are needed for the process or if solvents may affect the enzyme, complete cells must be used. The second problem is related to facing the limitations of the catalyst being possible even when there is a large number of available enzymes. In that case, a comprehensive literature review is recommended to find organisms or enzymes through bioinformatics and to develop the technical knowledge to be able to use them. The synthetic biology avoids conceiving totally new enzyme activities that can be developed by the design of proteins or through more classic genetic engineering methods like directed evolution or mutagenesis. To accomplish these tasks, the databases like the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) can be used to select family representatives and build models based on tridimensional structures.

In that line of thought, the biocatalysis cycle must be updated to introduce new biocatalytic reactions that are often originated in laboratories through new noticeable activities, overviewing the detection or identification of adequate enzymes that perform the process, thus accelerating the obtention of new molecules by bioconversion.

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CHAPTER

FOUR

Biotechnological applications of plant tissue culture

CHAPTER 4

BIOTECHNOLOGICAL APPLICATIONS OF PLANT TISSUE CULTURE

Tamayo-Ordoñez M.C.[1], Hernández-Delgado S.[2] Betancourt-Urbina G.A.[3], Bocanegra-Alonso A.[3], Ramos-García V.H.[3], Salazar-Bravo A.[2], Tamayo-Ordoñez Y.J.[4] and Ayil-Gutiérrez B.A.[5]*

[1]Laboratorio de Ingeniería Genética. Departamento de Biotecnología. Facultad de Ciencias Químicas. Universidad Autónoma de Coahuila. Ing J. Cardenas Valdez S/N, República, 25280 Saltillo, Coah.

[2]Centro de Biotecnología Genómica, Instituto Politécnico Nacional, Biotecnologia Vegetal. Blvd. del Maestro, s/n, Esq. Elías Piña, Reynosa 88710.

[3]Unidad Académica Multidisciplinaria Reynosa-Aztlán, Universidad Autónoma de Tamaulipas. Calle 16 y Lago de Chapala S/N Col. Aztlán, Reynosa Tamaulipas, 88740.

[4]Facultad de Ciencias Químicas. Universidad Autónoma de Coahuila. Ing J. Cardenas Valdez S/N, República, 25280 Saltillo, Coah.

[5]CONACYT- Centro de Biotecnología Genómica, Instituto Politécnico Nacional, Biotecnologia Vegetal. Blvd. del Maestro, s/n, Esq. Elías Piña, Reynosa 88710.

*Corresponding author: **benjamincgb_ipn@hotmail.com**

ABSTRACT

Plant tissue culture is a widely applied biotechnological tool for biomass production, elite material propagation, and for large scale reproduction with a number of purposes including propagation of stress-tolerant or producer of compounds of interest plants.

In this chapter, we discuss how this biotechnological tool has allowed obtaining biomass to be used to produce biofuel and how large scale propagation of threatened plants and food plants has contributed both to conservation of plant resources and to food security. Also discussed is how massive plant propagation revolutionized the obtention of compounds of interest currently applied in medicine and the pharmaceutical industry.

4.1 INTRODUCTION

Tissue culture is defined as a set of tools and methods that allow long-term growth under controlled, contaminant-free conditions of cells, tissues, organs, or any part of a plant structure, and to the regenerate whole plants from these cultured materials (Loyola Vargas and Vázquez, 2006).

The plant cells capable of growth and development to produce new individuals without the need for gamete fusion are known as *totipotent cells*, which is characteristic of meristematic cells present in plant organs (Segretín, 2006).

Based on that capability, tissue culture techniques have been devised to propagate plants by the processes of organogenesis and embryogenesis. Organogenesis implies the formation of a unipolar bud primordium and its development into a leafy vegetative shoot. The development of buds or root meristems can be made directly from explants (direct organogenesis) or from callus tissue (indirect organogenesis; Li, 1991). Embryogenesis consists in the development of embryos from cells not produced by gamete fusion during fertilization, in other words, produced through a process by which a bipolar structure (the embryo) is developed from a somatic cell. Using both processes (organogenesis and embryogenesis), the massive propagation of economically important crops like agave, sugarcane, and jatropha has been made possible.

Tissue culture has several current applications, for example, the clonal propagation of plants for their use as food, biofuel production, and for production of metabolites of pharmaceutical application. Tissue culture has also aided the development of plant models for basic study of several systems of plant signaling in biotic and abiotic stress responses like, for example, to freezing, drought, extreme temperatures, salinity, or soil acidity.

In this chapter, we discuss how this biotechnological tool has allowed obtaining biomass to be used to produce biofuel and how large scale propagation of threatened plants and food plants has contributed both to conservation of plant resources as to food security. Also discussed is how massive plant propagation revolutionized the obtention of compounds of interest currently applied in medicine and the pharmaceutical industry.

4.2 BIOMASS PRODUCTION BY PLANT TISSUE CULTURE

The production of biomass through plant tissue culture (Aghaali *et al.*, 2019; Rose *et al.*, 2017) has interest for food production (Flachowsky *et al.*, 2012), elite plant propagation, (Kataria *et al.*, 2013; Shiji *et al.*, 2018), production of secondary metabolites (Arias et al., 2009; Kapoor *et al.*, 2018; Zahir *et al.*, 2018), plant genetic improvement (Kundu et al., 2018) production of energy (Demura *et al.*, 2010; Lyczakowski *et al.*, 2017), and large scale production of ornamental plants (Batista *et al.*, 2017), among other applications.

Plant biomass may be obtained from any plant tissue (Rukh *et al*., 2019; Lyczakowski *et al*., 2017) because of its totipotent nature allowing to generate whole plant individuals from a single somatic cell (Verdeil *et al*., 2007; Karami *et al*., 2009). The production of biomass can be controlled by different conditions of light, stress, molecular factors, and growth regulators (Muñoz *et al*., 2003; Rukh *et al*., 2018; Santana-Buzzy, 2007; Demura *et al*., 2010) allowing cell division and development from plant tissues (Quiroz-Figueroa *et al*., 2002; Santana-Buzzy, 2007). Among the main causes that trigger plant biomass production is cutting signalling in plant tissues, which promotes cell reprogramming and gives place to stress responses that are followed by the activity of metabolic processes, protein synthesis, and the activation of cell cycle regulators (Ikeuchi *et al*., 2017). It has been demonstrated that synthetic growth regulators participate in the equilibrium of endogenic regulators (Ayil *et al*., 2013), making it possible to reversibly manipulate plant biomass to regulate differentiation and dedifferentiation (Aghaali *et al*., 2019).

In plants of *Coffea canephora*, a previous conditioning with synthetic auxins and cytokinins –like naphthaleneacetic acid (NAA) and kinetin (Kin)– triggers the increase of natural auxins –like indoleacetic acid (IAA) and indole butyric acid (IBA)– and when the leaf tissue is later cut and placed in a liquid medium in the presence of benzyl adenine (BA), the levels of natural auxin decreases again giving place to cell division and the initiation of somatic embryogenesis (Ayil *et al*., 2013). Among the strategies for in vitro manipulation, callus induction (Bibi *et al*., 2010), organogenesis (Bhusare et al., 2018), somatic embryogenesis (Sun *et al*., 2018), cell suspension, and transformed hairy roots (Vinterhalter *et al*., 2019) have facilitated the production of plant biomass.

Plant callus and undifferentiated tissues can originate somatic embryos (Zdravković-Korać *et al*., 2019) and organs by modification of the culture medium (Kazeroonian *et al*., 2018; Lakshmanan *et al*., 2016; Zhang *et al*., 2018) or by genetic transformation (Sharafi *et al*., 2014). Somatic embryogenesis can be of unicellular origin in a well-defined tissue structure, called direct or callus process, or in an indirect process from an unorganized structure (Zimmerman, 1993; Quiroz-Figueroa *et al*., 2002). The cell suspension and hairy root cultures have been important for the production of medicinal compounds while the concentration of these compounds has been increased by the use of genetic transformation use of genetic transformation has been important for the production of medicinal compounds and the use of genetic transformation has increased their concentration (Vinterhalter *et al*., 2019).

One of the examples of current use of tissue culture techniques is the production of plant biomass as a renewable source of biofuels (Somerville, 2007), crucial factors for their production having been found through molecular and genetic studies that are involved in meristematic activity, cell elongation, photosynthetic efficiency, and secondary wall biosynthesis (Demura *et al*., 2010). Cellulose is the main component of cell walls and has been used for producing biofuels (Somerville, 2006); therefore, there is interest in cell wall growth and the modification of the genes involved in this process could become a relevant source of biofuels

(Demura *et al.*, 2010). Genes coding for catalytic subunits of cellulose synthase (CesA) have been reported, as well as of other possible components like the endo-1,4-glucanase-like protein KORRIGAN, and the putative GPI-anchored protein COBL4. Regulation of these genes has shown defects in the secondary cell wall but results from their overexpression have not demonstrated an increase in cellulose content (Somerville, 2006). It has also been shown that sucrose synthase (SuSy) catalyzing the synthesis of UDP-glucose and fructose from sucrose is a component of the synthesis complex of the primary cell walls of *Vigna angularis* (Fujii *et al.*, 2010). In mutants of *A. thaliana*, alternative cellulose synthesis pathways have been suggested to exist because the elimination of four SuSy isoforms does not affect cellulose biosynthesis (Barratt *et al.*, 2009). An increased synthesis of cellulose has also been documented to occur when there is an overexpression of the membrane associated proteins TED6 and TED7, which interact with the cellulose synthase protein (CesA). The loss of this interaction has been shown to inhibit secondary cell wall formation suggesting that the identification of all the cellulose synthesis complex might help to increase the cell's content of biopolymers, which could be used as raw matter for the production of biofuels (Endo *et al.*, 2009).

The modification of several metabolic pathways and new genetic engineering techniques could be currently implemented in the manipulation and improvement of plant biomass production for different purposes.

4.3 PROPAGATION OF ELITE PLANT MATERIAL

Global needs for food, biodiversity conservation, and breeding of plant species have led to the propagation of elite plant. An assortment of plant tissue culture techniques have been used for in vitro growth, propagation, and breeding of useful plant species (Posada *et al.*, 2017, Cardoso *et al.*, 2018; Nguyen *et al.*, 2016; Uchendu *et al.*, 2017). Elite plants are selected in vitro according to their interest for commerce, the desired specific quality features, recovery from loss of habitat, species conservation, genetic improvement, and other purposes (Agrawal *et al.*, 2014; Khan *et al.*, 2018; Reyes *et al.*, 2017; Zheng *et al.*, 2010).

Among the examples of elite plant material of global interest is the white willow. The bark of *Salix alba* has been traditionally used as an anti inflammatory, antipyretic, and analgesic (Shara *et al.*, 2015). Salicin, a predecessor of salicylic acid, has been extracted from white willow bark (Pincock, 2005), whose extracts have been shown to act as mediators of the anti inflammatory metabolites factor α and nuclear factor-kappa B present in tumor necrosis processes (Shara *et al.*, 2015). The limited reproductive potential of the white willow constraints large scale production of salicin. The species is only known to have female individuals, which underscores the importance of its clonal propagation through tissue culture, added to the possibility of micropropagation of wild genotypes from buds and nodal segments in tissue culture to reintroduce individuals of interest to their places of origin (Skálová, 2012).

Another example of an economically important crop in the elite plant class is agave, and tequila made from *Agave tequilana* F.A.C. Weber has gained worldwide demand. In 2017, tequila production was 271.4 millions of liters of which 78.5% were exported (CRT, Tequila Regulatory Council, 2017). However, the low rates of asexual reproduction and growth, pollination problems, low seed viability, and overexploitation of *A. tequilana* have surpassed the capacity of the conventional propagation systems (Domínguez *et al*., 2008; Ramírez-Malagón *et al*., 2008; Trejo *et al*., 2018). New reliable alternative methods have been found to obtain genotypes of elite varieties or individuals through the use of the small offshoots that grow around a mother plant (Domínguez *et al*., 2008; Eguiarte *et al*., 2013; Ramírez-Malagón *et al*., 2008; Rodríguez-Garay *et al*., 2018), which allows accomplishing the parameters of authenticity that regulate the production of tequila by complying with the desired physicochemical properties based on quality parameters (Carreon-Alvarez *et al*., 2016). Beyond concerns for elite individuals for tequila production, in the past decades the issues involved in landscapes and restoration of the regions of origin have been also mentioned (Ramírez-Malagón *et al*., 2008).

One more globally important crop is coffee, with an estimated production of 164 million 60 kg bags between 2017 and 2018, in particular of *Coffea arabica* with 101.82 and *C. canephora* with 62.99 million 60 kg bags (Coffee Market Report - International Coffee Organization, 2018). The latter two species have been considered elite crops within the genus *Coffea* due to their organoleptic and high quality properties (Biggers *et al*., 1969). Both species are considered as biological models for tissue culture having been studied to generate basic knowledge about somatic embryo induction and development (Sattler et al., 2016; Bartos *et al*., 2018; Sanglard *et al*., 2018), the interaction of natural and synthetic growth regulators –and their combination– as possible responsibles for embryogenesis activators (Ayil *et al*., 2015), the identification of embryogenesis repression molecules (Nic-Can *et al*., 2015), the epigenetic regulation mechanisms (Nic-Can *et al*., 2013; De-la-Peña *et al*., 2015), the identification and isolation of genes involved in embryo formation and plant development (Nic-Can *et al*., 2013; Freitas *et al*., 2017; Jiménez-Guillen *et al*., 2018), and to gain insight of the proteomic profile in embryogenic cell suspensions (Campos *et al*., 2016). Research made with these well-established biological models (Quiroz-Figueroa *et al*., 2002; Santana-Buzzy *et al*., 2007) provides opportunities for applications and new strategies of analysis in the field of omics (Campos *et al*., 2017). Thus, tissue culture techniques have been of worth for generating knowledge of species of the genus *Coffea* (Los Santos-Briones et al., 2006; Santana-Buzzy *et al*., 2007) providing biological models free from interferences of other genomes in the axenic conditions required for omic studies. Coffee growers currently face issues related to climate change, pests, and diseases (Rice *et al*., 2018). Among examples of these issues is a 36% loss in global coffee production caused by root-knot nematodes (Hein and Gatzweiler, 2006), for which clones resistant to the pathogenic nematodes were obtained from orthotropic branches of *C. canephora* (Fatobene *et al*., 2017; Fatobene *et al*., 2018).

The use of tissue culture increases the probabilities for developing new biotechnolog-

ical strategies to generate pathogen-resistant cultivars and facilitates the cloning process without interfering with the organoleptic properties of the product wanted to be marketed. Also, because culture in vitro provides closed systems it may aid to the study of factors related to climate change and other issues, for example, the analysis of genes involved in resistance to pathogens or in plant responses to abiotic factors.

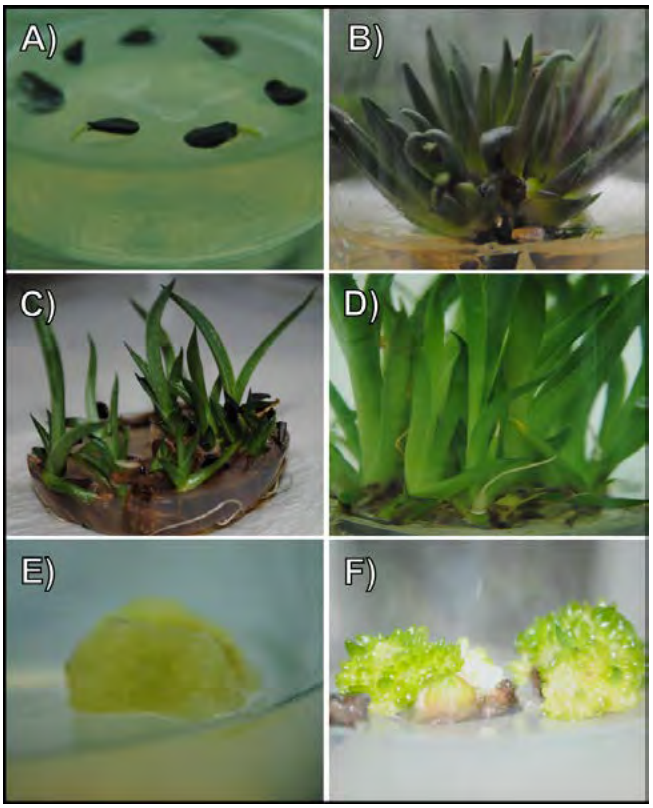

Figure 4.1.- In vitro propagation of elite material of ***Agave potatorum****. A) seed germination, B) in vitro propagation, C) and D) rooting. E) embryogenic callus, F) shoot formation from callus.*

4.4 BIOTECHNOLOGICAL APPLICATIONS IN MEDICINAL PLANTS CULTIVATED *IN VITRO*

Plants have been essential for traditional cultures' medicinal practices and their use for that purpose has persisted in developing countries (Aristyani et al., 2018; Suárez et al., 2019). Molecular biology techniques have allowed to disclose the mechanisms of action of plant compounds with pharmacological activity (Gruszka et al., 2013; Roufogalis et al., 2013), therefore increasing their scientific interest. Plant tissue culture has become a tool for basic and applied research of secondary metabolites (Loyola-Vargas et al., 2018). Currently, the modification of metabolic pathways for the production of secondary metabolites has been made possible through genetic engineering tools (Verma et al., 2017), elicitation (Shakya et al., 2019), variations in clonal propagation methods, the use of bioreactors (Mamun et al., 2015; Mittal et al., 2017), the induction of cell suspensions (Chung et al., 2018; Mahendran et al., 2018), and root transformation (Khezerluo et al., 2018). A large variety of plant species is presently studied in search of alternative ways of increasing the production of secondary metabolites (González et al., 2006), some examples of which are briefly discussed below.

Camellia sinensis is a plant with therapeutic potential that is rich in secondary metabolites like polyphenols, caffeine, amino acids, flavonoids, and catechin-like compounds. It has been attributed to be antioxidant and anticarcinogenic, to ease digestion, and to strengthen the immune system (Sharangi et al., 2009). The planting of cuttings of Camellia sinensis becomes impractical when

large quantities of new clones are required (Tahardi et al., 2003). However, biotechnology provides an alternative for quick, large-scale propagation by creating synthetic seeds through in vitro culture applying the encapsulation method, a technique to envelop plant tissue –callus, somatic embryos, meristem, explants– making possible to produce new plants from these protected tissues. The synthetic capsule acts as an endosperm having the function of providing a source of carbon, nutrients, and growth regulators (Muslihatin et al., 2018) maintaining the tissue until germination. Synthetic seed techniques would facilitate long-term storage, distribution, or dissemination and would protect seeds from infections (Rai et al., 2009; Ravi et al., 2012).

The plant *Melissa officinalis* has been found to contain high concentrations of rosmarinic acid and has been attributed to have antispasmodic, antibacterial, antioxidant, antiviral, and neuroprotection properties (Kamdem *et al.*, 2013; Pereira *et al.*, 2014; Bayat *et al.*, 2012). The production of rosmarinic acid by *M. officinalis* has been increased by the use of sodium nitroprusside (SNP) that acts as nitric oxide donor. Addition of 10 mM SNP to leaves cultivated in vitro produced a two-fold increase in the content of essential oils from 0.09% to 0.21% (v/w). In the case of nerol and geraniol, the increments were 1.4 and 4.1 times more, respectively, compared to plants cultivated ex vitro. The content of rosmarinic acid in plants varied from 3.12 to 4.82 mg/g, while untreated plants showed no significant increment (Esmaeilzadeh *et al.*, 2018; Hong *et al.*, 2007). Currently, the use of inductors, microorganisms, genetic manipulation, and synthetic enzymes of medicinal plants cultivated in vitro (Esmaeilzadeh *et al.*, 2018, Pannu *et al.*, 2019).

Moringa oleifera is a plant originally from India and presently found throughout the world in tropical and subtropical climates (Fuglie *et al.*, 1999; Devendra *et al.*, 2012) that is known to have a high content of vitamins A and C, Ca, Fe, and K (Fuglie *et al.*, 1999). It has been demonstrated that this plant species has medicinal properties attributed to be derived from its secondary metabolites –tannins, alkaloids, terpenes, flavonoids, and others. Its properties include antioxidant activity (Siddhuraju *et al.*, 2003), prevention of DNA damage in normal cells, reducing the load of diseases like cancer, diabetes, and obesity (Anwar *et al.*, 2007; Lin *et al.*, 2018; Hussain *et al.*, 2014). The extracts of *M. oleifera* have been found to contain antibacterial and antifungal properties (El-Mohamedy *et al.*, 2016; Maqsood *et al.*, 2017; Singh *et al.*, 2013) and several studies of the plant's extracts have demonstrated antitumor (Khalil *et al.*, 2014; Khor *et al.*, 2018) and anticarcinogenic activities in which the compound moringine acts exclusively against malignant cells not killing the healthy cells (Rajan *et al.*, 2016).

The resistance to antibiotics among pathogens throughout the world has currently become a threat to our capacity to combat infectious diseases. New biotechnological strategies have shown the viability of production from plants of proteins with therapeutic uses like antibodies, vaccines, and antivirals (Obembe *et al.*, 2010; Qiu *et al.*, 2014; Kolotilin *et al.*, 2014; Takeyama *et al.*, 2015; Vermij *et al.*, 2006). Antibodies have been produced from tobacco plants having activity against the ebola virus, of which the combination of monoclonal antibodies (ZMapp) was capable to rescue 100% of monkeys (*Macacos rhesus*) having high fever, viremia, and blood cell count anomalies prior to the administration of the

ZMapp antibodies. The documented diagnostic of advanced disease shows high levels of liver enzymes, mucosal hemorrhages, and generalized petequia, and that these symptoms can be reverted leading to a complete recovery. ELISA and neutralizing antibody tests indicate that ZMapp has a crossed reactivity wit the Guinea variety of the ebola virus, surpassing the efficacy of any other treatment described so far and the results justify its clinical application (Qui *et al*., 2014).

However, the search for profitable, efficient, and scalable strategies that allow producing large volumes of vaccines, prophylactics, antibodies (Kolotilin *et al*., 2014) and secondary metabolites is yet needed, and plant tissue culture is one such alternative. In the future, we consider necessary to collect seeds from plants with medicinal properties and to store them in different germplasm banks establishing plant tissue culture lineages for the study of mechanisms of action and DNA analysis to develop new strategies for clinical application.

Invitro culture has also had a great application for obtaining different metabolites and vaccines of commercial interest (Table 4.1).

Table 4.1. *Biotechnological applications in plant tissue culture to obtain metabolites and vaccines with biological activity.*

Species	Plant tissue technique	Metabolite	Activity	Reference
Iphiona mucronata	Embryogenic callus	Flavonoids	Anti-inflammatory, antioxidant, and anti-carcinogenic	Al-Gendy et al. (2013) Amer et al. (2018)
Piper cernuum, Piper crassinervium	Cell suspensions	Phenolic compounds	Antioxidantes	Delgado et al. (2013) Pajak et al. (2019)
Moringa oleifera	Synthetic seeds	Glucosinolates	Neuroprotective Anti-inflammatory Anti-carcinogenic	Muslihatin et al. (2018) Giacoppo et al. (2017); Jaafaru et al. (2018)
Camellia sinensis	Synthetic seeds	Glucosinolates	Cancer preventive	Muslihatin et al. (2018) Tan et al. (2010)
Fagopyrum tataricum	Protoplasts	Flavonoids	Antioxidante, anti-inflammatory, anti-carcinogenic	Zhang et al. (2018) Amer et al. (2018)
Gossypium hirsutum	Genetic engineering	Taninos	Reduce cholesterol levels	Lu et al. (2017)
Albizia lebbeck	Extractos, leaves; seed germination *in vitro*	Saponin	Anticancer	Desai et al. (2019); Sah et al. (2016)
Artemisia annua	Hairy root	Artemisinin	Antimalaria	Putalun et al. (2007); Pellicer et al. (2018)
Azadirachta indica	Hairy root	Azadirachtin	Anticancer	Satdive et al. (2007) Moga et al. (2018)

Species	Plant tissue technique	Metabolite	Activity	Reference
Beta vulgaris	Hairy root	Betalains	Antioxidants	Pavlov et al. (2006); Chhikara et al. (2018)
Salvia miltiorrhiza	Hairy root	Phenolic compounds; Rosmarinic acid	Antioxidant	Yan et al., 2006 Pajak et al. (2019)
Panax ginseng	Hairy root	Ginsenosides and triterpenes	Neuroprotection Anticancer Antidiabetic Hepatoprotective Immunomodulator	Murthy et al. (2017)
Tobacco	Cell suspension	Human glucocerebrosidase	Therapeutic human proteins	Obembe et al. (2010)
Tobacco	Cell suspension	Viral vaccine mixture	Vaccine	Obembe et al. (2010)
Tobacco	Cell suspension	HN protein of Newcastle disease virus	Vaccine	Vermij et al. (2006); Obembe et al. (2010)

4.5 CONCLUSIONES AND REMARKS

Tissue culture according to what is based on this book chapter presents great application in biomass production, propagation of elite plant material, obtaining compounds of interest from the propagation of medicinal plants. However, the limitation in the use of in vitro propagation techniques of plants of commercial interest has been limited by the protocols developed for the establishment of tissue culture. Sometimes reported protocols can be easily applied to a plant genus or species, but sometimes it is necessary to develop new *in vitro* plant establishment protocols. In this field it is still necessary to continue working on the establishment of protocols for the development of organogenesis or embryogenesis and to be able to apply it to native plants of the country.

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CHAPTER

FIVE

The impact of the creation of vaccines for disease control

CHAPTER 5

THE IMPACT OF THE CREATION OF VACCINES FOR DISEASE CONTROL

Diaz-Palafox G.[1], Bello-López J.M.[2], Tamayo-Ordoñez M.C.[1], Sanchez-Teyer L.F.[3]; Damas-Damas S.[4], Romero-Duran H.P.[5], and Tamayo-Ordoñez Y.J.[6*]

[1]Laboratorio de Ingeniería Genética. Departamento de Biotecnología. Facultad de Ciencias Químicas. Universidad Autónoma de Coahuila. Ing J. Cardenas Valdez S/N, República, 25280 Saltillo, Coah.

[2]División de Investigación, Hospital Juárez de México, Ciudad de México, Mexico.

[3]Centro de investigación Científica de Yucatán A.C. Unidad de Biotecnología. Mérida Yucatán México

[4]Facultad de Química, Universidad Autónoma del Carmen, Calle 56 No. 4 por Av. Concordia, Campus Principal, 24180 Ciudad del Carmen, Campeche.

[5]Facultad de Ciencias Económico Administrativas. Universidad Autónoma del Carmen, Calle 56 No. 4 por Av. Concordia, Campus Principal, 24180 Ciudad del Carmen, Campeche.

[6]Tacultad de Ciencias Químicas. Universidad Autónoma de Coahuila. Ing J. Cardenas Valdez S/N, República, 25280 Saltillo, Coah.

*Corresponding author: **yahatamayo@hotmail.es**

ABSTRACT

The World Health Organization (WHO) defines vaccines as any substance, the purpose of which is to generate immunity through the production of antibodies. Vaccines are a preventive method, although they do not have a curative role, the generation of vaccines has had a positive impact on disease control and during this COVID-19 pandemic that impacted the world, the relevance of vaccines was emphasized. In this book chapter it emphasizes how first, second and third generation vaccines have helped in the field of Health. In addition, we describe how the vaccines generated against the COVID-19 pandemic are an excellent biotechnology tool that has helped to counteract the deaths caused by the COVID-19 virus.

5.1 INTRODUCTION

The World Health Organization (WHO) defines vaccines as any preparation, the purpose of which is to generate immunity through the production of antibodies. The vaccines can be found in a suspension of dead or attenuated microorganisms or with the products derived from the microorganisms, in a usual way, their most common form of administration is by injection, although there are also versions with a nasal or oral spray. Vaccines are a preventive method, although they do not have a curative role, vaccines play an important role in disease control worldwide (San Miguel-Hernández *et al*., 2013).

The application of vaccines has played a key role in counteracting the death of patients exposed and infected by the different viruses that have occurred since the 18th centuries. Here we describe some of the relevant vaccine history issues.

At the beginning of the 18th century, Dr. Glacomo Pylarino began in Europe the inoculation of material from individuals affected by smallpox to healthy subjects; by 1750 the inoculation of pustular material to healthy people was considered an effective practice, but it was not universally accepted (de Micheli *et al*., 2011).

It is known that smallpox had become one of the most deadly diseases by the eighteenth century, causing approximately 10% of all deaths, and mainly affecting minors, it is estimated that about 50% of the people who they contracted the disease, died of smallpox. It was until Edward Jenner, managed to develop the vaccine against smallpox (Smith, 2011). After Jenner, the next big milestone for vaccine development was the discovery of the rabies vaccine (Tuells *et al*., 2011).

After these findings, in 1900, Camille Guérin and Albert Calmette had begun the development of an anti-tuberculosis vaccine; by 1940, various studies had already been carried out on the effectiveness and safety of BCG (Bacille Calmette-Guérin vaccune), and during World War II, Tuberculosis was already considered one of the major concerns worldwide, so the use of BCG was promoted by global organizations such as the United Nations Children's Fund (UNICEF) (Luca *et al*., 2013).

Starting in 1925, and thanks to the production of Tetanus antitoxin, great interest was aroused in the treatment for both humans and animals (Sordelli *et al*., 1936). During these years, the pertussis vaccine was developed in the same way, for the prevention of whooping cough, a childhood condition, produced by *Bordetella pertussis* (Standfast *et al*., 1973).

Another historical fact that marked the importance of vaccines was the effects caused by the influenza virus in 1557. Smith, Andrewes, and Stuart-Harris developed a subcutaneous vaccine, which was applied to the military force in England (Barberis *et al*., 2016). In the same way, Jonas Edward Salk to protect the US military force, in 1938, they applied this vaccine. Salk later in 1952 developed the polio vaccine (Barberis *et al*., 2016). In 1937 the yellow fever vaccine was successfully developed (Frierson, 2010).

In the early 1960s, the rubella virus was isolated, this is how the vaccines were licensed

in 1969 and suitable for pregnant women in 1970 (Plotkin, 2006). In the second half of the 20th century, the chickenpox vaccine was developed (Plotkin *et al*., 2004).

Since the development of the vaccine against Hepatitis B virus in 1986, there has been an enormous advance in understanding the clinical development of the disease (Pan *et al*., 2005).

Another advance in vaccines was the development of the human papillomavirus vaccine, these vaccines are produced from only proteins, they do not contain any biologically active product or DNA, so they do not cause infection (Cutts *et al*., 2007), the development of this vaccine has allowed an active fight against cervical cancer (Tejeda *et al*., 2007).

According to what has been described, the creation of vaccines has had a great impact on human health and in this chapter, we describe how the production of vaccines has progressed and its impact on human health.

5.2 FIRST, SECOND AND THIRD GENERATION VACCINES

The human body has the ability to resist pathogens, through immunity, which can occur in two ways: active immunity can be achieved naturally or acquired. This immunity refers to when the body comes into contact with an infection and its immune system is able to respond to it and generates protection for a prolonged period of time, probably for life. While passive immunity generates protection for a short period, which can be from weeks to 4 months, this immunity is achieved naturally or acquired. (Baxter, 2007).

Vaccines are a useful tool for the acquisition of immunity and to protect human beings from the pathogens that are present in our world today, the objective of vaccines is to be able to create a permanent immunity that allows us to eliminate the disease from permanently (Leroux-Roels, 2010).

The vaccines that are used in humans are found in different forms, live-attenuated vaccines, inactivated vaccines, and vaccine subunits. The most successful vaccines on the market today are generally those that use the live-attenuated pathogen, these vaccines are composed of viruses or bacteria that can replicate, inciting an immune response (Lee and Nguyen, 2015), these vaccines are also called first generation.

Louis Pasteur later started second-generation vaccines that consist of pathogens that are dead and inactive (San Miguel-Hernández *et al*., 2013). It has also been possible to produce purified antigen (subunit) vaccines consisting of inactivated toxins and antigens. These vaccines were produced to eliminate concerns around attenuated virus-bacteria.

Recently, so-called recombinant vaccines have also been generated, which are created from the cloning of genes that encode antigenic proteins. These confer immunity similar to inactivated vaccines. Similarly, we find DNA vaccines that consist of bacterial plasmids that contain cloned DNA that contains the antigen gene. This generation of vaccines

has more advantages over traditional vaccination methods, because there is no possibility of infection. (Buckley *et al*., 2015).

The following table shows some of the examples of vaccines that have been created from 1800 to date.

Table 5.1. *Examples of Vaccines developed in the fifties to date, which helped eradicate or control diseases in the human population*

Vaccine	Year of development	Technology used	Generalities of the disease	Reference
Smallpox	1796, developed by Edward Jenner	Attenuated vaccine	Smallpox was considered a highly contagious disease, initial treatments were considered risky and highly controversial. It was until 1853 when its application became mandatory that it favored its eradication for the year 1980.	Stewart and Devlin, (2006)
Measles	Developed by Maurice Hilleman, using Enders 1963's Edmonston B strain. Moraten strain marketed in 1968	Attenuated vaccine	Measles is one of the infectious diseases of greatest concern in the world due to its significant morbidity and mortality. In 2010, the OMS declared America free of measles, although exported cases have been reported.	Tuells, (2010); Garcés-Sánchez *et al*. (2015); Delpiano *et al*. (2015).
Parotitis	Developed by Maurice Hilleman in 1967	Attenuated vaccine	Parotiris is a viral disease in which the parathyroid glands become inflamed.	Tuells (2010). Latasa *et al*. (2019).
Rubella	Developed by Maurice Hilleman in 1969	Attenuated vaccine	Rubella is a condition caused by a virus, it is common to occur during childhood, although it is usually considered an uncomplicated disease, transient arthropathy is common in adults. The way to avoid the disease is vaccination. Antibodies generated by the vaccine generally last up to 21 years	Tuells J. (2010); García-León *et al*. (2018).
MMR vaccine (measles, mumps and rubella)	Developed by Maurice Hilleman in 1971	Attenuated virus	Vaccine designed for Mumps, Measles and Rubella. Currently there is a growing fear with the application of this vaccine and its relationship with autism, the same assumption has been denied on scientific grounds, but nowadays the outbreaks of contagion that exist are due to the non-application of this vaccine	Tuells (2010); Moyer-Gusé *et al*. (2018)

Vaccine	Year of development	Technology used	Generalities of the disease	Reference
Chickenpox	Developed by Maurice Hilleman in 1995 with the Japanese strain OKA	Attenuated virus	Chickenpox is one of the most contagious diseases caused by the varicella-zoster virus, it is a virus belonging to the herpes group, it is also responsible for herpes zoster. Chickenpox is a disease that is common during childhood, it can cause skin rashes but can present complications such as pneumonia or encephalopathy.	Latasa (2018).
Rotavirus	In 1973 discovery of the virus by Ruth Bishop	Attenuated virus	Previously, gastrointestinal diseases (diarrhea) were an important cause of mortality in minors. In 1973, rotavirus was discovered, currently there are two safe vaccines: Rotarix (GSK) and RotaTeq (Merck).	Bishop (2009)
Polio	Discovered in 1954 by Jonas Salk	Inactivated vaccine	The worldwide vaccination policy of the polio vaccine has resulted in an extremely significant reduction in the number of cases; Since the year 2000, the number of endemic cases has been reduced, it is estimated that the eradication of poliomyelitis will be achieved in the next few years.	San Miguel-Hernández, (2013); Khan *et al.* (2017).
Rabies	Vaccine developed in 1885 by Louis pasteur	Inactivated vaccine	Rabies is encephalitis caused by the *Lyssavirus*. Generally, the clinical signs of the disease are confusing to make an effective diagnosis.	San Miguel-Hernández, (2013); Hemachudha *et al.* (2002).
Typhoid fever	Developed in 1887 by Almroth Edward Wright	Inactivated vaccine	The superficial transmission of the causative agents of typhoid fever generally occurs in populations with limited access to drinking water. The clinical manifestations vary depending on the carrier, but it is possible to speak generally of persistent fever, general malaise and usually headaches.	Tuells (2009); Simon (2018).
Anthrax	Developed on February 28, 1888 by Louis Pasteur	Toxoid vaccine	This pathology is caused by *Bacillus antrachis*, characterized by febrile reactions. The rate of mostality when it causes anthrax is less than 2%, while when it causes meningitis it increases to 92%. This pathogen mainly affects herbivorous animals and humans after it comes into contact with the infected animal.	Smith (2012); Bower *et al.* (2019).

Vaccine	Year of development	Technology used	Generalities of the disease	Reference
Diphtheria	Developed by Emil von Behring in 1981	Toxoid vaccine	Two types of clinical manifestations of diphtheria can be found, the cutaneous and nasopharynx, in this the clinical signs are hypoxia and respiratory obstructions, while the cutaneous one is characterized by skin lesions.	Winau and Winau, (2002).
Tetanus	Developed in 1890 by Emil von Behring	Toxoid vaccine	This pathology is produced by *Clostridium tetani*, its symptoms are characterized by spasms, respiration becomes compromised.	Kaufmann (2017); de Oliveira *et al.* (2020).
Hepatitis A		Synthetic vaccine	It is characterized by being an acute disease, whose route of transmission is enteric (food)	Buckley et al. (2015).
Hepatitis B	Developed in 1986 by Baruch Blumberg	Synthetic vaccine	This pathology can trigger liver cirrhosis and hepatocellular carcinoma. It is one of the leading causes of death in Africa.	San Miguel-Hernández *et al.* (2013); Seto *et al.* (2018).
Human Papillomavirus (HPV)	Developed by Cervaxia in 2009	Synthetic vaccine	Recombinant antigen produced in yeast cells. In 2008, it was established that there is a relationship between the presence of the human papillomavirus and cervical cancer.	Buckley *et al.* (2019).
COVID-19	Corminaty® (Pfizer/ BioNTech) was approved on December 21, 2020.	mRNA Vaccine	The pandemic caused by the SARS-CoV-2 virus originating from Wuhan, China; It has caused more than 100,000 thousand deaths throughout the world. This virus causes COVID-19, currently the disease is not fully known, but signs of infection include fatigue, muscle aches, sneezing, sore throat, dry cough, high fever, breathing problems, etc. with some severe cases with pneumonia, severe respiratory syndrome	Casas and Mena (2021); Serrano-Castro *et al.* (2020); Ali and Alharbi (2020); Urbiztondo *et al.* (2020).
	Moderna® was approved on January 6, 2021	mRNA vaccine		
	AstraZeneca® was approves on January 29, 2021.	Viral vector		
	CanSino Biologicals	Adenovirus type 5 vector expressing protein S		

5.3 VACCINE GENERATION METHODS

5.3.1 CLASSIC VIRAL AND BACTERIAL TECHNOLOGY

Vaccines with attenuated live infective material can cause the development of the disease because it can be incompletely attenuated, microorganisms can also replicate, so it can become reactogenic, an example of this type of vaccine is the vaccine developed for smallpox in which the virus obtained in situ was used, and in fact this vaccine achieved the eradication of the disease (Henderson *et al*., 1977; Clark *et al*., 1988).

Starting in 1940, the second stage of this type of vaccine began, currently there is a vaccine against tuberculosis that contains the *Mycobacterium bovis* strain that was attenuated by 231 subcultures over 13 years (Guérin, 1957).

In vaccines with dead infective material, the pathogen cannot multiply or cause disease; so these vaccines are commonly better tolerated by most patients. These vaccines generally tend to produce less immunity, more doses may also be required. These vaccines promote humoral and memory immunity (Murdin *et al*., 1996).

Vaccines that are based on passive immunization, are made up of antibodies that bind to pathogens or the cell that is being affected, so that it is destroyed. They tend to cause adverse effects, so they are usually only administered when it is an emergency treatment (Seehofer *et al*., 2005).

Currently, there are also modern vaccines that consist of the deliberate modification of the genetic information of the cell by means of genetic engineering. Two strategies have been used; The first strategy for the development of these vaccines is to cause the virus to lose its ability to generalize the disease, through specific mutations within its genome, and the second strategy is to use a virus as an expression vector for immunogens or epitopes.

Regarding vaccines that consist of protein subunits, an example of the use of this technology consists in the production of the toxin in which its structure is preserved but the toxicity is eliminated and finally the nucleic acid vaccines consist of purified nucleic acids that encode a protective immunogen, for example injecting DNA or mRNA encoding an antigen (Criado *et al*., 2008).

5.4 THE CREATION OF EARLY DETECTION METHODS AND VACCINES AGAINST COVID-19

The world population is being seriously affected by the coronavirus pandemic (COVID-19), the cause of high mortality and economic crisis in Mexico and in the world. In mid-2020, the Secretary of Health has reported that 203k positive cases of coronavirus have been registered in the country, and of these, 25,060 deaths have been reported. These data are increasing day by day, causing a health emergency that affects our quality of life and has a negative effect on the economy of the country and worldwide.

Coronavirus disease 2019 (COVID-19), which began in December 2019 (Wuhan, China) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Guarner, 2020). Its mode of infection is by direct and indirect contact with the infected person, through fluids that are expelled when talking, coughing and sneezing, or by touching a surface or object that has the virus and later, enters the host when manipulating the virus. mouth, nose or eyes. Coronaviruses are particularly interesting because they contain the largest genome of RNA viruses, encoding a large number of gene products and using a discontinuous RNA synthetic process to produce messenger RNA molecules that they use during their replication.

The fatality rate of the coronavirus varies depending on the incubation phase of the virus and the existence of asymptomatic carriers (Llaneras and Domínguez, 2020). It is estimated that there is a lag of approximately 10 days between the moment in which a person can infect and the positive diagnosis; This means that the number of cases reported on a given date shows the number of people who could infect 10 days earlier. This means that there are many carriers of the virus that will infect other people for 10 days, until they go to the doctor and are diagnosed (Santillán, 2020). The long incubation time seems key to the spread of the virus and its difficulty in identification and detection, therefore, if health institutions are responsible for carrying out clinical tests on a greater number of people with suspected contagion for diagnosis timely of this disease.

Mexico and the countries in the framework of underdevelopment, present external economic dependence (commercial, financial and technological). Therefore, these countries are experiencing a lack of scientific technology and competition in the health sector for the diagnosis of this viral disease (COVID-19). Faced with this pandemic, Eastern and European countries have reported that the detection of SARS-CoV-2 RNA is necessary for the early diagnosis of COVID-19, beneficial to control the sources of infection and help patients prevent the progression of the disease. disease. Therefore, rapid, accurate and inexpensive detection of the coronavirus is of the utmost importance.

With the advancement in molecular biology technology, nucleic acid detection methods have developed rapidly and have become a revolutionary technology for the detection of coronovirus, especially the quantitative method based on the polymerase chain reaction (qPCR) and its coupling to CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats / Regularly Interspaced and Clustered Short Palindromic Repeats) (Liu *et al*., 2020; Broughton *et al*., 2020),

Thus, the methods of early detection of the virus, minimizes the infections and deaths caused by COVID-19. Table 5.2 illustrates some early detection methods for COVID-19 that have been developed until the end of 2021.

Table 5.2. COVID-19 Early Detection Methods (Sheridan, 2020)

Author	Test Name	Description	Status
Guangzhou Wondfo Biotech (Guangzhou, China)	Antibody test: Wondfo SARS-CoV-2	15-minute immunoassay to detect IgM and IgG antibodies directed against SARS-CoV-2	National Medical Products Administration EUA in China; CE mark in Europe
Innovita Biological Technology	Antibody test: SARS-CoV-2	15-minute immunoassay to detect IgM and IgG antibodies directed against SARS-CoV-2	National Medical Products Administration EUA in China
Jiangsu Medomics Medical Technologies (Nanjing, China)	Combined antibody set (IgM/IgG) for rapid detection of SARS-CoV-2	15-minute immunoassay to detect IgM and IgG antibodies directed against SARS-CoV-2	In transit
Mammoth Biosciences	SARS-CoV-2 DETECTR	30-minute rehearsal	In validation studies
Pharmact (Berlin)	SARS-CoV-2 Rapid Test	20-minute test to identify IgG and IGM antibodies against SARS-CoV-2, at the point of patient care	Approved, in transit
Sona Nanotech (Halifax, Nova Scotia)	Rapid SARS-CoV-2 antigen detection test	Test detecting an S1 domain of SARS-CoV-2 protein S	Trial development and validation with GE Healthcare Life Sciences, underway
Sherlock Biosciences, Cepheid	CRISPR-based rapid test to detect SARS-CoV-2 and other pathogens	Combines SHERLOCK Cas12 and Cas13 enzymes for nucleic acid detection with Cepheid's GeneXpert processing instruments	Designed as a proof of concept for a broad product development alliance in infectious diseases
Zhejiang Orient Gene Biotech (Zhejiang, China)	Rapid IgG/IgM test for COVID-19	Solid phase immunoassay	Aytu Bioscience has sublicensed the U.S. distribution rights of L.B. Resources (Hong Kong) and plans to obtain the U.S.; already has CE mark
Biomerica	Rapid IgM/IgG antibody test	Immunoassay with $10.00 dollar cost	FDA approved and in Europe, in transit
Caspr Biotech	Portable, ultra-fast and fast detection of the SARS-CoV-2 coronavirus sequence	Based on CRISPR-Cas12	Proof of concept evaluation
Sugentech (Daejeon, Corea del Sur)	SGTi-flex COVID-19 IgM/ IgG	10-minute immunoassay detecting IgM and IgG antibodies targeting SARS-CoV-2	Marked as CE in Europe

Author	Test Name	Description	Status
Cepheid	Xpert Xpress SARS-CoV-2	Rapid PCR test running on the GenXpert benchtop system – yields results in two hours from sampling to delivery of results	Received emergency authorization from the FDA
Xiamen AmonMed Biotechnology (Fujian, China),	COVID-19 IgM/IgG Testing Set (Colloidal Gold)	10-minute immunoassay detecting IgM and IgG antibodies targeting SARS-CoV-2	CE marking for use in Europe

5.5 THE ROLE OF DNA METHYLATION IN REGULATING COVID-19 VIRUS INFECTIONS

Methylation is one of the most common protein modifications. In prokaryotes and eukaryotes, proteins involved in translation have been shown to be methylated, including ribosomal proteins (RP) and translation factors (TF). In *Escherichia coli* and *Saccharomyces cerevisiae* it has been determined that the RP, L3 and L12, are methylated (Polevoda and Sherman, 2007).

The enzymes that catalyze methylation reactions are protein methyltransferases (MTases), generally use S-adenosylmethionine as a methyl donor to add one to three methyl groups (Egloff *et al.*, 2007; Ringeard *et al.*, 2019). The biological significance of RP and TF methylation is poorly understood, however, it is important for the correct translation of proteins to take place.

Methylation modulates intra- or intermolecular interactions of target proteins or affects their affinity for RNA and thus influences various cellular processes, including transcriptional regulation, RNA processing, ribosome assembly, nuclear protein trafficking and metabolism, and cell signaling. So the differential methylation of specific RP and TF in a number of organisms in different physiological states indicates that this modification can play a regulatory and also determining role for the treatment, diagnosis of diseases or search for a cure in some disease (Li *et al.*, 2019).

Viruses replicate only within living cells. Viruses have limited genomes, so during viral infection, they must employ a variety of host cellular factors to survive and produce new viral particles. PRs play critical roles in the life cycle of viruses. In virus-infected cells, after infection by some viruses (e.g., herpes virus), cell mRNA translation is often selectively suppressed, while transcription of RP mRNAs increases and persists (Li *et al.*, 2019; Lee, 2013), this regulation of RPs' mRNAs, is the result of virus-host interaction, to preferably produce RPs to maintain viral spread. Due to the importance of RPs in viral spread, many studies have been directed to use RPs as potential targets for antiviral therapeutics, and it has even been proposed as a tool to block the replication of the SARS-COV 2 virus, as a prospective treatment (Rofeal and Abd El-Malek, 2020).

At least in the literature it has been reported that 14 RPs proteins are important for the reg-

ulation of mRNA and the translation of viral proteins when the virus is in contact with the host. RPL18, RPL10 (0) and RPL24 have been shown to play a major role in viral spread (Wang *et al.*, 2018; Hafren *et al.*, 2013). For example, infectious pouch disease (IBD) is an acute, highly contagious and immunosuppressive avian disease caused by ibD virus (IBDV) and within this disease IBDV VP3 which is a multifunctional protein has been shown to be key in virus assembly and pathogenesis. VP3 has been shown to interact with chicken ribosomal protein L18 (chRPL18) in host cell experiments. In addition, the absence of chRPL18 transcription by RNAi inhibited virus replication (IBDV) (Wang *et al.*, 2018).

Regarding RPL10 (0), it has been shown to be important for PVA (potato A virus) infection. RPL10 is important for protein translation and VPG has been shown to direct PVA RNA to a specific gene expression pathway that protects viral RNA from degradation and facilitates its translation (Hafren *et al.*, 2013). In addition, due to the importance of RPs, a study has recently been published where the study of RPL9 is proposed for use in therapeutic treatment (Rofeal and Abd El-Malek, 2020).

Viral protein biosynthesis involves the interaction of numerous RPs with viral mRNA, proteins that are necessary for the regulation of virus replication and infection within host cells. Most of these interactions are crucial for the activation and accumulation of viruses. However, only a small percentage of these proteins are specifically responsible for the protection of host cells by activating the immune pathway against the virus and unfortunately regarding the interaction RPs and the CONVID virus (SARS-CoV-2), there are no current reports indicating that RPs are paramount for the replication of the virus within the host cell. However, using information reported in other viruses, it has been shown that RPL10, RPL18 and RPL24, are good candidates to study and know if they can be used in early diagnosis of CONVID (SARS-CoV-2).

5.6 CONCLUSIONS AND REMARKS

The generation of vaccines since the 1950s has helped fight and eradicate many diseases that have resulted in deaths in the human population. Over the years the generation of vaccines has evolved, and with recombinant DNA strategies it has been possible to create nucleic acid vaccines. The pandemic that is still present in the world (COVID-19), showed us the importance of continuing to develop new recombinant DNA technologies that allow us to create successful vaccines against the variety of viruses that cause various diseases. Viruses will always be in constant evolution and if we want to be successful with vaccines to eradicate or control them, it is necessary that advancement in the knowledge of vaccine generation always be at the forefront.

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CHAPTER

SIX

The scope of algal biotechnology in the production of biofuels

CHAPTER 6

THE SCOPE OF ALGAL BIOTECHNOLOGY IN THE PRODUCTION OF BIOFUELS

Ruiz-Marín A.[1*], Canedo-Lopez Y.[1], Tamayo-Ordoñez Y.J.[2], and Tamayo-Ordoñez M.C[3].

[1]Facultad de Quimica, Universidad Autónoma del Carmen. Calle 56, No.4. Esq. Av. Concordia. Colonia: Benito Juárez, CP. 24180. Ciudad del Carmen, Campeche. México.

[2]Universidad Autónoma de Coahuila. Ing J. Cardenas Valdez S/N, República, 25280 Saltillo, Coah.

[3]Laboratorio de Ingeniería Genética. Departamento de Biotecnología. Facultad de Ciencias Químicas. Universidad Autónoma de Coahuila. Ing J. Cardenas Valdez S/N, República, 25280 Saltillo, Coah.

*Corresponding author: **aruiz@unacar.mx**

ABSTRACT

The production of biodiesel and biohydrogen by microalgae has shown potential as an alternative to renewable energy, however the large volumes of water used during cultivation leads to increased costs and cannot compete with fossil fuels, and an alternative is the use of waste and urban wastewater. Several strategies have been used to increase the accumulation of lipids, as well as techniques for the production of hydrogen including biophotolysis, photo/ dark fermentation and electrochemical processes, but few have evaluated the used wastewater. In this study, a review will be presented on the biotic and abiotic factors that control the production of biomass, as well as the manipulation of cultures to increase the accumulation of lipids for biodiesel production purposes and according to international quality standards. Likewise, the potential use of microalgae for the treatment of wastewater, production of algal biomass and energy (biodiesel and biohydrogen) were analyzed.

6.1 INTRODUCTION

The renewable energies have received attention in many countries worldwide. The search for sources of renewable energy is commonly linked to economic development and prosperity, quality of life, and global stability because of which it is essential to take into consideration the responsibility of all parts involved in decision-making and to establish short, medium, and long term strategies in that regards. For example, many countries and regions throughout the world have established objectives for reducing CO_2 emissions to accomplish more sustainable goals in agreement to the Kyoto Protocol. Many options are being studied and implemented and each is achieving different results and levels of satisfaction along the different stages of the research, like for example the use of sources of energy as solar, thermal, photovoltaic, hydroelectric, geothermal, eolic, and biofuel; each one with advantages and disadvantages, but the best choice of technology depending on the geographic area in which it is implemented.

At the global level, the main objective is to substitute fossil with renewable sources of energy because of which biofuels are contemplated as an opportunity to meet the challenge of reducing contaminants in a short time period (Mata *et al.*, 2010). The most common biofuels are biodiesel and ethanol, which use has been suggested to replace the diesel and gasoline consumed by combustion engines and requiring none or slight automotive or mechanical engineering modifications. These biofuels are mainly produced from biomass, using sources of renewable raw materials –like agroindustrial wastes– therefore contributing to reduce contaminant emissions in comparison with the use of fossil fuels.

Although currently biofuels are more expensive than fossil fuels, their production has increased in cities throughout the world. According to the European Commission, the global biofuel production has been estimated to be above 35 billion liters. Biodiesel is commonly obtained from vegetable oil or animal fat but in the case of plant oils that are also used for human consumption, it could result in the increase in price of food grade oils, and therefore, in a rise in the price of biodiesels making impossible their competition against fossil fuels.

One of the opportunity areas for obtention of biodiesels is the use of photosynthetic microorganisms. Microalgae –photoautotrophic microorganisms– are commercially exploited as sources of food given their high content in protein (39 – 71% dry weight), pigments, and other bioactive components as dietary fiber (in some species with a high value of dry weight biomass of 74.6%), carotenoids, carbohydrates, omega-3 fatty acids, and other lipids used by the food and pharmaceutical industries (Balasubramanian *et al.*, 2011).

Many applications have been given to microalgal cultures and the research made to effort to lower the costs of biomass production has focused in the use of industrial and urban wastewaters. Algal technology systems for wastewater treatment has had the goal to use and take advantage of the removal of the nitrogen and phosphorus present in them. This means that culturing algae in wastewaters does not generates additional pollution when the biomass is harvested, making the system more attractive than the biological treatment systems (ac-

tivated sludge) (Ruiz-Marín *et al*., 2010). One of the challenges involved in the production of biomass as a raw material for biofuel is to lower the costs and urban wastewaters offer an alternative to reduce the final production expenses by 30% compared with conventional methods (Sivers *et al*., 1994). However, the high variability in nitrogen and phosphorus contents of wastewater poses a technical problem that involves factors like the design, operation mode, and efficiency of production facilities.

Another benefit derived from algal technology is its contribution for lowering the atmospheric levels of CO_2, one of the main greenhouse gases. Despite a variety of strategies exist for CO_2 mitigation, its biological fixation has represented an innovation in algal biotechnology (Wang *et al*., 2010; Zeng *et al*., 2011) compared to the physicochemical, chemical conversion, and injection into deep ocean bottoms or other geological formations technologies.

The non-conventional sources of diesel have been used since the 30's and 40's decades; however, the continued growth of the worldwide demand for energy has led to the continued search for alternative sources of energy, in particular in cities and countries in which conventional fuels are scarce or costly, opening the opportunity for the biofuel industry to supply the current market's demand (Ryan *et al*., 2006). The environmental advantages of biodiesel over conventional diesel must be acknowledged, the former contributing to the decrease in carbon dioxide emission, reducing the emissions of Sulphur in 10% and of carbon monoxide in 30%, and lacking aromatic compounds known to pollute the environment (Hill *et al*., 2009; Cho *et al*., 2011).

Compared with terrestrial plant seeds, the production of biofuels from algal biomass has advantages because the algae being considered to be among the most efficient solar converting organisms and not competing for cropland use due to their technological production (Soto-León *et al*., 2014; Lim *et al*., 2010; De Godos *et al*., 2010).

The capability for accumulation of lipids has been reported from many autotrophic microalgae as *Chlorella vulgaris, Botryococcus braunii, Navicula pelliculosa, Scenedesmus acutus, Crypthecodinium cohnii, Dunaliella primolecta, Monallanthus salina, Neochloris oleoabundans, Phaeodactylum tricornutum,* and *Tetraselmis suecica* (Liang *et al*., 2009). These and other microalgal species have different oil production capacities depending on culture conditions such as temperature light intensity, light quality, pH, salinity, available minerals, nitrogen sources used, and culture age.

The lipid content in microalgae varies broadly, some species being able to synthesize medium-sized fatty acid chains (C_{10}, C_{12} and C_{14}), while others produce very long fatty acid molecules (>C_{20}). Nevertheless, most species of algae commonly synthesize fatty acids with chain sizes ranging from C_{16} to C_{18} containing one to three double bonds like palmitic acid ($C_{16:1}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), and linoleic acid ($C_{18:2}$). The capacity for oil production in algae is usually assessed by its contents in C_{16} and C_{18} molecules, which makes microalgae a promising raw material for biodiesel production (Hu *et al*., 2008; Converti *et al*., 2009; Tan and Lin 2011). But many challenges need to be overcome before the industrial scaling of algal oil becomes financially viable. The main concerns are maintaining a high oil produc-

tion and a sufficient supply of nutrients. The lipid content of microalgae can increase due to environmental stress factors –such as the limited availability of essential nutrients like nitrogen, phosphorus, and some metals– that may stimulate the cell carbon flow from protein synthesis from 20 to 40%, as has been observed in *Chlorella vulgaris* (Illman *et al*., 2000; Liu *et al*., 2008).

A problem to take into account is that the high content of lipids produced under limited nutrient availability is related to a low algal biomass productivity. Hence, one of the challenges is to produce enough biomass with an improved content of lipids and a proposal to achieve that goal is the use of two-stage culture methods (Ho *et al*., 2010; Li *et al*., 2008a; Li *et al*., 2008b; Converti *et al*., 2009; Widjaja *et al*., 2009; Tan and Lin, 2011; Mutjaba *et al*., 2012; Robles-Heredia *et al*., 2016).

The production of H_2 from cultivated microalgae is another currently explored source of renewable energy. Several mechanisms exist for producing H_2 from microalgae: a) Direct biophotolysis, b) indirect biophotolysis, and c) dark fermentation. The *direct biophotolysis* is a biological H_2 production process that uses the photosynthetic system to transform solar energy into chemical energy in the form of H_2 in the presence of the Fe-hydrogenase enzyme (Ni *et al*., 2006). During *direct biophotolysis*, there must be a low level of O_2; by maintaining the level of oxygen below 0.1%, the efficiency of the conversion of a photon of visible light to H_2 is about 22% (Hallenbeck and Benemann, 2002).

In *indirect biophotolysis* for H_2 production, the issue of the lack of sensitivity to O_2 of Fe-hydrogenase during the process is overcome by separating the evolution of O_2 and H_2 in reactions occurring at different stages, which are coupled through the fixation and evolution of CO_2. This process that is mainly used in cyanobacteria –although some microalgae are capable of producing H_2 by it– involves two stages: 1) Biomass production through photosynthesis with a 10% efficiency and the storage of carbohydrates (starch), 2) The concentration of the biomass, 3) Aerobic dark fermentation producing four moles of H_2 for each mole of glucose inside the cell and two moles of acetate, and 4) The conversion of two moles of acetate into eight moles of H_2.

The chemical reactions that take place are:

Finally, *dark fermentation* is performed by anaerobic bacteria (Lin and Jo, 2003), some microalgae like *Chlorococcum littorale*, and the green algae that use substrates rich in carbohydrates to produce H_2 in darkness (Ueno *et al*., 1998; Ni *et al*., 2006). The reaction that occurs is:

In this report, we analyze the potential of microalgae for the production of energy, the biotic and abiotic factors involved in each culture technology for improving both the production of H_2 and the quality of biodiesel, and we discuss the opportunities and challenges in algal technology for the production of the two latter forms of bioenergy.

6.2 STRATEGIES FOR PRODUCTION OF ALGAL BIOMASS

6.2.1 ALGAL CULTURE SYSTEMS (AUTOTROPHIC, MIXOTROPHIC, AND HETEROTROPHIC)

The microalgae are photosynthetic microorganisms that convert natural or artificial visible light to chemical energy through photosynthesis. The commonest procedure for culturing microalgae is the autotrophic growth. This is due to many microalgae being photosynthetic and particularly efficient in the transformation of solar to chemical energy, for that reason, they can be cultivated in environments with natural or artificial illumination. Under autotrophic culture, the cells harvest the luminous energy and use CO_2 as a carbon source. One of the initial technologies applied since the 1950's for the massive production of algal biomass was based on the use of large open outdoor ponds, the most common of which being the raceway ponds (Oswald, 1992). While these systems offer some advantages, they have inherent disadvantages as well, mainly for the control of the environmental parameters in the cultures; therefore, many photobioreactors (PBR) were designed having a diversity of configurations, whose main goal is to reduce the cost of biomass production (Molina *et al.*, 1999; Perez-Garcia *et al.*, 2011).

The use of differently shaped PBRs with various volume capacities ensure the optimal functioning of the process by the control of parameters like pH, temperature, and gas diffusion, with it achieving a higher biomass production. However, the high initial investment in infrastructure and operation of the culture remain to be the main inconveniences. One alternative for culture is to take advantage of the capacity of some species to grow in darkness. These species grow heterotrophically using organic compounds synthesized by other organisms (D´Este *et al.*, 2017).

Microalgae are capable of adapting to different environments potentializing the use of the resources present in the environment. During growth, microalgae essentially depend on sufficiently abundant sources of carbon and light for performing the photosynthesis. According to the environmental conditions, microalgae can adjust or transform their internal structure (their biochemistry and their physiological acclimatization) such that many of them may survive in extreme environments. For that reason, the microalgae can display autotrophic, heterotrophic, mixotrophic, or photoheterotrophic metabolisms and are capable of metabolic changes in response to modifications in the environmental conditions.

According to these growth characteristics, microalgae can be cultured either in raceway pond or in high-rate algal pond and photobioreactors. In both cases, the cultures are aerated or exposed to the open air, which allows microalgae to maintain cell growth by means of the capture of carbon dioxide from the atmosphere; however, the CO_2 concentration in the atmosphere is low (0.03 to 0.06%) and it is thought that the mass transfer in the culture media is limited, which affects the microalgae cell growth. In autotrophic cultures, the CO_2 acts as the only carbon source being incorporated in the formation of protein chains (Wijffels and Barbosa, 2010). Because of that, the CO_2 capturing capacity of photosynthetic organisms makes them an alternative for the global reduction of this greenhouse gas.

It is difficult to obtain a high concentration of algal biomass in autotrophic systems because the continued growth in the culture obstructs light penetration, the latter being an essential control factor in these cultures. Self-shading might result in insufficient light capture that leads to low nutrient consumption and, hence, to lower lipid productivity by algal biomass unit (Borowitzka, 1999).

The assimilation of light in heterotrophic cultures is null, sugars and organic acids acting as carbon sources instead of the CO_2 obtained from the atmosphere in autotrophic condition; therefore, in heterotrophic systems, the cell density and productivity can be significantly incremented using some species of microalgae. Some microalgae might grow both in autotrophic and in heterotrophic conditions and under different photoperiods –periods of light and darkness– the culture becoming mixotrophic. This means that, under heterotrophic conditions, the microalgae assimilate organic compounds as a carbon source, releasing CO_2 during respiration. These same released CO_2 is once more assimilated by the microalgae as a carbon source during autotrophic conditions. Noticeably, mixotrophic cultures are rarely used for lipid production from microalgae and this is a little explored area of algal technology (Chen *et al.*, 2011).

There are scarce information of photoautotrophic conditions in the literature and, commonly, it is difficult to distinguish from mixotrophic growth conditions (Mata *et al.*, 2010). In photoautotrophic culture, the microalgae requires light as a source of energy while using organic matter as a carbon source. In contrast, mixotrophic growth requires organic carbon and CO_2 as essential carbon sources and a source of light (Chojnacka and Márquez-Rocha, 2004).

Although the photoautotrophic conditions are commonly used in microalgal culture, some reports remark that the highest biomass content and lipid production in *Chlorella* sp. could be obtained in heterotrophic and mixotrophic conditions (Liang *et al.*, 2009; Chen *et al.*, 2011;), suggesting that the content of lipid and the biomass of some microalgae could largely be dependent on the culture system (Mata *et al.*, 2010; Yeh and Chang, 2012) and that it could be enhanced under the four culture conditions (Table 6.1).

Table 6.1. *Characteristics of the different culture conditions.*

Cultivation condition	Energy source	Carbon source
Phototrophic	Light	Inorganic
Heterotrophic	Organic carbon	Organic
Photoheterotrophic	Light	Organic
Mixotrophic	Light and organic carbon	Inorganic and organic

According to Yeh and Chang (2012), the microalgae *C. vulgaris* ESP-31 in photoheterotrophic culture can increase the biomass content up to 15 times compared to heterotrophic and mixotrophic conditions using basal and Bristol's modified media, in which luminous energy is required to efficiently assimilate organic carbon sources. This means that the metabolism of glucose varies under different light conditions, the pentose phosphate pathway (PPP) is dominant in darkness and it is replaced by the Embden-Meyerhof pathway (EMP) in the presence of light. Some species of microalgae are unable to metabolize glucose under anaerobic-dark conditions because of

low levels of the lactate dehydrogenase enzyme and some species cannot assimilate glucose even if the enzymes are available.

The photoheterotrophic organisms can grow only in the presence of glucose and light meaning that light is used as a source of energy that is transformed into chemical energy like ATP and NADPH molecules (Chojnacka and Marquez-Rocha, 2004; El-Sheekn *et al.*, 2012). *Chlorella vulgaris* and *Scenedesmus obliquus* have the ability to utilize organic substrates under both light and dark condition, in particular, *C. vulgaris* possesses an inducible active hexose transport system, and this is constitutive in *S. obliquus* (Combres *et al.*, 1994). Table 6.2 describes the maximal lipid content and productivity of some microalgae and indicates that the changes in growth and biochemical composition are strongly dependent on the culture conditions.

Studies have shown variations in the quantity and quality lipids in cells as a result of changes in the growth conditions (temperature and light intensity) or the concentration of nutrients (nitrogen, phosphorus, iron) (Liu *et al.*, 2008; Converti *et al.,* 2009). Because microalgae being autotrophic organisms, light and quality (wavelengths) play an essential role and has effects in their growth and chemical composition. Das *et al.* (2011) assessed the growth and lipid of *Nannochloropsis* sp. At different wavelengths in phototrophic and mixiotrophic culture and found that the highest production of fatty acid methyl ester (FAME) occurred under blue LED light, being of 55.13 mg $L^{-1}d^{-1}$ and 111.96 mg L^{-1} d^{-1} in the former and the latter conditions, respectively (Table 6.2).

Table 6.2. *Lipid productivity of microalgae grown on different media under different cultivation condition (phototrophic, heterotrophic, photoheterotrophic and mixotrophic cultivation).*

Culture medium / Species	Unit	Lipid productivity				
		A	B	C	D	Reference
Bold´s Basal Medium at 25 °C/*C. vulgaris*	mg L^{-1} d^{-1}	20.22 ± 0.6	-	-	-	Converti *et al.* (2009)
Bold´s basal medium at 25 °C/ *N. oculata*	mg L^{-1} d^{-1}	10.10 ± 2.09	-	-	-	Converti *et al.* (2009)
Glicerol/Nannochloropsis sp.	mg L^{-1} d^{-1}	55.13 ± 2.42	-	-	111.96 ± 3.77	Das *et al.* (2011)
Basal/ *C. vulgaris* ESP-31	mg L^{-1} d^{-1}	56.2± 2.9	3.5 ± 0.5	69.0 ± 2.3	67.4 ± 2.5	Yeh and Chang (2012)
Modified Bristol´s / *C. vulgaris* ESP-31	mg L^{-1} d^{-1}	40.2 ± 0.8	3.5 ± 0.1	115.4 ± 24.7	143.9 ± 0.8	Yeh and Chang (2012)
MBL / *C. vulgaris* ESP-31	mg L^{-1} d^{-1}	51.2 ± 5.5	7.7 ± 0.4	20.7 ± 3.9	104.9 ± 7.2	Yeh and Chang (2012)
Glucose/C. vulgaris	mg g^{-1} DW	-	20	-	15	El-Sheekh *et al.* (2012)

Culture medium / Species	Unit	Lipid productivity				
		A	B	C	D	Reference
Glucose/ S. obliquus	mg g^{-1} DW	-	11	-	10	El-Sheekh *et al.* (2012)
Vinasse wastewater anaerobic digested / *Chlorella vulgaris*	mg L^{-1} d^{-1}	-	-	-	12	Marques *et al.* (2013)
BG11 / *C. vulgaris* FACHB-31	mg L^{-1}	65.1	93.33	-	-	Zhang *et al.* (2014)
Artificial wastewater/ C. vulgaris	mg L^{-1} d^{-1}	37.34 ± 0.23	-	-	38.91 ± 0.15	Canedo-Lopez *et al.* (2016)
Urban wastewater/C. vulgaris	mg L^{-1} d^{-1}	40.37 ± 0.02	-	-	108.1 ± 0.023	Canedo-Lopez *et al.* (2016)
House wastewater / *Chlorella vulgaris*	mg L^{-1} d^{-1}	20.25	-	-	-	Lu *et al.* (2016)
Fermented molasses wastewater / *Scenedesmus sp.*	mg L^{-1} d^{-1}	-	64.8	-	-	Ren *et al.* (2018)
Food wastewater-Anaerobic digested wastewater / *Chlorella sorokiniana* / *Scenedesmus obliquus* / *Scenedesmus abundans*	mg L^{-1} d^{-1}	23.4 / 27.5 / 20.2	-	-	-	Gupta and Pawar (2018)

A: *Phototrophic culture;* **B**: *Heterotrophic culture;* **C**: *Photoheterotrophic culture;* **D**: *Mixotrophic culture*

El-Sheekh *et al*. (2012) suggest the mixotrophic culture offering advantages for algal protein production. For example, Hu and Gao (2003) reported a higher protein content in *Nannochloropsis* sp. In mixotrophic culture; however other studies suggest the opposite, as reported by Orús et al. (1991) for *C. vulgaris* UAM 101 in mixotrophic culture, in those conditions, the microalgae reduced the protein content and had high lipid concentration in glucose-containing media. This phenomenon was also observed under heterotrophic conditions by Ogbonna and Tanaka (2000), who reported the decrease in protein content of *Chlorella* in heterotrophic conditions. That indicates that the production of protein and lipids in algae and their growth depend on the concentration of the light and carbon sources. Liu *et al.* (2010) suggest that glucose is the best carbon source in mixotrophic and heterotrophic cultivation stimulating growth and lipid production in microalgae and many other microbe species.

Studies by Martinez *et al.* (1991) indicate that glucose as a carbon source promotes physiological changes in *Chlorella vulgaris* that strongly affect the carbon assimilation metabolic pathway, cell size, starch, and lipid storage, and contents of protein, chlorophyll, vitamins, and other molecules (Perez-Garcia *et al*., 2011).

Although the heterotrophic cultivation is inappropriate for many obligate autotrophic microalgae, some species grow efficiently in total darkness in conventional fermenters (Chen and Chen, 2006). The costs and rela-

tive simplicity of operation are the main advantages for the use of heterotrophic growth. Studies have demonstrated that, in heterotrophic culture and depending on the strain used, the growth rate, biomass production, generated ATP (mg of biomass generated for each mg of ATP), and lipid content were significantly higher than in autotrophic cultivation conditions (Perez-Garcia *et al.,* 2011). It is interesting that heterotrophic conditions favor the production of saturated fatty acids relative to autotrophic conditions, while in the latter conditions, a higher quantity of polyunsaturated fatty acids (C16:3 and C18:3) are produced, which in case of biofuel production are directly associated to the quality of the product (Day *et al*., 1991; Chen *et al*., 2007).

However, some limitations of heterotrophic cultures must be taken into consideration for the production of biomass like:

1. The limited number of microalgae capable of heterotrophic growth.
2. The costs are higher due to the addition of organic substrate.
3. The contamination by and competition with other microorganisms.
4. The inhibition of growth because of the excessive organic substrate.
5. The incapability of producing light-induced metabolites.

Recent studies show the rising interest of heterotrophic cultures for the production of an ample variety of microalgal metabolites both in experimental batch as at industrial scales (Yang *et al*., 2000; Li *et al*., 2007; Brennan and Owende, 2010). The obtention of biodiesel from microalgae is considered to be an attractive technology a feasible alternative because some species can significantly increase lipid production and also because it is now possible to cultivate microalgae in low-cost heterotrophic cultivation systems. It is a fact that the productivity of many microalgae exceeds the oil produced from seeds, the oil content of many species of microalgae grown heterotrophically is usually of 80% of the dry weight.

The microalgae *C. protothecoides* appears to have potential for heterotrophically produce biodiesel from organic carbon. The species is capable of producing about 50% of the total biomass dry weight. According to Perez-Garcia *et al*. (2011), the production of biodiesel using microalgae cultures in heterotrophic conditions is a rather new research area having scarce available robust information; however, the possibility of the use of inexpensive carbon sources gives this approach a high commercial potential for future research. The production and processing of biofuel can be economically feasible using the available technology and the process is environmentally sustainable because the cultivation is not seasonal and the product can be harvested daily.

Some examples of microalgae that can grow in photoautotrophic, heterotrophic, and mixotrophic conditions are *Arthrospira platensis*, *Chlorella vulgaris*, and *Haematococcus pluvialis* and other strains of microalgae like *Scenedesmus acutus* and *Selenastrum capricornutum* might grow photoautotrophically, heterotrophically, or autotrophically (Chojnacka and Marquez-Rocha, 2004; Rizwan *et al*., 2018). These changes in metabolism occurring in the microalgae as a result of the variation in environmental conditions offer a biotechnological opportunity for the generation of new products like biofuels.

6.2.2 BIOTIC AND ABIOTIC FACTORS IN MICROALGAE CULTIVATION

The microalgae are essential for aquatic ecosystems contributing almost 50% of the atmospheric oxygen, in that way supporting the flow of energy in ecosystems. Some species have low requirements and can live in extreme conditions like in caves, desert soils, hypersaline, acidic, or alkaline lakes, and at high temperatures. Despite their simplicity and ample distribution, microalgae only live where there are conditions favorable to colonizing and developing. Microalgae possess an efficient biological system capable of using solar light for producing organic compounds (Rizwan *et al*., 2018).

The photosynthetic organisms depend on a series of biotic and abiotic conditions that modulate the primary productivity that include:

a) Availability and concentration of nutrients
b) Availability, quality, orientation, and intensity of light
c) Temperature
d) Salinity
e) pH
f) Availability of oxygen and carbon dioxide.

The source and concentration of nitrogen in the culture medium are two of the most known critical factors affecting the biochemical composition of microalgae (Yeh and Chang, 2011). The manipulation of culture conditions and media composition has been used to accomplish an improved production of lipids in microalgae. Producing sufficient biomass with enhanced lipids contents can be done using a two-stage culture strategy (Ho *et al.,* 2010). In this strategy, the alga is first grown under nutrient-sufficient conditions to allow maximum cell density, and then deprived of certain nutrients to trigger lipids accumulation (Mujtaba *et al*., 2012).

However, algae being photosynthetic organisms, light plays an essential role in their cultivation having influence in their growth and chemical composition; also the quality (wavelength) of light has shown to be an important factor of the pigment content, growth, and biochemical composition. Seyfabadi *et al.* (2011) suggest that the contents of pigments and other cell components like fatty acids, carbohydrates, and proteins can be modified by the photoperiod and the wavelength. Romero-Romero and Sánchez-Saavedra (2016) observed that the biochemical composition can be improved and modified through the manipulation of the light spectrum, which brings advantages in aquaculture applications.

Studies suggest that some kinds of illumination can provide a specific wavelength but this not necessarily brings an improved algal biochemistry (Mohsenpour *et al*. (2012). Other studies have assessed the effect of the wavelength in phototrophic and mixotrophic cultures. For example, cultivation of the microalgae *Nannochloropsis* sp. At different wavelengths (red, green, blue, and white) showed that, in both types of culture conditions, the highest growth rates (0.64 d^{-1} and 0.66 d^{-1}, respectively) were observed when using blue light (Das *et al.,* 2011).

Chávez-Fuentes *et al*. (2018) reported for *Chlorella vulgaris* in two-stage cultures under different light qualities –white, blue, yellow, and violet at 140 µE m^{-2} s^{-1}– that the maximal biomass and cell density at 140 µE m^{-2}

s^{-1} were obtained with white light (0.69 g L^{-1} and 6.5 × 10^6 cells mL^{-1}, respectively) and with blue light (0.65 g L^{-1} and 8.0 × 10^6 cells mL^{-1}, respectively) compared to violet and yellow light. However, the maximal lipid content (% w/w) were present under violet light (83.87) and yellow light (70.92) at 140 µE m^{-2} s^{-1}.

Thus, the available research reports agree in that the intensity of the incident light has a direct impact on the biochemical composition of microalgae, including modification of the fatty acid profile (Amini-Khoeyi *et al.,* 2012), which indicates that the effect of light on lipid production is species-specific because it has been observed that an optimal light condition for growth is not favorable for lipid accumulation (Cheirsilp and Torpee, 2012).

6.2.3 MICROALGAL BIOMASS PRODUCTION (PHOTOBIOREACTORS AND RACEWAY PONDS)

The cultivation of microalgae can be made in open culture like in raceway ponds or "high-rate algal ponds, and in closed systems like photobioreactors (PBR). PBR are usually used for having a better control, these being considered as production units in which the biological conversion is total. This means that a PBR is a reactor in which microorganisms (bacteria or fungi) grow or perform a photobiological reaction.

Commercial monoculture of large quantities of microalgal biomass is usually carried out outdoors in closed continuous run tubular loop bioreactors with tubes that are typically less than 0.08 m in diameter. These photobioreactors occupy a large land surface and are expensive to build and operate (Sánchez-Mirón *et al*., 2002). An alternative to conventional tubular reactors is to use relatively large-diameter (> 0.1 m) vertical column reactors such as bubble columns and airlift bioreactors (Chisti, 1989). Vertical column reactors are compact, low cost, and easy to operate monoseptically. In addition, vertical column reactors are a realistic option for producing large quantities of microalgal biomass.

The raceway ponds or high-rate algal ponds were first developed in the 1950s for treating wastewater. Since the 1960s, outdoor open raceways have been used in commercial production of microalgae and cyanobacteria (Terry and Raymond 1985; Oswald 1988; Borowitzka and Borowitzka 1989; Becker 1994; Chisti 2012); such production does not use wastewater. Compared to closed systems, open culture systems are normally less expensive to build and operate, last longer, and have high production capacity. However, studies made by Richmond (2004) show that ponds use more energy for nutrient homogenization and the water level cannot be under 15 cm in depth (150 L m^{-2}) so that algae receive enough light to grow.

Ponds are, in general, more susceptible to climatic conditions and there is no control of water temperature, evaporation, and illumination. Also, these systems can produce large amount of biomass but occupy large extensions of land and are highly vulnerable to contamination by other microalgae or bacteria. Furthermore, because the atmosphere only contains 0.03 – 0.06% CO_2, mass transference is limited causing a decrease in algal growth (Mata *et al.* 2010).

Jebali *et al*. (2018) cultivated *Scenedesmus* sp. In semicontinuous mode in pilot-scale

outdoor raceways (7.2 m^2) using flue gas for CO_2 supply and centrate from the anaerobic digestion of urban wastewater as the sole nutrient source. Under optimal condition of 30% centrate, 0.3 d^{-1} dilution rate and a 15 cm culture depth, the maximal biomass productivity obtained was 22.9 g m^{-2} d^{-1}. Nitrogen and phosphorus removal rates of 3 g N m^{-2} d^{-1} and 0.6 g P m^{-2} d^{-1} (90.6%) were recorded, respectively, these being higher removal rates than those reported by Qi *et al.* (2018) from microalgae and bacteria co-culture using fermentation wastewater. This suggests that there is a double benefit by removing nutrients and using the CO_2 from flues, therefore contributing to mitigate the greenhouse gas effect.

In addition, the PBR are flexible systems that can be optimized according to the biological and physiological characteristics of the algae species allowing single-species cultivation. The benefits of a PBR are that there is a better control of the culture parameters (pH and temperature), favoring the transference of gases and nutrients (CO_2 and O_2), allowing to obtain high cell density, and minimizing the invasion by microorganisms. The main limitations include overheating, bio-fouling, oxygen accumulation, difficulty in scaling up, the high cost of building, operating and of algal biomass cultivation, and cell damage by shear stress and deterioration of material used for the photo-stage, and others as reported in the table 3 (Molina *et al.,* 2001; Chisti, 2016).

It has been observed that a higher cell density can be attained in microalgae cultured in photobioreactors that in other culture systems. Zhang *et al.* (1999) compared three culture systems for *Chlorella* sp., reporting a lower cell density of 2,880 x 10^4 cel mL^{-1} in aerated ponds compared to the use of conical containers (8,000 x 10^4 cel mL^{-1}) and photobioreactors (13,000 x 10^4 cel mL^{-1}).

It may be difficult to compare the yield achieved by PBR and open ponds because the assessment depends on a number of factors among which are the microalga species and productivity. Three parameters are commonly used to evaluate the productivity of algal units: Volumetric productivity (g L^{-1} d^{-1}), areal productivity (g m^{-2} d^{-1}), and illuminated surface productivity (ISP, g m^{-2} d^{-1}). In table 6.3, a comparison is made between PBR and ponds for the different culture conditions and growth parameters.

Large-scale production of microalgal biomass generally uses continuous culture during daylight periods. During this method of operation, fresh culture medium is fed at a constant rate and the same quantity of microalgal broth is continuously withdrawn. Feeding ceases during the night, but the mixing of broth must continue to prevent settling of the biomass. As much as 25% of the biomass produced during daylight can be lost during the night because of respiration. This loss depends on the light level under which the biomass was grown and on the growth temperature (Molina *et al.,* 1999; Chisti, 2007).

Because if that, the challenges in the study of reactors implies characterization of the fluid – dynamics and transference of matter, quantification of the stress phenomena to which cells are exposed, determination of light availability, average light intensity (I_{av}) inside reactors, operation variables (pH, temperature, oxygen), and the influence of the above-mentioned variables in the systems' productivity.

Table 6.3. *Comparative analysis between open and closed microalga culture systems.*

Culture Systems	PBRs	Ponds
Contamination control	Easy	Difficult
Contamination risk	Reduced	High
Sterility	Achievable	None
Process control	Easy	Difficult
Species control	Easy	Difficult
Mixing	Uniform	Very poor
Area/bliquu ratio	High (20-200 m^{-1})	Low (5 – 10 m^{-1})
Cell density	High	Low
Investment	High	Low
Operation costs	High	Low
Light utilization efficiency	High	Low

Culture Systems	PBRs	Ponds
Temperatura control	More uniform	Difficult
Productivity	3-5 times more productive	Low
Hydrodynamic stress	Low – high	Very low
Gas transfer control	High	Low
O_2 inhibition	High	PBRs > Ponds
Biomass concentration	3-5 times in PBRs	PBRs > Ponds
Scale – up	Difficult	Difficult

Mata *et al.* (2010)

The knowledge of all these factors allowed the optimal design and operation of photobioreactors and their scaling to industrial production levels, and is one of the most relevant research areas of biotechnology (Molina *et al.,* 1999; Ogbonna and Tanaka, 2000; Molina *et al.,* 2001).

6.3 ALGAL CULTURE AND WASTEWATER TREATMENT

Within wastewater treatment processes, the primary and secondary stages are involved, which are widely used with the purpose of removal of easily sedimentable particles (primary treatment) and the oxidation of the organic matter present in wastewater (secondary treatment). The final result of each stage is a seemingly clean effluent that, in general, is discharged to natural bodies of water (rivers, lakes, and the ocean). However, the secondary effluents still have high concentrations of inorganic nitrogen (N) and phosphorus (P) that might cause eutrophication and other long-term issues due to the persistence of heavy metals and organic compounds (De la Noüe *et al.,* 1992; Sala and Mujeriego, 2000; Vaillant *et al*., 2002).

Many proposals have been focused in solving the eutrophication issues having economic and technical inconveniences. The conventional treatments can result in an insufficient level of removal of the nitrogen and phosphorus to prevent eutrophication in the receptor

bodies of water. Also, the implementation of the physical and chemical processes is generally costly and their use leads to secondary contamination (Pittman *et al.*, 2011).

The tertiary treatment –sometimes also called advanced treatment– is defined as a series of processes aimed at achieving an effluent quality above that of the conventional secondary treatment (Ramalho, 1991) with the purpose of removing suspended solids remaining in wastewater. These remnants can be of organic matter or other solids and their nature might vary from being relatively simple inorganic ions like calcium, potassium, sulfate, nitrate, and phosphate, to a growing number of very complex synthetic organic compounds (Metcalf and Eddy, 1995).

The stabilization ponds are among the treatment systems capable of attaining tertiary treatment. These systems reach a high oxidation rate for domestic wastewater treatment, taking advantage of the metabolic activity of bacteria and the photosynthetic activity of the microplankton (microalgae) that remove important quantities of nutrients from effluents (Maynard *et al.,* 1999).

Microalgae-based processes offer a promising option for an eco-friendly wastewater bioremediation process; however, this need to be accompanied of the production of a profitable by-product to offset the operation costs (Park *et al.,* 2011). For that reason, numerous studies have assessed microorganism growth and nutrient removal from wastewater taking advantage of the tolerance of some microalgae to grow in such extreme environments (Jebali *et al.*, 2018; Qi *et al.*, 2018; Zhu *et al.*, 2017; Ruiz-Marin *et al.,* 2010). This represents an option of tertiary treatment of wastewater due to the capacity of microalgae for using inorganic nitrogen and phosphorus to grow and to remove the heavy metals like Cu, Ni, Pb, Fe, Zn, and CH_3-Hg, which can be found in enriched or polluted marine systems and in wastewater (Oswald, 1992; De la Noüe *et al.,* 1992; Pascucci and Kowalak, 1999; Pickhardt *et al.,* 2002), and because of their efficiency for reducing the biological oxygen demand (BDO) (Caldwell, 1946).

The combination of tertiary treatment of wastewaters and the culture of microalgae may counterbalance the operational costs related, first by reducing the cost of wastewater treatment due to low energy consumption 0.5 KWh m^3 and, second, by producing microalgal biomass that could be used as a soil conditioner or to produce high-value chemical products as biodiesel. Yang *et al.* (2011) estimate that the use of wastewater to cultivate microalgae could save over 90% of freshwater and would provide with 94% needed nitrogen and 100% of the micronutrients.

The production of microalgae linked to wastewater treatment and the resulting mitigation of CO_2 emissions has not yet been commercialized; however, the alga biomass could be used as raw material to produce biofertilizers, biohydrogen, biodiesel, bio-oil, bio-materials, and other biochemical derivatives (Rinna *et al.,* 2017; Park *et al.*, 2013).

6.3.1 SUSPENDED CELL CULTURE

The massive cultivation of microalgae and the removal of nutrients have been studied for over 50 years (Lavoie and De la Noüe, 1985) showing a high potential to be profitable in cost and effectivity terms, both due

to the simple technology it involves and to the recycling of nutrients; i.e., conserving them as a valuable material with a high protein content (Nuñez *et al.,* 2001).

Even that there is little acceptance of the use of wastewater for cultivating microalga due to public health concerns (Becker, 1994), the microalgae culture systems have shown a versatility that allows them to be used in different processes as wastewater treatment and the production of animal food, fertilizer, and chemical compounds (De la Noüe and De Pauw, 1988). Studies by Sandbank and Hepher (1980) have demonstrated the feasibility of using wastewater for cultivating microalgae and the use of the biomass as fish food. The animal wastes can also be treated by algal systems for the production of high protein content biomass. Therefore, the efforts made to cultivate microalgae in wastewater seek a double benefit, biomass production and treated effluents (Shelef *et al.,* 1980).

Recent studies confirm the potential of algal treatment systems, particularly in the removal of nutrients. There is ample information regarding the cultivation of freshwater algae for treatment of domestic wastewater, for which the microalgae genera more commonly used are *Chlorella* and *Scenedesmus*, although there are also reports of the use of cyanobacteria in the genus *Phormidium* (Pouliot *et al.,* 1989) and of the marine algae *Phaeodactylum tricornutum, Dunaliella tertiolecta*, and *Thalassiosira pseudonana* (Goldman *et al.,* 1974; Goldman, 1976; Craggs *et al.*, 1995). Table 6.4 includes some studies that have shown the efficient removal of nitrogen and phosphorus using cultures in wastewater.

Table 6.4. *Efficiency of nutrient removal in cultures of* ***Chlorella vulgaris*** *and* ***Scenedesmus bliquus*** *in wastewater.*

Algae	Culture	Removal	Medium	Reference
Chlorella vulgaris	Batch	$N\text{-}NH_4$: 47.5%-92.5%	Wastewater	Lau *et al.* (1995)
Chlorella vulgaris	Batch	$N\text{-}NH_3$: 95% $P\text{-}PO_4$: 57%	Artificial Wastewater	Gonzalez *et al.* (1997)
Chlorella + obliquus + macrophyte	Batch	N: 72% P: 28%	Diluted industrial wastewater	Valderrama *et al.* (2002)
Chlorella vulgaris	Batch Immobilized (carrageenan) and free cells	$N\text{-}NO_3$: 100% $P\text{-}PO_4$: 100%	Bristol Medium	Lau *et al.* (1998)
Scenedesmus obliquus	Batch and semi continuous	$N\text{-}NH_4$:100% $P\text{-}PO_4$: 75%	Wastewater-secondary effluent	Lavoie and De la Noue (1985)
Scenedesmus obliquus (SC0.I)	Semi-continuous	NH_4: 70% Protein: 25 y 33%	Artificial wastewater	Nuñez *et al.* (2001)
Scenedesmus obliquus	Batch	N: 99 % P: 98% Protein: 11.8 %	Sterilized wastewater	Martinez *et al.* (2000)

Algae	Culture	Removal	Medium	Reference
Scenedesmus obliquus (SCx2)	Semi-continuous	NH_3: 8 a 13 % NO_3: 69 a 79 % PO_4: 29 a 43 %	Artificial wastewater	Voltolina *et al.* (2004)
Scenedesmus obliquus and *Chlorella sp.*	Batch	NH_3: 100% PO_4: 100 %	Digested swine manure	De la Noue and Basseres (1989)
Scenedesmus obliquus and *Chlorella sp.*	Semi-continuous	NH_4: 60-100% PO_4: 50-85 %	Wastewater-secondary effluent	Ruiz-Marin *et al.* (2010)
Chlorella species	Batch	COD : 63 % TN : 90 % TP : 62%	Food processing industrial wastewater	Gupta *et al.* (2016)
Chlorella vulgaris	Batch	COD : 77.05 % TN : 60.5 % TP : 20.23 %	House urban	Lu *et al.* (2016)
Chlorella vulgaris	Bacth	COD : 80.62 % TN : 85.47 % TP : 65.96 %	Anaerobic digested wastewater	Choi (2016)
Chlorella sorokiniana *Scenedesmus obliquus* *Scenedesmus abundans*	Batch	COD : 84.3 % TN : 84.7 % TP : 54.7 % COD : 88.5 % TN : 86.9 % TP : 69.3 % COD : 86.1 % TN : 85.3 % TP : 70.1 %		Gupta and Pawar (2018)

In comparison to the activated sludge treatment, the main advantage of the algal treatment systems is that the biomass generated could be recycled. However, one of the greatest practical limitations of this system is the harvesting or separation of the biomass from the treated effluent. An efficient separation of the algal biomass is essential for the final discharge of water treatment residuals and their reutilization. Numerous efforts have been made to develop a profitable technology for biomass recovery that goes from simple filtration through sand, centrifugation, self -flocculation (addition of cells) and, immobilized cell systems (Lau *et al.,* 1998; Mallick, 2002).

Of the different separation methods, the immobilized cell systems are the most studied and used with the purpose of removing nitrogen and phosphorus. The immobilized cell systems have not only avoided cell washing in bioreactors, but also offered a high degree of operational flexibility and ease of separation (Mallick, 2002). Bioreactors can be used at dilution rates exceeding the maximal specific growth rate (μ_{max}) thus, the cell washing is avoided.

Although the main objective of wastewater treatment systems is to obtain water depurated to the desired quality standards to be discharged into bodies of water or to be reused, it is also important to obtain biomass with optimal characteristics for its use and its assessment. The biochemical composition of microalgae is essential to determine its possible use as food, soil conditioner, extraction of chemicals, or as biofuel because of which numerous factors need to be considered including nutrient concentration, growth media composition, temperature, light intensity, and photoperiod, to mention a few. These factors can be changed to manipulate the growing conditions. Only a few studies have used the manipulation of light intensity to obtain different growth rates and nutrient content of algae (Correa-Reyes *et al.,* 2001).

Records have been obtained in *Scenedesmus* of 50%-56% protein, 12-14% lipids, and 10-17% carbohydrates, showing the potential as a protein source of this microalgae (Soeder and Hegewald, 1988). Likewise, Oh-Hama and Miyachi (1988) reported content of 51-58%, protein 14-22% lipids, and 12-17% carbohydrates in *Chlorella vulgaris*, and shown that when this microalgae grows in media with nitrogen limitations it accumulates larger quantities of lipids (52.8%).

Martínez *et al.* (2000) observed that protein synthesis is directly related to the N content in the culture medium. The average protein content in *Scenedesmus obliquus* cultured in wastewater (22.6 mg N l^{-1}) was 11.8%, while its carbohydrate content was 10–17%. With the use of protein, it is also possible to obtain a higher quality of water by reaching 90%- 99% removal of nitrogen and 79%-80% removal of phosphorus at a temperature of 35ºC. Voltolina *et al*. (2004) used a mixotrophic culture of *Scenedesmus* sp. in semicontinuous culture with a light:darkness period of 14:10 h, reported a content of 62.80 mg l^{-1} of protein and of 127.76 mg l^{-1} of biomass, with the 12% of the NH_3, 79% of the NO_3, and 43% of the PO_4 removed.

Núñez *et al.* (2001) reported direct measurements of nitrogen use through increased protein in *Scenedesmus obliquus* in semicontinuous culture and recorded 56.76 - 75.36% consumption of nitrogen with a protein content of 34.06% and 34.60% of the dry weight biomass, which suggested that only between 25% and 33% of the total nitrogen was recycled as protein and the gaseous ammonia was two to three times larger than the quantity of nitrogen recovered as protein. Similar results was reported by Ruiz-Marin and Mendoza-Espinosa (2008) where the amount of stripped nitrogen by effect of high pH values (9-9.5) was between 35% - 59% of the total nitrogen, suggested that part of the available nitrogen can be incorporated into new protein; this is not due only to ammonia uptake, since one part of this is lost to the atmosphere, showing that an important role of microalgae in wastewater treatment is that of facilitating NH_3 stripping due to the photosynthesis-induced pH increases.

6.3.2 IMMOBILIZED CELL CULTURE

An insoluble immobilization matrix can protect cells and improve the photosynthetic activity, which may lead to an increased metabolic activity that also increased nutrient removal. Cell immobilization may stabilize the metabolic activity for long periods and there is the possibility of efficiently incorporating symbiotic bacteria (Travieso *et al*., 1996; De Bashan *et al.,* 2002).

One of the main problems of immobilized systems is the high cost of the polymeric matrices used for immobilizing cells, like alginate, agarose, carrageenan, and agar, making less feasible their use in large scale applications. Other polymers used like polyacrylamides have shown toxicity to cells. Quitosan promises to be a viable immobilization medium and has been use satisfactorily for immobilizing species of the cyanobacteria genus *Phormidium* (Mallick, 2002).

The success of wastewater treatment with immobilized microalgae depends on factors as the species, the immobilization matrix, the cell concentration, the morphology and size of spheres, aeration, and retention time, among many others (Tam and Wong, 2000). Alginate is the most popular microalgae immobilization matrix for wastewater treatment, probably mostly due to the simplicity of its preparation. The studies made have shown that the use of calcium alginate allows a uniform distribution of cells in spheres and long term viability (Tam *et al*., 1994; Lau *et al.,* 1997). However, because of the ionotropic of alginate its stability is largely dependent on the ionic conditions of the environment. The integrity of the alginate is highly vulnerable to chelating agents like phosphate and citrate that cause the dissolution of the matrix and the suspension of cells (Robinson *et al.,* 1985), something that must be taken into account because of the high concentration of phosphate in wastewater.

While many studies have been made with the goal of assessing the nutrient removal capacity both in suspended and in immobilized cell cultures, few works have used as a growing medium real wastewater from treatment plants in which the competition for nutrients and the lowering of the N/P ratio by bacteria could affect the growth of the microalgae (Lau *et al.,* 1995). However, species like *Chlorella vulgaris* and *Scenedesmus obliquus* have shown an extraordinary vitality in urban wastewaters displaying growth rates similar to those reported for synthetic media (Martínez *et al.,* 2000; Ruiz-Marin *et al.,* 2010). Table 6.5 describes some of the most relevant studies to assess the nutrient removal in different immobilization matrices.

Gonzalez and Bashan (2000) evaluated the growth of *Chlorella vulgaris* co-immobilized in alginate with *Azospirillum brasilense* –a plant growth promoting bacteria– in artificial wastewater culture, obtaining a significant growth of *C. vulgaris* from the first to the sixth days demonstrating that the co-immobilization in the same matrix of these two microorganisms can result in an increased production of pigment and biomass similar to that reported by De Bashan *et al.* (2002). However, some associations are not favorable, as reported by González *et al.* (2000) in cultures of *Chlorella vulgaris* co-immobilized with the nitrogen-fixing bacteria *Phyllobacterium myrsinacearum*.

Besides the immobilized systems, recent studies have used co-immobilized systems with the purpose of increasing the production of biomass and the removal of nutrients from residual treatment water. The co-immobilization consists in including two species of microorganisms within the same matrix so that the association can be positive for some of the microorganism; in this case, for the microalgae.

Table 6.5. *Matrices used to immobilize microalgae for wastewater treatment.*

Algae	Culture	matrix	Reference
S. obliquus	Batch	carrageenan	Chevalier and de la Noüe (1985)
C. vulgaris and C. kessleri	PBR	alginate	Travieso *et al.*, (1992)
C. vulgaris	PBR	alginate	Tam *et al.*, (1994)
C. Vulgaris	PBR	carrageenan, alginate, Polyurethane and polystyrene	Travieso *et al.*, (1996)
C. vulgaris	Batch	carrageenan and alginate	Lau *et al.*, (1997)
Chlorella vulgaris	Batch	alginate	Tam and Wong, (2000)
Chlorella vulgaris	Batch Semi continuous Continuous	alginate	De Bashan *et al.*, (2002)
Chlorella vulgaris + *Azospirillum brasilense* (Coimmovilized)	Batch Semi continuous Continuous	alginate	De Bashan *et al.*, 2002
Scenedesmus obliquus and *Chlorella sp.*	Semi continuous	alginate	Ruiz-Marin *et al.* (2010)

6.4 MICROALGAE AND BIOFUEL PRODUCTION

Algal biomass has been considered as one of the raw materials requiring treatment different from that given to commonly used biomass for bio-refineries because they grow in liquid media and several steps are required to obtain biomass useful for biochemical extraction. The more important stages during the processing of algal biomass include cultivation, harvesting, and extraction.

A bio-refinery has been defined as a facility for the production of biofuels as by-products with high value obtained from algal biomass that implies low environmental impact bioprocesses and chemical technologies. Microalgae can also produce a variety of biochemical components that have been used in the research of food and drug production technologies.

The microalgal biomass contains lipids, starch, cellulose, and proteins because of which it has been considered as a raw material for producing several kinds of renewable fuels like biodiesel, bioethanol, biohydrogen, and methane (Chisti, 2008). The content of lipids in a diversity of microalgae have attracted the attention as a future raw material for the synthesis of biodiesel due to its potential of the high productivity of than

it is possible to obtain from the lipid sources present in seeds (Chisti, 2007; Griffiths and Harrison, 2009). Several strategies have been applied to improve the growth of microalgae and their lipid content. These strategies include the optimization of the culture medium (carbon source, vitamins, minerals, etc.), the physicochemical variables (pH, temperature, and light intensity), and the type of metabolism (phototrophic, heterotrophic, mixotrophic, and photoheterotrophic) (Mata *et al.,* 2010). Research made has proven that these strategies contribute to improving the lipid content but the method of culture using different sources of energy (light) and carbon (inorganic or organic) is always acknowledged to be a key factor having strong influence on the growth and lipid accumulation of microalgae (Mata *et al.,* 2010; Chen *et al.,* 2011).

6.4.1 BIODIESEL PRODUCTION

The biodiesel is a clean-burning fuel currently being produced from grease, vegetable oils, or animal fats. Its chemical structure is that of fatty acid alkyl esters. Biodiesel is produced by transesterification of oil with short-chain alcohols or by the esterification of fatty acids. The transesterification reaction consists of transforming triglycerides into fatty acid alkyl ester, in the presence of an alcohol, such as methanol or ethanol, and a catalyst, such as an alkali or acid, with glycerol as a byproduct.

Replacing the fossil fuel by biodiesel continues to be one of the most relevant global challenges. It has become clear that the oil from seeds cannot significantly contribute to replace the fossil fuels in a near future. This scenario may change successfully if the microalgae are used to produce biodiesel because it has been estimated that their productivity by hectare of biofuel is larger than that obtained from other sources (Table 6.6).

Table 6.6. *Comparison of some sources of biodiesel (Chisti, 2007).*

Crop	Oil yield ($L\ ha^{-1}$)	Land area needed (M ha)[a]
Corn	172	846
Soybean	446	326
Canola	1190	122
Jatropha	1892	77
Coconut	2689	54
Oil palm	5950	24
Microalgae[b]	136 900	1.1
Microalgae[c]	58 700	2.5

[a] For meeting 50% of all transport fuel needs of the United States; [b] 70% oil (by wt) in biomass; [c] 30% oil (by wt) in biomass

Researches made agree in that the microalgae offer an alternative as a source of biodiesel with high potential for replacing fossil diesel. Unless other sources of oil extracted from seeds, the microalgae grow extremely quickly and some of them accumulate high oil contents. The microalgae commonly duplicate their biomass in the first 24 hours, with a short time of cell replication during the 3.5 h exponential phase (Chisti, 2007).

Depending on the species, microalgae produce different types of lipids, hydrocarbons, and other complex oils. Because of that, not all of the produced oil is satisfactory for biodiesel production and it is necessary to be able to evaluate the quality of the biodiesel according to standards. In the United States, that standard is the ASTM D6751 biodiesel standard and in the European Union the EN 14214 stan-

dard is applied for biodiesel used for vehicles. The attractive of using algal biomass is that the production of oil by microalgae does not compromise the production of food and other products derived from seeds (Table 6.7).

Table 6.7. *Oil content of some microalgae*

Microalga	Oil content (w/w % dry wt)
Botryococcus braunii	25 -75
Chlorella sp.	28 – 32
Crypthecodinium cohnii	20
Cylindrotheca sp.	16 – 37
Dunaliella primolecta	23
Isochrysis sp.	25 – 33
Monallanthus salina	> 20
Nannochloris sp.	20 – 35
Nannochloropsis sp.	31 – 68
Neochloris oleoabundans	35 – 54
Nitzschia sp.	45 – 47
Phaeodactylum tricornutum	20 – 30
Schizochytrium sp.	50 – 77
Tetraselmis suecica	15 – 23

Chisti (2007).

Many species of microalgae can be induced to accumulate a substantial amount of lipids (Sheehan *et al.,* 1998), under certain culture conditions, the lipid content varying between 1% and 70% (w/w biomass dry weight) (Chisti 2007; Li *et al*., 2008a, Canedo- López *et al.,* 2016).

Some differences can be observed in the lipid content and productivity between the studied species (Table 6.8). For example, the lipid content of *Botryococcus braunii* can reach 75 % (w/w_{dw}) although it can have a low productivity depending on the culture conditions. Algunas microalgas como *Chlorella, Dunaliella, Isochrysis, Nannochloris, Nannochloropsis, Neochloris, Nitzschia, Phaeodactylum* and *Porphyridium sp.,* poseen niveles de aceite entre 20% y 50%, con una interesante productividad; en particular *Chlorella* ha mostrado ser una microalga calificada como un buen candidato para la producción de biodiesel (Amaro *et al.,* 2011).

Table 6.8. *Lipid content and productivity of various freshwater microalga species (Mata* ***et al.,*** *2010)*

Microalgae species	Lipid content (% W/W_{DW})	Lipid productivity (mg L^{-1} d^{-1})
Botryococcus sp.	25.0-75	-
Chaetoceros muelleri	33.6	21.8
Chaetoceros calcitrans	14.6-16.4/39.8	17.6
Chlorella emersonii	25.0-63.0	10.3-50.0
Chlorella protothecoides	14.6-57.8	1214
Chlorella sorokiniana	19.0-22.0	44.7
Chlorella vulgaris	5.0-58.0	11.2-40.0
Chlorella sp.	10.0-48.0	42.1
Chlorella pyrenoidosa	2.0	-
Chlorella sp.	18.0-57.0	18.7
Chlorococcum sp.	19.3	53.7
Ellipsoidion sp.	27.4	47.3
Haematococcus pluvialis	25.0	-
Scenedesmus obliquus	11.0-55.0	-
Scenedesmus quadricauda	1.9-18.4	35.1
Scenedesmus sp.	19.6-21.1	40.8-53.9

Besides considering a high productivity, the selection of the most adequate species for biodiesel production must take into account other factors like the capability for nutrient removal or for growing under specific environmental conditions. Also relevant is the fatty acid profile because these compounds

condition the quality of the biodiesel and, therefore, the proper function of the combustion engines.

According to Amaro *et al.* (2011), the selection of an adequate wild microalgae must be made through a multicriteria analysis including a balance of the following items: growing rate, lipid quantity and quality, weak response to environmental disturbances (temperature, nutrient input and light, competition with other microalga and bacterial species), possibility of obtaining high added-value chemicals, uptake rate, and ease of biomass harvesting.

A commonly used alternative to increase the lipids content is to subject the cultures to nitrogen-limiting conditions; however, the high lipids contents produced under nutrient limitation conditions are usually associated with low algal biomass productivity. Thus, a replenishing nitrogen source is generally necessary to maintain a high cell growth rate and achieve high cell density. Producing sufficient biomass with enhanced lipids contents can be done using a two-stage culture strategy (Ho *et al.,* 2010). In this strategy, an alga is first grown under nutrient-sufficient conditions to allow maximum cell density, and then deprived of certain nutrients to trigger lipids accumulation (Mujtaba *et al.,* 2012).

The most important variable to maximize in the biodiesel production from microalgae cultures is the lipid productivity P_L (grams of lipids per liter of culture per day) (Rodolfi *et al.*, 2009; Khozin-Goldberg *et al.,* 2006), calculated by:

$$P_L = \frac{d(wX)}{dt}$$

Also, the biomass production rate can be expressed in terms of the growth rate μ so that $dX/dt = \mu X$, and during the logarithmic phase, the specific growth rate remains constant, so that

$$\mu = \frac{\ln(X_2) - \ln(X_1)}{t_2 - t_1}$$

where t_1 and t_2 are cultivation times during the logarithmic phase. In batch cultures the overall lipid productivity can be approximated by:

$$P_L = \frac{\Delta(wX)}{\Delta t}$$

where $\Delta(wX)$ represents the accumulated lipids from inoculation to harvest, which occurs in the time Δt.

In terms of lipid productivities and fatty-acid profiles, *Chlorella vulgaris* and *Scenedesmus obliquus* have been identified as two species with potential for biodiesel production (Niels *et al*., 2012). Several works have reported lipid productivity of these two species. From the values shown in Table 6.9, it is noteworthy that the higher productivities were achieved by limiting the nitrogen source, at 180 mg $L^{-1} d^{-1}$ and 140 mg $L^{-1} d^{-1}$ for *C. vulgaris* and *S. obliquus*, respectively (Gouveia and Oliveira, 2009; Ho *et al.,* 2012). Studies made by Tan and Lin (2011) observed that in cultures with limited nitrogen (3 mM) and phosphorus (20 μM), it is possible to increase the lipids productivity of *Scenedesmus rubescens* (426.9 ± 134.7 mg L^{-1}) at the end of the cultivation period.

Table 6.9. *Reported lipid productivities for* ***C. vulgaris*** *and* ***S. obliquus*** *in photoautotrophic cultures*

Species	P_L (mg $L^{-1} d^{-1}$)	Culture conditions and reference
C. vulgaris	180.0	Nitrogen limitation (Gouveia and Oliveira, 2009)
	147.0	Semi-continuous cultivation in bubble column photobioreactors (Feng *et al.*, 2011)
	77.1	Nitrogen limitation (Mujtaba *et al.,* 2012)
	67.0	Nitrogen limitation (Griffiths et al., 2011)
	54.0	Nitrogen starvation of *Chlorella* sp. BUM11008 (Praveenkumar *et al.,* 2012).
	40.0	Optimized productivity limiting nitrogen source, adding CO_2 and varying the light intensity (Lv *et al.,* 2010)
	16.9	Nitrogen starvation (Widjaja *et al.,* 2009)
	20.4	Reduced nitrate culture (Converti *et al.*, 2009)
S. obliquus	140.4	Five-day nitrogen starvation in *S. obliquus* CNW-N (Ho *et al.*, 2012)
	106.0	Nitrogen limitation (Griffiths *et al.*, 2011)
	90.0	Nitrogen limitation (Gouveia and Oliveira, 2009)
	78.73	Nine-day nitrogen starvation in *S. obliquus* CNW-N (Ho *et al.,* 2010)
	426.9	Nitrogen and phosphorus limitation (Tan and Lin, 2011)

Because of the latter, when considering microalgae culture within the process of biodiesel production, it is important to quantify and assess the influence of each physicochemical parameters and nutrients –as well as the interactions between each of these parameters– in order to be able to establish management criteria.

6.4.2 ALGAL BIODIESEL QUALITY

Biodiesel quality (fuel characteristics) such as saponification value (SV), cetane number (CN), iodine value (IV), long chain saturated factor (LCSF) and cool filter plugging point (CFPP) has been determined based on the fatty acid composition of the microalgal using the following empirical equations described by Vidyashankar *et al*. (2015) and Gulhe *et al.* (2016): where *D*, *M* and *N* denote the number of double bonds, molecular mass and % mass fraction of each fatty acid component, respectively, and

Where, *DU* is degree of unsaturation, calculated from the fatty acid profile (Nascimento *et al.,* 2013; Guldhe *et al.,* 2016). The properties of synthesized biodiesel is compared with the specifications given by the ASTM 6751 and EN14214 standards.

A subject of interest is the content of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids present in the oil, since these have a high impact on the quality of the biodiesel. The high proportion of SFA compared with MUFA and PUFA in microalgae contributes to increase oxidative stability. An analysis of oil containing high concentrations of PUFA and MUFA on SFA tends to originate a biodiesel with high iodine value, a condition that determines the generation of a biodiesel prone to oxidation.

The cetane number value has been associated with the efficient combustion properties of biodiesel, which is related to the presence of high proportions of SFA (Vidyashankar *et al.*, 2015). This is beneficial since a high percentage of SFA affects the flow properties causing crystallization/solidification of the fuel in the engine's filters under colder climatic condition; therefore, there must be balance between the content of SFA and PUFA. One of the problems that causes a high proportion of PUFAs is to affect the oxidative stability of the fuel.

In summary, a high value of SFA with respect to PUFA may be the best condition for a good ignition of biodiesel; on the contrary, a high percentage of SFA affects the flow properties causing crystallization/solidification of fuel in the engine's filters under colder climatic condition. Because of that, the analysis of biodiesel quality must be the base of the international standards (Table 6.10).

Table 6.10. *International standards (EN and ASTM).*

Biodiesel properties	Units	ASTM D6751	EN 14214
CN	Min	47	51
IV	g I_2 /100 g	-	120
CFPP	°C	-	≤5/≤-20
SV	mg KOH/g	-	-
DU	% wt	-	-
OS	h	3	6

Abbreviations: CN, cetane number; IV, iodine value; CFPP, cold filter plugging point; SV, saponification value; DU, degree of unsaturation; OS, oxidative stability.

6.5 MOLECULAR HYDROGEN PRODUCTION

Several studies were carried out in recent years to obtain energy sources that can replace fossil fuels and have no negative environmental effects. Hydrogen is an alternative substitute of fossil fuels and is considered to be an 'energy carrier' with a promising future. Hydrogen has a high energy content of 122 Kj/g, which is 2.75 times higher than the content in hydrocarbon fuels.

Hydrogen plays an essential role as a contribution to a clean and renewable energy. It is environment-friendly, clean energy, and in its ionic form is the most abundant element. When used as fuel, this gas does not produce air pollution, on the contrary, its combustion produces water as a byproduct. Hydrogen gas can be produced by biological processes becoming an interesting and promising alternative because it can be operated under ambient temperatures and pressures with minimal energy expense (Mohan *et al.,* 2007).

Many microorganisms are known to produce hydrogen under certain conditions including microalgae like cyanobacteria that use light energy to split water for hydrogen formation and other that usually use carbohydrates to store energy from photosynthesis to produce hydrogen from water (Hallenbeck and Ghosh, 2002). Microalgae produce hydrogen by adopting a two-stage process: In stage 1, the CO_2 is fixed in the presence of sunlight through photosynthesis, and in stage 2, hydrogen is produced by the deg-

radation of stored organic compounds via fermentation (Rashid *et al*., 2013).

Photosynthesis in green microalgae and cyanobacteria can operate under oxygenic and anoxic conditions. Oxygenated photosynthesis occurs in cyanobacteria, algae and vascular plants, while anoxigenic photosynthesis occurs in microorganisms such as *Chromatiales* and *Chlorobias* bacteria. Many microalgae, particularly green algae, produce hydrogen after a period under anaerobic conditions, during which the enzyme [FeFe] -hydrogenase is activated and synthesized, which is highly sensitive to oxygen (Benemann *et al*., 1997).

The simplest method to induce the production of hydrogen in green algae is by creating anaerobic conditions by physically or chemically removing oxygen from the culture medium. An alternative is chemically induced with the addition of sodium dithionite, reducing the oxygen content, another alternative is physically removing the oxygen by aerating the culture with an inert gas such as nitrogen (N_2) or Argon (Ar) (Wünschiers *et al*., 2003). Alternatively, hydrogen production can be induced by creating an anaerobic environment during dark incubation. Under this condition, photosynthesis and oxygen production will stop and respiration will deplete the remaining oxygen in the crop (Skjånes *et al.,* 2013).

The biological processes of hydrogen production include biofotolisis (direct and indirect) carried out by microalgas and cyanobacteria and; the production of hydrogen by fermentation (with light and dark) that is mainly carried out by photosynthetic bacteria (Azwar *et al.,* 2014).

6.5.1 DIRECT BIOPHOTOLYSIS

Direct biophotolysis is a biological process that can produce hydrogen directly from water using the photosynthetic systems of microalgae to convert solar energy into chemical energy in the form of hydrogen, according to the following reaction (Azwar *et al.,* 2014):

$$2H_2O + Energía\ solar \xrightarrow{Fotosíntesis} 2H_2 + O_2$$

Direct biophotolysis by microalgae is the most studied method for dividing water into H_2 and O_2 using sunlight as an energy source (Mathews and Wang, 2009, Khetkorn, 2017). When the light is absorbed by the photosynthetic microalgae, oxidation of the H_2O molecules by the PSII is improved, and the electrons and protons released are transported to the ferroxine (Fd) of the chloroplast through the PSI (Melis *et al*., 2007). Reduced ferredoxin acts as an electron donor for [FeFe] -hydrogenases (Ghirardi *et al*., 2009), which reversibly facilitates the reduction of protons (H^+) to H_2 molecules (Eroglu and Melis, 2011; Melis *et al*., 2000).

6.5.2 INDIRECT BIOPHOTOLYSIS

In indirect biophotolysis, the problem of the inactivation of [FeFe] -hydrogenase by the presence of O_2 is potentially eliminated by the temporal or spatial separation of the evolution of O_2 and H_2 (Hallenbeck and Benemann, 2002; Manish and Banerjee, 2008). In the first stage, microalgae are allowed to grow under normal light conditions as a source of driving energy to fix CO_2 in the

form of carbohydrates and proteins (Benemann, 2000). During this process, microalgae are allowed to reproduce as much as possible to increase the total carbohydrate in the cells while producing O_2 as a by-product (Mathews and Wang, 2009).

In the second stage, commonly called dark anaerobic fermentation, the carbohydrate and proteins stored in the algal cells, is consumed by the microalgal metabolism, creating an excess of electrons to be eliminated (Nagarajan *et al.,* 2016). The NADPH electrons are transferred directly to the plastoquinones, without passing through the PSII. The electron transport chain will work until oxygen is present in the medium. Oxygen depletion will create an anaerobic condition in the culture, the electrons are then transported through PSI, ferredoxin and finally when the conditions in the culture medium are anaerobic, the [FeFe] -hydrogenase is activated, beginning the production of H_2 (Show *et al.,* 2012). When the light period starts, the growth becomes photosynthetic and inhibits the [FeFe] -hydrogenase. The general reactions for the first and second stages are shown below in the equations.

Compared with direct Biophotolysis, indirect Biophotolysis has the advantage of using water as an electron donor and inorganic carbon as a carbon source (Brentner *et al*., 2010, Oh *et al.,* 2011). It should be noted that the two-stage process uses sulphate deprivation to create anaerobic conditions for H_2 production.

6.5.3 PHOTO-FERMENTATION

Photo-fermentation is a fermentative conversion of organic substrates by a diverse group of photosynthetic bacteria that use sunlight as energy to convert organic compounds to hydrogen and CO_2. This process takes place under anoxic or anaerobic conditions and through the use of photosynthetic bacteria and sunlight as energy (Azwar *et al.,* 2014). In principle, photosynthetic bacteria are capable of completely converting organic compounds into hydrogen, even against a relatively high partial pressure of hydrogen, because the evolution of hydrogen is driven by ATP-dependent nitrogenase and the ATP formed is the energy of the light captured through photosynthesis (Azwar *et al.,* 2014). The reduction of ATP and reduced ferredoxin lead to hydrogen protons with nitrogenase. The microorganism cannot obtain electrons from water, therefore, the use of organic compounds, usually organic acids, as substrates is required. The global reaction for the production of hydrogen through the photo-fermentation process is given in the following equation (Argun and Kargi, 2011).

6.5.4 DARK FERMENTATION

Dark fermentation is the fermentative conversion of organic substrate and biomass materials to produce biohydrogen that takes place under anaerobic conditions and without the presence of light. It is a complex process that is manifested by several groups of bacteria by involving a series of biochemical reactions. Dark fermentation has several advantages compared to other biological methods of hydrogen production, due to its ability to produce hydrogen continuously without the presence of light, higher rate of hydrogen production, simplicity of the process, lower net energy consumption and utilization of low value waste as raw materials. Most of the production of microbial hydrogen is driven by the anaerobic metabolism

of pyruvate, formed during the catabolism of various substrates. The decomposition of pyruvate is catalyzed by one of two enzyme systems (Hallenbeck and Ghosh, 2002).

1. Pyruvate: Formiato-lyase.
2. Pyruvate: ferredoxin (flavodoxin) oxidoreductase.

Therefore, in both biological systems, pyruvate generated by glycolysis is used, in the absence of oxygen, to produce acetyl CoA, from which ATP derives, and in turn reduce the ferredoxin from which hydrogen is derived.

The use of immobilized cells for the production of hydrogen is more attractive than suspended cell cultures. In culture systems, cell immobilization has attracted a considerable attention due to the versatility in its application. The immobilized cells help to avoid sedimentation, which inhibits their growth. Although qualitatively feasible, commercial exploitation of H_2 requires quantitatively better yields. Recent advances and the state of art in photobiological hydrogen production research has been reported. Topics of interest in the quest to increase the production of hydrogen include areas such as bioreactor design, hybrid, and integrated systems, metabolic engineering, and associated genetic manipulations that would be needed to make hydrogen a commercially viable fuel for the global economy.

6.6 CONCLUSIONS AND REMARKS

This document shows a review of the different strategies of microalgae cultivation for the viable production of biodiesel and biohydrogen, as well as the use of wastewater. Research concludes that the prospects for the use of wastewater for the production of algal biomass and bioenergy are promising. The objective in numerous studies is to take advantage of the nutrient content of organic wastes, in addition to an opportunity for the cultivation of microalgae, it is the reduction of cultivation costs, coupled with obtaining a double benefit; energy production and effluent treatment, as well as helping to minimize occasional impacts on the environment. The major limitation / challenge to the use of microalgae for wastewater treatment in an open pond system at outdoor site is the environmental stability of the culture. The optimum temperature and light intensity required for microalgae growth have been observed in the ranges of 18–24 ° C and 80–200 µmol m^{-2} s^{-1}, respectively. On the other hand, greater control of crops can be achieved with the use of photobioreactors, where most of the physical and chemical parameters can be controlled and prevent the growth of undesirable species.

Therefore, the control of the cultivation variables and the manipulation of some factors such as light, temperature, and nutrients, among others, has shown efficient removal of nutrients, increased biomass production, lipid accumulation, and biohydrogen production in photobioreactors. It is evident that the production of bioenergy using microalgae and wastewater is promising; however, further research is needed to satisfy the demand and production costs for the implementation of bio-refineries.

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CHAPTER

SEVEN

Production of biohydrogen in algae through culture optimization and genetic engineering

CHAPTER 7

PRODUCTION OF BIOHYDROGEN IN ALGAE THROUGH CULTURE OPTIMIZATION AND GENETIC ENGINEERING

Tamayo-Ordoñez Y.J.[1]; Sanchez-Teyer L.F.[2], Cordova-Quiroz A.V.[3]; Vital-Ocampo A.G.[3], Montoya-Hernandez D.J.[3]; Milan-Cardenas M.C.[3]; Robles-Heredia J.C.[3] and Tamayo-Ordoñez M.C.[4*]

[1]Facultad de Ciencias Químicas. Universidad Autónoma de Coahuila. Ing J. Cardenas Valdez S/N, República, 25280 Saltillo, Coah.

[2]Centro de investigación Científica de Yucatán. Unidad de Biotecnología. Mérida Yucatán México

[3]Faculty of Chemical Engineering, Universidad Autónoma del Carmen. Calle 56, No.4. Esq. Av. Concordia. Colonia Benito Juárez, C.P. 24180. Ciudad del Carmen, Campeche. México.

[4]Laboratorio de Ingeniería Genética. Departamento de Biotecnología. Facultad de Ciencias Químicas. Universidad Autónoma de Coahuila. Ing J. Cardenas Valdez S/N, República, 25280 Saltillo, Coah.

*Corresponding author: **mtamayo@uadec.edu.mx**

ABSTRACT

The current early stage of development of bioprocesses to produce molecular hydrogen provides us with opportunities to develop and innovate strategies allowing to increase the production of molecular H_2 in different microorganisms. Genetic manipulation of algae has proven to be a viable option for improving H_2 production. This book chapter explores the advances made in algae to make H_2 production more efficient and describes an approach to the enzymes involved in photosystem II in algae that are currently being a cost-effective option to produce H_2 in algae.

7.1 INTRODUCTION

The negative effects of fossil fuels on the environment have led to developing bioprocesses that can be used in the production of clean and environmentally sustainable energy. Within this context, molecular hydrogen (H_2) has proven to be a viable alternative that can be accomplished by microorganisms through processes such as bio-photolysis of water using microalgae and cyanobacteria (Eroglu and Melis, 2011; Fakhimi *et al.*, 2020; Jiménez-Llanos *et al.*, 2020).

The current early stage of development of bioprocesses to produce molecular hydrogen provides us with opportunities to develop and innovate strategies allowing to increase the production of molecular H_2 in different microorganisms. Genetic manipulation of algae has proven to be a viable option for improving H_2 production in *Chlamydomonas reinhardtii* (Esquível *et al.*, 2011) and *Chlorella* sp. (Yang *et al.*, 2019).

Because we know that HYD and FDX act in coordination during H_2 production in algae, their regulation and impact on the production of H_2 in algae are currently of research interest. There are more studies related to [FeFe]-HYD in algae than studies related to FDX. FDX enzymes function as electron carriers in various metabolic pathways and three different types have been reported: [2Fe-2S], [4Fe-4S], and [3Fe-4S]. In algae, the [2Fe2S] type ferredoxin PetF is characterized by accepting electrons from photosystem I (PSI), directing them towards several reducing biosynthetic pathways taking place in chloroplasts. Several isoforms of FDX have been described. The unicellular green algae *Chlamydomonas reinhardtii* contains at least six genes that encode plant-type FDX proteins, including PetF and FDX2-FDX6, but not FDX4 (Long *et al.*, 2008; Winkler *et al.*, 2010; Zalutskaya *et al.*, 2018)

As previously mentioned, despite the approaches that have been made to know the regulation of FDX and HYD in algae, there are few studies aiming to know the concomitant regulation of HYD and FDX during the production of H_2 in algae. In this book chapter we describe the advances made in some algal genus, -through the optimization of culture and through genetic engineering- to increase the production of H_2. We also performed an *in silico* analysis that allowed the characterization of the enzymes hydrogenase and ferredoxin between algal genera.

7.2 ADVANCES IN PRODUCCION OF H_2 IN ALGAE GENUS

In at least 9 algal genera it has been described that they can produce molecular hydrogen (Table 7.1). Within these genera *Chlorella* spp. and *Chlamydomonas* spp. they have proven to be the best producers of molecular hydrogen.

Tabla 7.1. *Algae used to produce molecular hydrogen (Tamayo-Ordoñez et al. 2022)*

Specie or strain	Molecular hydrogen values	Production conditions	Reference
Chlorella sp. DT	30 mL L^{-1}	Homologously overexpress hydA to enhance H_2 production in Chlorella sp. DT (DT) under aerobic and S-supplied (pS) conditions	Chien *et al.* (2012)
Chlorella AH1	170 mL L^{-1}		
Chlorella BH2	350 mL L^{-1}		
Scenedesmus obliquus	204.8 ± 61.2 (mL H_2/L/day) and 128.0 (mL H_2/L)	Culture under conditions of exposure in violet light	Ruiz-Marin *et al.* (2020)
Chlorella vulgaris	60.4 mL H2/L) o 39.18b ± 23.1 (mL H2/L/day)		

Specie or strain	Molecular hydrogen values	Production conditions	Reference
Chlorella sp	7.13 ml/g VS/ 144 h at the ISR	Culture in dark fermentation, anaerobic and 35 °C	Sun *et al.* (2011)
Chlorella vulgaris	34.8 mL / h / L	Cultures of immobilized, sulfur-deprived cells were purged with N2 and either kept in the dark for 72 h, kept in the dark for 24 h before being exposed to light (intensity of 120 µ mole/m2/s) for 72 h, exposed to light for 72 h, or kept in the light for 24 h before being subjected to darkness for 48 h.	Rashid *et al.* (2011)
Chlorella sp. LSD-W2	66.47 ± 5.44 and 67.64 ± 1.77 nmol H_2 mg dry weight	White light, 30 µmol fotones m^{-2} s^{-1} a 30°C, 4 days, anaerobic.	Timpranee *et al.* (2016)
Chlorella vulgaris	7.9 and 10.8 mL H_2 /g VS	Culture in dark fermentation, anaerobic and 357°C	Lakaniemi *et al.* (2011)
Chlorella vulgaris	19 ± 2.94 – 135 ± 3.11 mL H_2 g-VS with enzymatic pretreatment	Culture in dark fermentation and thermophilic	Wieczorek *et al.* (2014)
Chlorella vulgaris BEIJ strain G-120	The average H_2 production rate was 4.98 mL L^{-1} h^{-1} for the illuminated culture and 2.08 mL L^{-1} h^{-1} for the one maintained in the dark.	The strain can grow either heterotrophically or photo autotrophically. The total output of 896 mL of H_2 was attained for illuminated culture and 405 mL for dark cultures.	Touloupakis *et al.* (2021)
Scenedesmus obliquus	67.1 mL H_2 g_V^{S-1}	Culture under dark fermentation	Ferreira *et al.* (2019)
Scenedesmus obliquus *Scenedesmus obliquus*	4.7 mL (mL PCV)-1 (packed cell Volumen) por 5 days 12.3 mL (mL PCV)-1(Packed cell Volumen) por 5 days	Kinetic of hydrogen production from Scenedesmus cultures in N_2-atmosphere (oxygen depletion at the onset of the experiment) incubated with and 3,4-dcp. Kinetic of hydrogen production from 2,3-dcp treatment in nitrogen atmosphere, (grey triangle) 2,3-dcp treatment with additional glucose doping in air atmosphere.	Papazi *et al.* (2012)
Scenedesmus acuminatus *Scenedesmus acuminatus* *Scenedesmus acuminatus*	0.149 nmolH_2/g chl/h, in cells incubated under anaerobic adaptation for 4 hours 1.99 nmolH_2/µg chl/h 0.45 nmolH2/µg chl/h,	*S. acuminatus* was heterotrophically grown in PLEM at room temperature. Algae cells were incubated under anaerobic condition for 2 to 36 hours in darkness before hydrogen production measurement. Same conditions described above and culture under light Same conditions described above and culture under light	Unpaprom *et al.* (2017)

Specie or strain	Molecular hydrogen values	Production conditions	Reference
Tetraspora sp. CU2551	12.8 ± 0.9 mL H_2 25 mL^{-1} of medium 182 ± 20 nmol mg^{-1} DW h^{-1}	Immobilized cells were incubated in S-deprived media which could be repeated up to six times when using refreshed media	Maswanna *et al.* (2020)
Dunaliella tertiolecta	8.4 and 12.6 ml H_2/g VS	Culture in dark fermentation at 37°C	Lakaniemi *et al.* (2011)
Chlamydomonas reinhardtii	OP68; 25.1 ± 5.1 µmol H_2/ mg Chl^{-1}/h^{-1} R2D2 30 ± 5.9 µmol H_2/ mg $Chl^{-1}/^{h-1}$	Dark fermentation and 150µE luz by 5 días	Weiner *et al.* (2018)
	D66; 12.6 ± 3.69 µmol H_2/ mg Chl^{-1}/h^{-1} CC124: 21.4 ± 4.2 µmol H_2/ mg Chl^{-1}/h^{-1}		
Chlamydomonas reinhardii mutant L159I-N230Y	166 mL H2 $(g\ chl\ h)^{-1}$	Culture with sulfur depletion	Torzillo *et al.* (2009)
Chlamydomonas reinhardii	7 mmol H2 $(mg\ chl\ h)^{-1}$	Culture with sulfur depletion	Tsygankov *et al.* (2012)
Monoraphidium sp.	1.32 H_2 mL/L 2.04 H_2 mL/L	Culture in TAP-S medium Culture in TAP-S (0.7 mM NH4Cl) medium	Pongpadung *et al.* (2015)
Nannochloropsis oceanica	183.9 mL/g-TVS and 39 mL/g-TVS	Dark fermentation, microwave heat, hydrolysis with H_2SO_4	Xia et al. (2013)
Tetraselmis GSL1 *Tetraselmis QNM1*	68.34 ± 2.46 nmol H_2 x µg chl^{-1} x h^{-1} 78.25 ± 0.89 nmol H_2 x µg chl^{-1} x h^{-1}	Photoproduction	D, Adamo *et al.* (2014)
Tetraselmis GSL1 *Tetraselmis QNM1*	44.81 ± 4.06 nmol H_2 x µg chl^{-1} x h^{-1} 32.56 ± 4.38 nmol H_2 x µg chl^{-1} x h^{-1}	Dark fermentative production	D, Adamo *et al.* (2014)
Micractinium reisseri YSW05	3.07 mL/h and 191.2mL/L 2.06 ml/h and 118 ml/L	Culture under continuous light condition by 24h Culture under Light/dark 12/12h	Hwang *et al.* (2014)
C. reinhardtii (DP-H2)	150mL/L	Media: pre-treated olive mill wastewater (50%) + Sulphur derived TAP, pH: 7.2, PI: 70 µmol photon m-2 s-1, T: 28 ℃, IT: 168h	Faraloni *et al.* (2011)

Specie or strain	Molecular hydrogen values	Production conditions	Reference
C. reinhardtii CC124 and D1 protein mutant strain (D239-40, D240, D240-41)	CC 124: 243 ± 10 mL L^{-1} D240: 165 ± 10 mL L^{-1} D239-40: 490 ±10 mL L^{-1} D240-41: 388 ± 10 mL L^{-1}	Media: Sulphur derived TAP, pH: 7.2 ± 0.2, PI: 50-200 μmol photon m-2 s-1, T: 27 ± 0.5 °C, IT: 168h	Oncel *et al.* (2014)

In these algal genera, research has been carried out that includes crop optimization and genetic engineering. *Scenedesmus obliquus*, *Chlorella vulgaris*, *Tetraspora* sp. CU2551, *Dunaliella tertiolecta*, *C. reinhardtii*, and *Nannochloropsis oceanica* have been shown to produce H_2, under different optimized conditions such as variable light intensities (Ruiz-Marin *et al.* 2020), dark fermentation (Unpaprom *et al.*, 2017; Lakaniemi *et al.*, 2011), sulfide-deprived anaerobic cultures (Faraloni *et al.* 2011, Oncel *et al.*, 2014; Maswanna *et al.*, 2020), alternating dark and light periods (Hwang *et al.* 2014), and presence of photosystem blockers (Papazi *et al.*, 2012). Future increase in biohydrogen yields in these algae could be obtained through genetic engineering strategies.

At the genetic level, through heterologous expression of the *Shewanella oneidensis* MR-1 HYD operon (hydA, hydB, hydE, hydF, and hydG) in *Anabaena* sp. PCC 7120, Khetkorn et al. (2013) produced 3.4 nmol H_2 μg chl α-1h-1. Dubini and Ghirardi (2015) increased H2 production 7-fold by homologous overexpression of the HydA gene in *Chlorella* compared to the unmutated strain, and Weiner et al. (2018) increased H_2 production 4.5 times through expression of a ferredoxin-hydrogenase fusion enzyme (Fd-Hyd) in *Chlamydomonas reinhardtii* compared to the mutant strain without HYD. This latter report suggests HYD and FDX act concomitantly during H_2 production in *Chlamydomonas reinhardtii*. A recent study of Tamayo-Ordoñez et al. (2021) allowed identifying genes coding for HYD in *Chlorella vulgaris* and *Scenedesmus obliquus* and highlights the possible presence in both microalgae of isoforms of *hyd* genes which are transcribed during the early stages of H_2 production.

7.3 EVOLUTIONARY RELATIONSHIP OF THE MICROALGAE GENOME

7.3.1 GENETIC RELATIONSHIP BETWEEN ALGAE

To establish evolutionary patterns in microalgae species, the NCBI genome database was explored and the relatedness determined through similarity of alignments with a selected subset of 49 clusters of orthologous groups (COG) domains of gene families of *Coccomyxa subellipsoidea* (GCF_000258705.1), *Auxenochlorella protothecoides* (Krüger) Kalina and Puncochárová (GCF_000733215.1), *Volvox carteri F.* Stein (GCF_000143455.1), *Chlamydomonas reinhardtii* (GCF_000002595.1), *Micromonas pusilla* (GCF_000151265.2), *Micromonas commoda* (GCF_000090985.2), *Bathycoccus prasino* (GCF_002220235.1), *Ostreococcus tauri* (GCF_000214015.2), and *Ostreococcus lucimarinus* (GCF_000092065.1) (Table 7.2). The phylogenetic relationships were constructed by the rapid estimation

method to approximate maximum likelihood phylogeny in the software Species Tree Builder version 2.1.10 (Price *et al.* 2010). Comparative analysis of nine microalgae genomes was made in the Clara Genome Comparison SDK (v.0.0.7) program.

Table 7.2. *The COGs domains used in the estimate of relatedness evolutive microalgae*

Código KBASE*	Familie gene	Descripción
COG0012	COG0012	Predicted GTPase, probable translation factor [Translation, ribosomal structure and biogenesis].
COG0013	AlaS	Alanyl-tRNA synthetase [Translation, ribosomal structure and biogenesis].
COG0016	PheS	Phenylalanyl-tRNA synthetase alpha subunit [Translation, ribosomal structure and biogenesis].
COG0018	ArgS	Arginyl-tRNA synthetase [Translation, ribosomal structure and biogenesis].
COG0030	KsgA	Dimethyladenosine transferase (rRNA methylation) [Translation, ribosomal structure and biogenesis].
COG0041	PurE	Phosphoribosylcarboxyaminoimidazole (NCAIR) mutase [Nucleotide transport and metabolism].
COG0046	PurL	Phosphoribosylformylglycinamidine (FGAM) synthase, synthetase domain [Nucleotide transport and metabolism].
COG0048	RpsL	Ribosomal protein S12 [Translation, ribosomal structure and biogenesis].
COG0049	RpsG	Ribosomal protein S7 [Translation, ribosomal structure and biogenesis].
COG0051	RpsJ	Ribosomal protein S10 [Translation, ribosomal structure and biogenesis].
COG0052	RpsB	Ribosomal protein S2 [Translation, ribosomal structure and biogenesis].
COG0072	PheT	Phenylalanyl-tRNA synthetase beta subunit [Translation, ribosomal structure and biogenesis].
COG0080	RplK	Ribosomal protein L11 [Translation, ribosomal structure and biogenesis].
COG0081	RplA	Ribosomal protein L1 [Translation, ribosomal structure and biogenesis].
COG0082	AroC	Chorismate synthase [Amino acid transport and metabolism].
COG0086	RpoC	DNA-directed RNA polymerase, beta' subunit/160 kD subunit [Transcription].
COG0087	RplC	Ribosomal protein L3 [Translation, ribosomal structure and biogenesis].
COG0088	RplD	Ribosomal protein L4 [Translation, ribosomal structure and biogenesis].
COG0089	RplW	Ribosomal protein L23 [Translation, ribosomal structure and biogenesis].
COG0090	RplB	Ribosomal protein L2 [Translation, ribosomal structure and biogenesis].
COG0091	RplV	Ribosomal protein L22 [Translation, ribosomal structure and biogenesis].
COG0092	RpsC	Ribosomal protein S3 [Translation, ribosomal structure and biogenesis].
COG0093	RplN	Ribosomal protein L14 [Translation, ribosomal structure and biogenesis].
COG0094	RplE	Ribosomal protein L5 [Translation, ribosomal structure and biogenesis].
COG0096	RpsH	Ribosomal protein S8 [Translation, ribosomal structure and biogenesis].
COG0097	RplF	Ribosomal protein L6P/L9E [Translation, ribosomal structure and biogenesis].
COG0098	RpsE	Ribosomal protein S5 [Translation, ribosomal structure and biogenesis].
COG0099	RpsM	Ribosomal protein S13 [Translation, ribosomal structure and biogenesis].
COG0100	RpsK	Ribosomal protein S11 [Translation, ribosomal structure and biogenesis].
COG0102	RplM	Ribosomal protein L13 [Translation, ribosomal structure and biogenesis].
COG0103	RpsI	Ribosomal protein S9 [Translation, ribosomal structure and biogenesis].
COG0105	Ndk	Nucleoside diphosphate kinase [Nucleotide transport and metabolism].
COG0126	Pgk	3-phosphoglycerate kinase [Carbohydrate transport and metabolism].
COG0127	COG0127	Xanthosine triphosphate pyrophosphatase [Nucleotide transport and metabolism].

Código KBASE*	Familie gene	Descripción
COG0130	TruB	Pseudouridine synthase [Translation, ribosomal structure and biogenesis].
COG0150	PurM	Phosphoribosylaminoimidazole (AIR) synthetase [Nucleotide transport and metabolism].
COG0151	PurD	Phosphoribosylamine-glycine ligase [Nucleotide transport and metabolism].
COG0164	RnhB	Ribonuclease HII [DNA replication, recombination, and repair].
COG0172	SerS	Seryl-tRNA synthetase [Translation, ribosomal structure and biogenesis].
COG0185	RpsS	Ribosomal protein S19 [Translation, ribosomal structure and biogenesis].
COG0186	RpsQ	Ribosomal protein S17 [Translation, ribosomal structure and biogenesis].
COG0215	CysS	Cysteinyl-tRNA synthetase [Translation, ribosomal structure and biogenesis].
COG0244	RplJ	Ribosomal protein L10 [Translation, ribosomal structure and biogenesis].
COG0256	RplR	Ribosomal protein L18 [Translation, ribosomal structure and biogenesis].
COG0343	Tgt	Queuine/archaeosine tRNA-ribosyltransferase [Translation, ribosomal structure and biogenesis].
COG0504	PyrG	CTP synthase (UTP-ammonia lyase) [Nucleotide transport and metabolism].
COG0519	GuaA	GMP synthase, PP-ATPase domain/subunit [Nucleotide transport and metabolism].
COG0532	InfB	Translation initiation factor 2 (IF-2; GTPase) [Translation, ribosomal structure and biogenesis].
COG0533	QRI7	Metal-dependent proteases with possible chaperone activity [Posttranslational modification, protein turnover, chaperones].

Our comparative analysis of nine microalgae genomes grouped the proteins present in this set of genomes into families, indicating 89,517 coding genes, of which 53,814 corresponded to homologous families and 35,703 to unique families (not conserved) (Table 7.2 and Table 7.3). The results of this analysis indicated that *Auxenochlorella protothecoides* has homologous families with *Bathycoccus prasinos* (1089), *Chlamydomonas reinhardtii* (1701), *Coccomyxa subellipsoidea* (1890), *Micromonas pusilla* (1199), *Ostreococcus lucimarinus* (1007), *O. tauri* (977), *Micromonas commode* (1235), and *Volvox carteri* (1694). In *Coccomyxa subellipsoidea*, homologous families were found with *Auxenochlorella protothecoides* (1890), *Bathycoccus prasinos* (1242), *Chlamydomonas reinhardtii* (2049), *Micromonas pusilla* (1459), *Ostreococcus lucimarinus* (1186), *O. tauri* (1140), *Micromonas commode* (1499), and *Volvox carteri* (2049). In *Micromonas pusilla*, we found homologous families with *Auxenochlorella protothecoides* (1199), *Bathycoccus prasinos* (2262), *Chlamydomonas reinhardtii* (1394), *Coccomyxa subellipsoidea* (1459), *Ostreococcus lucimarinus* (2382), *O. tauri* (2277), *Micromonas commode* (4739), and *Volvox carteri* (1399). We found the lowest number of homologous families relative to the microalgae species analyzed in *Ostreococcus lucimarinus* and *O. tauri*. In *Ostreococcus lucimarinus* we identified 1007, 2292, 1128, 1186, 2382, 4797, 2412, and 1145 homologous families with *Auxenochlorella protothecoides*, *Bathycoccus prasinos*, *Chlamydomonas reinhardtii*, *Coccomyxa subellipsoidea*, *Ostreococcus tauri*, *Micromonas commode*, *Micromonas pusilla* and *Volvox carteri*, respectively. Finally, *Ostreococcus tauri* showed 977, 2239, 1117, 1140, 2277, 4797, 2380 and 1125 homologous protein groups with *Auxenochlorella protothecoides*, *Bathycoccus prasinos*, *Chlamydomonas reinhardtii*, *Coccomyxa subellipsoidea*, *Micromonas pusilla*, *Ostreococcus lucimarinus*, *Micromonas commode*, and *Volvox carteri*, respectively.

Table 7.3. *Identification of homologous genes and families in the microalgae genome.*

Genome	G1	G2	G3	G4	G5	G6	G7	G8	G9
Auxenochlorella protothecoides	2186	1089	1701	1890	1199	1007	977	1235	1694
Bathycoccus prasinos	1089	2828	1180	1242	2262	2292	2239	2381	1168
Chlamydomonas reinhardtii	1701	1180	6497	2049	1394	1128	1117	1460	5848
Coccomyxa subellipsoidea	1890	1242	2049	2845	1459	1186	1140	1499	2049
Micromonas pusilla	1199	2262	1394	1459	5072	2382	2277	4739	1399
Ostreococcus lucimarinus	1007	2292	1128	1186	2382	5056	4797	2412	1145
Ostreococcus tauri	977	2239	1117	1140	2277	4797	5002	2380	1125
Micromonas commoda	1235	2381	1460	1499	4739	2412	2380	5143	1446
Volvox carteri f. nagariensis	1694	1168	5848	2049	1399	1145	1125	1446	6517

G1 - Auxenochlorella protothecoides, G2 - Bathycoccus prasinos, G3 - Chlamydomonas reinhardtii, G4 - Coccomyxa subellipsoidea, G5 - Micromonas pusilla, G6 - Ostreococcus lucimarinus, G7 - Ostreococcus tauri, G8 -Micromonas commoda, G9 - Volvox carteri f. nagariensis.

Table 7.3. *Identification of homologous genes and families in the microalgae genome*

Genome	Genes	Genes in Homologs	Genes in Singletons	Homolog Families
Ostreococcus lucimarinus	7640	5618	2022	5056
Ostreococcus tauri	8114	5533	2581	5002
Volvox carteri f. nagariensis	14437	10474	3963	6517
Chlamydomonas reinhardtii	14488	9553	4935	6497
Auxenochlorella protothecoides	7016	2620	4396	2186
Bathycoccus prasinos	7959	3535	4424	2828
Coccomyxa subellipsoidea	9915	3957	5958	2845
Micromonas commoda	10184	6035	4149	5143
Micromonas pusilla	10248	6489	3759	5072

Our phylogenetic analysis showed that the more closely related species in terms of number of shared homologous families were *Volvox carteri* with *Chlamydomonas reinhardtii* and *Micromonas commoda* with *Bathycoccus prasinos* and *Ostreococcus tauri* (Fig. 7.1 and Table 7.3). In *Volvox carteri*, homologous families were identified with *Chlamydomonas reinhardtii* (5848), *Auxenochlorella protothecoides* (1694), *Bathycoccus prasinos* (1168), *Coccomyxa subellipsoidea* (2049), *Micromonas pusilla* (1399), *Ostreococcus lucimarinus* (1145), *O. tauri* (1125), and *Micromonas commode* (1446), *Micromonas commoda* showed the highest number of shared families with *M. tauri* (4739), followed by *Micromonas pusilla* and *Ostreococcus tauri* with close to 2380 homologous families, while the numbers of related families with the other species of microalgae were 1235, 1460, 1499, 2412, and 1446 with *Auxenochlorella protothecoides*, *Chlamydomonas reinhardtii*, *Coccomyxa subellipsoidea*, and *Ostreococcus lucimarinus*, respectively.

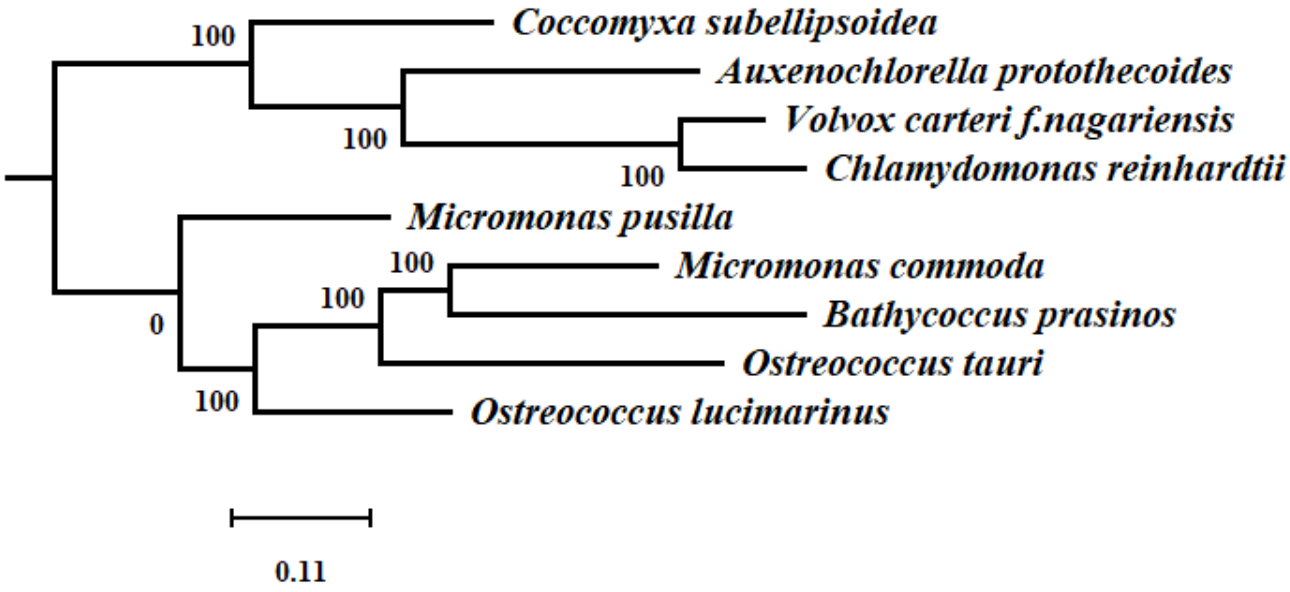

Figure 7.1. Evolutionary relationship of the microalgae genome. The phylogenetic relationship was constructed by method to quickly estimate approximate maximum likelihood phylogeny (version 2.1.10) the software SpeciesTreeBuilder. The analysis included ***Coccomyxa subellipsoidea*** *C-169 (GFC_000258705.1),* ***Auxenochlorella protothecoides*** *(GCF_000733215.1),* ***Volvox carteri f. nagariensis*** *(GFC_000143455.1),* ***Chlamydomonas reinhardtii*** *(GCF_000002595.1),* ***Micromonas pusilla*** *CCMP1545 (GFC_000151265.2),* ***Micromonas commoda*** *(GFC_000090985.2),* ***Bathycoccus prasinos*** *(GFC_002220235.1),* ***Ostreococcus tauri*** *(GFC_000214015.2), Ostreococcus lucimarinus CCE9901 (GFC_000092065.1).*

7.3.2 GENETIC RELATIONSHIP BETWEEN ALGAE, PLANTS, AND FUNGI

The microalgae are included within a monophyletic taxon related to green plants and ascomycete fungi (Gladieux *et al.*, 2014) a relationship that was corroborated by our results (Fig. 7.2.). The genomes more closely related to microalgae were those of the fungal species *Spizellomyces punctatus*, *Lobosporangium transversal*, *Aspergillus fischeri*, *Diplodia corticola*, *Postia placenta*, *Auricularia subglabra*, *Trametes versicolor*, and *Heterobasidion irregulare*. Among the less related genomes, we found plant species like *Momordica charantia* L., *Morus notabilis*, *Herrania umbratical*, *Sorghum bicolor*, and *Arabidopsis lyrate*.

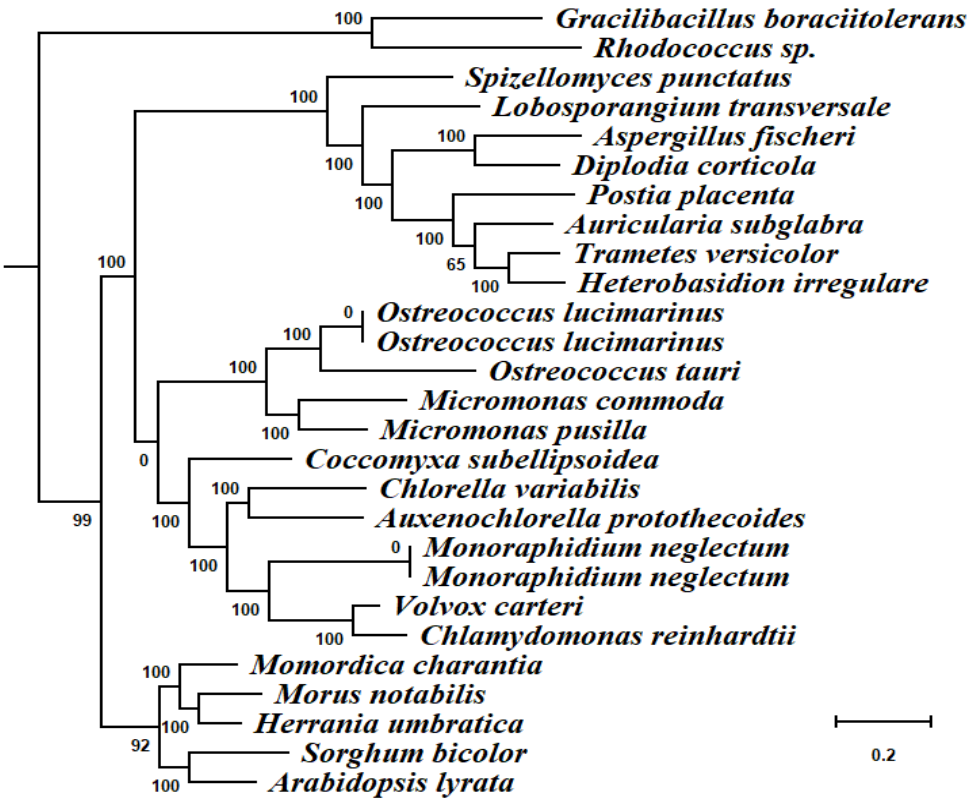

Figure 7.2. Phylogenetic relationship of the genome of fungi, microalgae and plants. The phylogenetic relationship was constructed by method to quickly estimate approximate maximum likelihood phylogeny (version 2.1.10) the software SpeciesTreeBuilder. Fungi species: ***Gacilibacillus boraciitolerans*** *(GCF_000521485.1),* ***Rhodococcus*** *sp. (GCF_001312925.1),* ***Spizellomyces punctatus*** *(GCF_000182565.1),* ***Lobosporangium transversale*** *(GCF_002105155.1),* ***Aspergillus fischeri*** *(GCF_000149645.1),* ***Diploidia corticola*** *(GCF_001883845.1),* ***Postia pacenta*** *(GCF_000006255.1),* ***Auricularia subglabra*** *(GCF_000265051.1),* ***Trametes versicolor*** *(GCF_000271585.1), and* ***Heterobasidium irregulare*** *(GCF_000320585.1). Microalgae species:* ***Coccomyxa subellipsoidea*** *C-169 (GFC_000258705.1),* ***Auxenochlorella protothecoides*** *(GCF_000733215.1),* ***Volvox carteri*** *f. nagariensis (GFC_000143455.1),* ***Chlamydomonas reinhardtii*** *(GCF_000002595.1),* ***Micromonas pusilla*** *CCMP1545 (GFC_000151265.2),* ***Micromonas commoda*** *(GFC_000090985.2),* ***Bathycoccus prasinos*** *(GFC_002220235.1),* ***Ostreococcus tauri*** *(GFC_000214015.2), and* ***Ostreococcus lucimarinus*** *CCE9901 (GFC_000092065.1). Plant species:* ***Momordica charantia*** *(GCF_001995035.1), Morus notabilis (GCF_000414095.1),* ***Herrania umbratica*** *(GCF_002168275.1),* ***Sorghum bicolor*** *(GCF_000003195.3), and* ***Arabidopsis lyrata*** *(GCF_000004255.2).*

7.3.3 PANGENOME ANALYSIS OF ALGAE

The construction and analysis of the pangenome of these nine microalgal species allowed us assessing the consistency of functional assignments for highly homologous proteins and the degree to which each protein family has been conserved across the entire set of genomes. *Volvox carteri* and *Chlamydomonas reinhardtii* with a large genome size (14437 and 14488 genes, respectively) contained the highest numbers of genes (10474 and 9553, respectively) and homologous families (6517 and 6497, respectively). A similar pattern was observed in *Micromonas commoda and M. pusilla*, *Coccomyxa subellipsoidea* presented the highest number of divergent genes (5958) demonstrating a distant evolutionary relationship (Table 7.3).

Figure 7.3, illustrates orthologous groups present in all the analyzed genomes (core proteins) and non-conserved or non-redundant orthologous proteins in non-core proteins, sometimes called accessory proteins. These proteins are likely related to more recent phylogenetic adaptations of species to their environment. The set of orthologous genes suggests the probable existence of conservation of certain ancestral lineage genes in modern genomes. Furthermore, since the gene has been retained by all modern species, it is likely that it is necessary, or at least beneficial enough for the lifestyle (or biotechnological application) of each species of microalgae (Kim *et al*. 2014; Tamayo-Ordoñez *et al*. 2017; Krasovec *et al*. 2018; Tamayo-Ordoñez *et al*., 2021). The species of *Ostreococcus* show conservation in probably ancestral orthologous families, even though their genome exhibits a greater genetic distance. In the pangenome of microalgal species, regions of the genome corresponding to vertically inherited paralogue genes are observed in opposition to expansions derived from classified duplication lineages (non-redundant ortholog sets), as in the species of *Micromonas*. Within this category are those groups of orthologs present in more than one genome, but not in all (Fig. 7.3).

We identified a large proportion of non-homologous genes on the microalgal genomes, which suggests the presence of new functions as a consequence of horizontal transfer from proximal distal lineages (Fig. 7.3), as well as genetic events that derived in the evolution of complementary functionalities, neo-functionalization, and functional specialization in groups of proteins of commercial interest (Wei *et al*., 2013; Kim *et al*., 2015).

In general, the clade made up of the species *Micromonas pusilla*, *M. commoda*, *Bathycoccus prasinos*, *Ostreococcus lucimarinus*, and *O. tauri* showed the presence of shared and non-redundant orthologous groups, suggesting a specialization of genomes in the species *Chlamydomonas reinhardtii*, *Coccomyxa subellipsoidea*, and *Volvox carteri*, which are microalgae mostly used for commercial purposes, which indicates that extrinsic pressure plays a major role in the evolution of microalgal genomes.

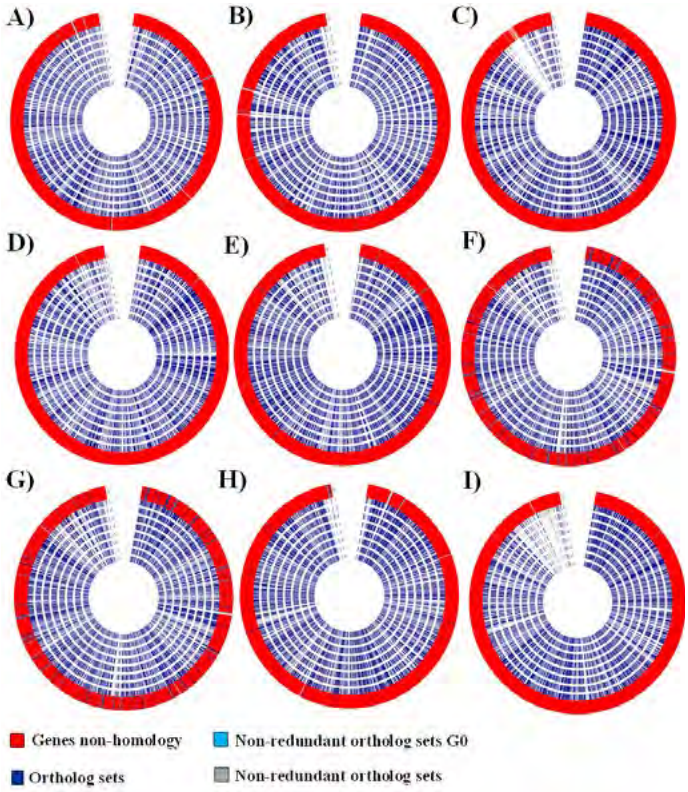

Figure 7.3. *Representation of the pangenome of microalgae species. Comparison of the reference genome with other species of microalgae. a)* ***Auxenochlorella protothecoides*** *(G0);* ***Coccomyxa subellipsoidea*** *(G1);* ***Chlamydomonas reinhardtii*** *(G2);* ***Volvox carteri*** *(G3);* ***Micromonas commode*** *(G4);* ***Micromonas pusilla*** *(G5);* ***Bathycoccus prasinos*** *(G6);* ***Ostreococcus lucimarinus*** *(G7); and* ***Ostreococcus tauri*** *(G8). b)* ***Bathycoccus prasinos*** *(G0);* ***Micromonas commoda*** *(G1);* ***Ostreococcus lucimarinus*** *(G2);* ***Micromonas pusilla*** *(G3);* ***Ostreococcus tauri*** *(G4);* ***Coccomyxa subellipsoidea*** *(G5);* ***Chlamydomonas reinhardtii*** *(G6);* ***Volvox carteri*** *(G7); and* ***Auxenochlorella protothecoides*** *(G8). c)* ***Chlamydomonas reinhardtii*** *(G0);* ***Volvox carteri*** *(G1);* ***Coccomyxa subellipsoidea*** *(G2);* ***Auxenochlorella protothecoides*** *(G3);* ***Micromonas commoda*** *(G4);* ***Micromonas pusilla*** *(G5);* ***Bathycoccus prasinos*** *(G6);* ***Ostreococcus lucimarinus*** *(G7); and* ***Ostreococcus tauri*** *(G8). d)* ***Coccomyxa subellipsoidea*** *(G0);* ***Volvox carteri*** *(G1);* ***Chlamydomonas reinhardtii*** *(G2),* ***Auxenochlorella protothecoides*** *(G3);* ***Micromonas commoda*** *(G4);* ***Micromonas pusilla*** *(G5);* ***Bathycoccus prasinos*** *(G6);* ***Ostreococcus lucimarinus*** *(G7); and* ***Ostreococcus tauri*** *(G8). e)* ***Micromonas pusilla*** *(G0);* ***Micromonas commoda*** *(G1);* ***Ostreococcus lucimarinus*** *(G2);* ***Ostreococcus tauri*** *(G3);* ***Bathycoccus prasinos*** *(G4);* ***Coccomyxa subellipsoidea*** *(G5);* ***Volvox carteri*** *(G6);* ***Chlamydomonas reinhardtii*** *(G7); and* ***Auxenochlorella protothecoides*** *(G8). f)* ***Ostreococcus lucimarinus*** *(G0);* ***Ostreococcus tauri*** *(G1);* ***Micromonas commoda*** *(G2);* ***Micromonas pusilla*** *(G3);* ***Bathycoccus prasinos*** *(G4);* ***Coccomyxa subellipsoidea*** *(G5);* ***Volvox carteri*** *(G6);* ***Chlamydomonas reinhardtii*** *(G7); and* ***Auxenochlorella protothecoides*** *(G8). g)* ***Ostreococcus tauri*** *(G0);* ***Ostreococcus lucimarinus*** *(G1);* ***Micromonas commoda*** *(G2);* ***Micromonas pusilla*** *(G3);* ***Bathycoccus prasinos*** *(G4);* ***Coccomyxa subellipsoidea*** *(G5);* ***Volvox carteri*** *(G6);* ***Chlamydomonas reinhardtii*** *(G7);* ***Auxenochlorella protothecoides*** *(G8). h)* ***Micromonas commoda*** *(G0);* ***Micromonas pusilla*** *(G1);* ***Ostreococcus lucimarinus*** *(G2);* ***Bathycoccus prasinos*** *(G3);* ***Ostreococcus tauri*** *(G4);* ***Coccomyxa subellipsoidea*** *(G5);* ***Chlamydomonas reinhardtii*** *(G6);* ***Volvox carteri*** *(G7); and* ***Auxenochlorella protothecoides*** *(G8). i)* ***Volvox carteri*** *(G0);* ***Chlamydomonas reinhardtii*** *(G1);* ***Coccomyxa subellipsoidea*** *(G2);* ***Auxenochlorella protothecoides*** *(G3);* ***Micromonas commoda*** *(G4);* ***Micromonas pusilla*** *(G5);* ***Bathycoccus prasinos*** *(G6);* ***Ostreococcus lucimarinus*** *(G7); and* ***Ostreococcus tauri*** *(G8).* ***Auxenochlorella protothecoides*** *(GCF_000733215.1),* ***Bathycoccus prasinos*** *(GFC_002220235.1),* ***Chlamydomonas reinhardtii*** *(GCF_000002595.1),* ***Coccomyxa subellipsoidea*** *C-169 (GFC_000258705.1), GFC_002220235.1),* ***Ostreococcus tauri*** *(GFC_000214015.2),* ***Micromonas commoda*** *(GFC_000090985.2), and* ***Volvox carteri*** *f. nagariensis (GFC_000143455.1). The representation of the set of characteristics corresponding to the pangenomes (Core, non-core, singleton) was constructed by the kb_phylogenomics v.1.4.0 program.*

Performing mapping genes related to these ferredoxins in genomes from accessions nine, the presence of 10 genes related to ferredoxins were demonstrated, but are not represented equally in each genome analyzed (Table 7.4). The CHLREDRAFT_174881 relative to 2Fe-2S ferredoxin, was found in a single genome and F751_5255 was found in 5 genomes. This would suggest the presence of different isoforms of these genes encoding ferredoxins.

Table 7.4. *Mapping of ferredoxins genes in genome of microalgae*

Family	Function	Protein Coding Gene Count	Genome Count
CHLREDRAFT_188740	Ferredoxin	1	1
CHLREDRAFT_174881	2Fe-2S ferredoxin	1	1
F751_0153	Ferredoxin	1	1
F751_5255	2Fe-2S ferredoxin	5	5
F751_3058	Ferredoxin-1	2	2
COCSUDRAFT_42152	Ferredoxin reductase-like protein	1	1
COCSUDRAFT_52836	Ferredoxin reductase-like protein	1	1
COCSUDRAFT_31164	Ferredoxin	1	1
COCSUDRAFT_33121	Ferredoxin reductase-like protein	1	1
MICPUN_61126	Ferredoxin, chloroplast precursor	2	2

Unfortunately, only a few algal genomes have been sequenced so far, which limits these studies, however according to comparative genomics analysis it appears that each microalgae may present different genes that encode isoforms of enzymes that favor the production of a compound of interest according to the characteristics of their habitat allowing their survival. Apparently, each species, according to its genetics and evolution, could present different capacities to produce molecules like fatty acids and H_2.

7.4 CONCLUSIONS AND REMARKS

At least 9 genera of algae have shown that by optimizing culture (varying light intensities, different sources of nitrogen and carbon, and even using blockers from photosystems I and II), they improve H_2 production. At the level of genetic engineering, in algae such as *Chlorella* and *Chlamydomonas*, the importance of genetic manipulation of genes encoding ferredoxin and hydrogenase has been highlighted, which have been shown to participate positively in increasing H_2 production in the algae. Despite these advances, few algal genera have been studied to try to understand the biological factors involved in the production of H_2. It is necessary in the future to carry out studies that involve a greater number of species of algae other than those described in the literature. It is estimated that in genetically manipulated algae, only 15% of the theoretical production is being reached, suggesting that it is still necessary to optimize both strategies; optimization of crops and genetic engineering methods, which allow us to achieve higher values in the production of H_2.

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CHAPTER

EIGHT

Plant metabolites and the generation of biotechnological compounds

CHAPTER 8

PLANT METABOLITES AND THE GENERATION OF BIOTECHNOLOGICAL COMPOUNDS

Estrada-Alfaro N.A.[1], Córdova-Alvarado A.A.[2], Tamayo-Ordoñez M.C.[3] Rodríguez-Zapata L.C.[1], Poot-Poot W.[4], Ramos-Garcia V.H.[5], and Ayil-Gutierrez B.A.[6]

[1]Centro de investigación Científica de Yucatán A.C. Unidad de Biotecnología. Mérida Yucatán México.

[2]Centro de investigación Científica de Yucatán.
Unidad de Bioquímica y Biología Molecular de Plantas. Mérida Yucatán México.

[3]Laboratorio de Ingeniería Genética. Departamento de Biotecnología. Facultad de Ciencias Químicas.
Universidad Autónoma de Coahuila. Ing J. Cardenas Valdez S/N, República, 25280 Saltillo, Coah.

[4]Laboratorio de Biotecnología, Facultad de Ingeniería y Ciencias,
Universidad Autónoma de Tamaulipas, Centro Universitario, 87120 Cd Victoria, Tamps.

[5]Unidad Académica Multidisciplinaria Reynosa-Aztlán, Universidad Autónoma de Tamaulipas.
Calle 16 y Lago de Chapala S/N Col. Aztlán, Reynosa Tamaulipas, 88740.

[6]CONACYT- Centro de Biotecnología Genómica, Instituto Politécnico Nacional, Biotecnologia Vegetal.
Blvd. del Maestro, s/n, Esq. Elías Piña, Reynosa 88710.

Corresponding author: **benjamincgb_ipn@hotmail.com**

ABSTRACT

Plants have been widely used to obtain different compounds of commercial interest, including metabolites. This book chapter describes some plant metabolites that have been widely used in medicine, food, and research. It also describes the most widely used techniques and methods for obtaining a wide variety of plant metabolites.

8.1 INTRODUCTION

Being immobile organisms, plants are exposed to a wide variety of threats, of biotic origin (insects, viruses, fungi, etc.) or abiotic (high temperatures, soil salinity, water availability, among others), so they must develop highly effective systems to mitigate the adverse effects that occur in plant-environment interactions. Among these strategies is the production of substances with different activities, mainly defense, although there are also energy storage, with reducing properties, osmoregulators, etc.

These substances are products of plant metabolism (hence the name metabolite) and in many cases, production can be induced by the environment or other factors (tender); however, not being part of the biomolecules involved in primary functions such as growth and photosynthesis (central or primary metabolism), these have been classified as secondary metabolites and in more recent years, they are referred to as part of specialized metabolism.

Plants produce an enormous diversity of these compounds, which have been documented for several years, for example, in 1988 there were, reported, about 88 thousand of these in the NAPRALERT database and it is estimated that each year approximately 4 thousand new ones are reported (Verpoorte, 2000).

In general, the term metabolite was used to refer to substances of low molecular weight, but includes a wide range of chemical species. The classification of these compounds can be done by means of their structural characteristics (for example if they contain nitrogen such as alkaloids, if they have aromatic rings or have a linear structure), although they can also be classified by means of the biosynthetic route from which they come (biogenetic origin); while in various texts three main groups are mentioned: Nitrogenous compounds, phenolic compounds and terpenes.

Phenolic compounds are bioactive substances, mainly known as potent antioxidants that are present in a large number of plants, including simple phenols, phenolic acids, hydroxycinnamic acid derivatives and flavonoids; all of them contain at least one aromatic ring and one hydroxyl group (Tungmunnithum *et al.*, 2018). Among the nitrogenous compounds are alkaloids, this group presents certain characteristics such as a complex structure and its basic character related to a heterocyclic tertiary nitrogen in its structure, which comes from the metabolism of amino acids (Verpoorte, 2005). Terpenes are another family of compounds with varied structure, ranging from simple to complex, their main characteristic is that they are composed of five-carbon units (isoprene) and can be divided into subgroups according to the number of these isoprene units (Huang and Osbourn, 2019). These last two groups of metabolites correspond to the most numerous; on the one hand there are alkaloids with approximately 12,000 compounds, related to a limited number of plant families (Newman and Cragg, 2016) and in turn terpenoids have a number close to 27,000 compounds (Verpoorte, 2000).

As for the economic aspect, secondary metabolites represent a great source of value, since their uses are applied in many fields

such as food, medicine and cosmetics. In Western medicine, they represent about 25% of the drugs currently used and the estimated value for this item is close to 250 billion dollars a year (Verpoorte, 2000).

Because they are compounds that are not synthesized in large quantities (being derived from primary metabolism are not required in vital processes), but have a great diversity and high economic value, an extensive study of plant species related to their production and accumulation has been implemented, in order to obtain established systems of large-scale production. These works include the culture of plant tissues, organs and cells (Karuppusamy, 2009; Isah *et al*., 2018), the tendering of plants to stimulate production (Namdeo, 2007; Narayani and Srivastava, 2017; Thakur *et al*., 2019), genetic transformation (Srivastava and Srivastava, 2007; Moharrami *et al*., 2017) and metabolic engineering (Sathlhut *et al*., 2015; Eichenberg *et al*., 2017).

8.2 METABOLITES FOR THE FORMULATION OF BIOINSECTICIDES

As part of agricultural practices, it is necessary to ensure crop yield on plantations; for a long time this has been achieved through the use of synthetic substances for pest control, however excessive use and its high persistence in the environment generate problems of accumulation and exposure, making it a dangerous practice for health, which has led to the need to find ecologically friendly alternatives that present a lower environmental impact.

In recent years, plant-derived compounds, such as some secondary metabolites, have attracted attention as a viable option to synthetic insecticides, since they have certain advantages such as lower toxicity, less persistence and easier degradation, being ecologically friendlier to humans and non-target organisms.

Some of the main phytochemical agents that are being studied, in recent years, for these purposes are the essential oils of plants. These compounds have extensive biological activity against different pests and the vast majority exhibit low toxicity to mammals, which is why they are recognized as safe to use. They are located within the group of terpenoids, and in plants, they are found in variable quantity depending on the species and some environmental factors, ranging between 0.1% and 10% (Walia *et al*., 2017). Widely known plants such as mint *(Mentha piperita)*contain essential oils effective against flies, ants, moths and some other insects. According to the review made by Hikal and collaborators in 2017, some essential oils also have ideal characteristics to replace synthetic insecticides, such as growth inhibition, oposition and repellent; therefore, work is carried out for the development of formulations that can be used in the field (granulations, aerosols, among others).

8.3 METABOLITES IN OBTAINING PHARMACEUTICAL PRODUCTS

Due to their great diversity in nature and the ability to synthesize products with complex structures, plants have been an intensively used resource in the search for products that meet the needs of human health. For a long time now, traditional medicine has figured as the main tool for the relief of the diseases that afflict us and it is from this same, where modern medicine has managed to find several compounds with characteristics of great potential to act as novel therapeutic agents. Two of the largest and best documented records of the use of medicinal plants are found in the compendiums of traditional Chinese medicine and traditional Hindi medicine. Many natural compounds have been studied to test their ability as an active ingredient in formulations, since it is presumed that they have activity/ against various agents among which can be mentioned: antiparasitic, antiviral, antimicrobial, anticancer, among others (Aye *et al*., 2019).

The use has been so wide and widespread that some species of plants are even known which have been cataloged as medicinal plants, and in some cases can serve as edibles, among these can be mentioned mint (*Menta* sp.), turmeric (*Curcuma longa),* mandrake (*Mandragora officinarum*), ginseng (*Panax ginseng)* and several other species (Patra *et al*., 2018).

In science there are two main disciplines, which are ethnobotany and ethnopharmacology, which are responsible for studying the aspects related to plants, their use and obtaining products that contain beneficial elements for the relief of diseases from them (Anand *et al*., 2019). Together with these disciplines and the advantages they offer, in addition to the interesting and attractive properties of plant-derived products, there is a panorama of hope in the face of one of the biggest problems that concern worldwide, the next bacterial apocalypse and the uncertain future that awaits us with bacteria super resistant to multiple drugs; in which the search for new biologically active products is the key role for the innovation of new therapies.

In this field, plant-derived products are in growing demand worldwide and are a good prospect to contribute in a great way, since there are records that an amount above 50% of the compounds used in modern medicine come directly (isolated from different parts) or indirectly (modified after isolation) from plants (Shakya, 2016).

One of the most promising examples is the antimalarial drug artemisinin (obtained from *Artemisia annua* L.), which could be discovered thanks to the extensive study of plants used in traditional Chinese medicine, which earned its discoverer, the Nobel Prize in medicine in 2015.

Recently with the massive outbreak of the Wu Han coronavirus in 2019 (Sars-Cov-2), which spread around the world and is a current health problem, plant-derived products are still in scientific interest being able to provide possible treatments such as the compounds crocin, digitoxygenin and β-eudesmol; phytochemical agents recognized for their antiviral activity, as mentioned in the study conducted by Aanouz *et al*. (2020).

8.4 METABOLITES AS NUTRACEUTICALS

Nutraceuticals are oral dietary components found naturally in foods and are thought to have a medical or health benefit. This term was coined in 1989 by Dr. Stephen Defelice, who combined the words "nutrition" and "pharmacist" (Souyoul *et al.*, 2018). Some plant-derived metabolites and phytochemicals have been shown to be beneficial to health, nutraceuticals are designed to improve physical, mental health, increase longevity, etc. Traditionally, these are referred to to a greater extent as nutrients, but they also have health benefits and, therefore, represent the intermediate part that exists between those that are considered as food and those contemplated as drugs (Tanna and Mishra, 2018). The popularity of these products has increased among people and health providers, especially towards natural nutraceuticals, which are characterized by coming directly from nature, without any change in their natural form, among these are, probiotic microorganisms, enzymes, chemical constituents and phytochemical compounds, which we will focus on in this section (Chanda *et al.*, 2019). Flavonoids and other phenolic compounds are perhaps the main biologically active metabolites that have been studied as nutraceuticals, occur naturally in numerous plant species, and more than 8,000 of these compounds have been reported, whose known activities range from compounds that have antioxidant properties, such as the best known, to compounds with anti-inflammatory activity, skin protection, antibacterials, and others (Tungmunnithum *et al.*, 2018). There is a greater evidence to suggest that various plant-based foods may increase cellular resistance to aging, inflammation, autophagy, and oxidative stress. Various phytochemical compounds such as turmeric, resveratrol, lycopene, carotenoids, among others have demonstrated various beneficial activities for health (Davinelli and Scapagnini, 2019).

8.4.1 TURMERIC

Turmeric (Curcuma longa) belongs to the Zingiberaceae family and has been traditionally used for centuries in Asian cuisine, it is used as an additive for a variety of products containing a medically acceptable intense yellow color. The most important part of the turmeric tuber is a group of bioflavonoids, i.e. curcumins (curcumin (77%), bisdemetoxicurcumin and demethoxycumin). Curcumin has important anti-inflammatory, antioxidant, chemoprotective, anticancer and gastroprotective properties. It affects the neurological system and is one of the most researched bioflavonoids (Maxine, 2018).

Curcumin shows antioxidant properties by 80%, this is because turmeric has the property of donating electrons to neutralize free radicals by creating stable products, prevents oxidation and modification of lipids of low-density lipoproteins, and as a consequence, the inhibition of prostacyclin (PGI2), which contributes to the formation of thrombosis and arteriosclerosis and therefore contributes to the prevention of the same. It also reduces tumor necrosis factor alpha (TNF-α), interleukins (IL-1, IL-2, IL-6, IL-8, IL12), chemokines, through the inhibitory effect on NF-κB (kappa cell factor B) so it presents anti-inflammatory activity (Jovičić *et al.*, 2017).

8.4.2 RESVERATROL

Resveratrol (3,5,4′-trihydroxy-trans-stylbene) belongs to the group of stilbenoids of polyphenols, which has two phenol rings joined together by an ethylene bridge. This natural polyphenol has been detected in more than 70 plant species, especially in the skin and seeds of grapes, and was found in discrete amounts in red wines and various human foods. It is a phytoalexin that acts against pathogens, including bacteria and fungi. As a natural food ingredient, numerous studies have shown that resveratrol possesses a very high antioxidant potential. Resveratrol also exhibits antitumor activity, and is considered a potential candidate for the prevention and treatment of several types of cancer (Salehi *et al*., 2018). Resveratrol's effects are attributed to its ability to interact with a wide range of targets including kinases, receptors, and signaling molecules. Clinical and preclinical data have shown that it can modulate numerous signaling molecules, including Wnt, nuclear factor -κB (NF- κB), cytokines, caspases, Notch, matrix metalloproteinases (MMP), 5′-AMP. activated protein kinase (AMPK), intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), sirtuin type 1 (SIRT1), tumor necrosis factor α (TNF-α), peroxisome proliferator-activated receptor coactivator γ-α 1a (PGC -1α), insulin-like growth factor 1 (IGF -1), insulin-like growth factor binding protein (IGFBP -3), among others (Singh *et al*., 2019).

8.4.3 LYCOPENE

Lycopene is the main pigment of tomato, it can also be found, to a lesser extent, in watermelon, pink guava, papaya and fruit-ween. It is an antioxidant carotenoid that has been proven to have beneficial effects for diseases that present oxidative damage of lipids, proteins and DNA such as cardiovascular diseases, cancer and osteoporosis (Vera Guerrero *et al*., 2019).

The following table summarizes some of the main secondary metabolites, the species where they have been found and their use as an agent to obtain pharmaceutical products, as a nutraceutical or with a specific activity (Kumar *et al*., 2017; Koh and Pan, 2018). In Table 8.1. Some plant-derived metabolites and the application they currently have are described.

Table 8.1. Plant-derived metabolites and some of their best-known uses.

Metabolite	Related species	Application	Reference
Capsaicin	*Capsicum* spp.	Anti-microbial agent/ Nutraceutical for weight control/ Insect repellent/ Analgesic and anti-inflammatory	Oyedemi *et al*. (2019) Reddi and Manjappara, (2020) Li *et al*. (2020) Ghiasi *et al*. (2019)
6-gingerol	*Zingiber officinale*	Anti-tumor and anti-inflammatory/ Anti-oxidant nutraceutical agent	de Lima *et al*. (2018) Li *et al*. (2019)
*Flavopiridol	*Dysoxylum gotadhora*	Anticancer and antitumor	Deep *et al*. (2018)
Resveratrol	*Vitis* spp. *Veratrum album*	Neuro and cardioprotective nutraceutical	Navarro *et al*. (2018)

Metabolite	Related species	Application	Reference
Limonene	*Citrus* spp.	Bioinsecticide/ Anti-inflammatory agent	Showler *et al.* (2019) Klimek-Szczykutowicz et al. (2020)
3,3'-diindolilmetano	*Brassica* spp.	Anti-inflammatory and anti-apoptotic/ Intestinal protective nutraceutical agent	Luo *et al.* (2018) Kim *et al.* (2019)
Timol	Family plants *Lamiaceae* •Thymus vulgaris	Agente cicatrizante y antiinflamatorio	Jiji *et al.* (2019)
Carvacrol	Family plants *Lamiaceae* •*Origanum vulgare*	Anti parasitic/ Anti-inflammatory agent/ Anti-oxidant nutraceutical agent	Borges *et al.* (2020) Ezz-eldin *et al.* (2020) Rúa *et al.* (2019)
curcumin	*Curcuma longa* L.	Neuroprotective and anti-oxidant nutraceutical/ Anti-tumor agent	Sarker and Franks, (2018) Moghtaderi *et al.* (2018)
Hexadecanoic acid Piperine	*Alangium salvifolium* *Piper* spp.	Bioinsecticida Bio insecticide/ Anticancer and antitumor/ gastroprotective nutraceutical	Thanigaivel *et al.* (2017) Samuel *et al.* (2016) Si *et al.* (2018b) Pongkorpsakol *et al.* (2015)
Eugenol	Plants of families *Lamiaceae, Lauraceae, Myrtaceae* y *Myristicaceae* *Syzygium aromaticum* L.	Bio insecticide and repellent	Reis *et al.* (2016)
Kaempferol	Multiple plant families •*Crocus sativus* L.	Anti-oxidant and neuroprotective nutraceutical	Zeka and Arroo (2016)
Coumarin and derivatives	Multiple plant families •*Dipteryx odorata* *Cinnamomum* spp.	Bio-insecticide	Si *et al.* (2018a)
Podophyllothoxyone and derivatives	•*Sinopodophyllum hexandrum*	Bio-insecticide	Che *et al.* (2019)
Linalol	Multiple plant families *Aniba rosaeodora* •*Ocimum basilicum*	Bio-insecticide/Nutraceutical/ anti-oxidant	Campos *et al.* (2018) Jabir *et al.* (2018)
Alin	*Allium sativum* L.	Antioxidant and anti-inflammatory nutraceutical	Sánchez-Sánchez *et al.* (2020)
Azadirachtin	*Azadirachta indica*	Bio-insecticide	Shah *et al.* (2019)
Estragol	*Satureja hortensis* L.	Bio-insecticide	Ebadollahi 2020
Eucalyptol	*Eucalyptus* spp.	Bio-insecticida/ Nutraceutical anti oxidant and anti inflammatory agent	Sarma *et al.* (2019) Kennedy-Feitosa et al., 2016

**Synthetic derivative, • Among the most common*

8.5 METHODS OF EXTRACTION AND IDENTIFICATION OF METABOLITES AND THEIR USE IN PLANT METABOLOMICS

8.5.1 SECONDARY METABOLITE EXTRACTION METHODS IN PLANTS

The secondary metabolites of plants are molecules of great interest due to their great applicability in various industrial sectors. For the isolation of these compounds it is important to know their chemical nature and, in this way, select the extraction method that guarantees the purity of the desired metabolite. During the extraction of secondary metabolites in the plant material, it is important to minimize the co-extraction of unwanted compounds, avoid the decomposition of metabolites and / or avoid the formation of artifacts derived from the conditions of the isolation methodology. This section describes in a general way the extraction methods most used today, as well as their advantages and disadvantages.

Extraction is the first step in separating the desired metabolites from the raw materials. The extraction methods can be generally classified into three types, liquid-liquid extraction, solid-liquid extraction and supercritical fluid extraction, which in turn have other sub-classifications.

8.5.1.1 Liquid-liquid extraction

Liquid-liquid extraction is a widely used method for the recovery and purification of the desired metabolite in solution, in this methodology the compound of interest (solute) is transferred from one solvent to another due to the difference in solubility or the distribution coefficient of two solvents that are immiscible or partially miscible with each other (Chen and Wang, 2017). In this methodology, the solute to be extracted will pass into the solvent with the polarity value close to the polarity of the compound to be extracted, so the selection of the solvent is crucial and its selectivity and safety must be considered. Also, it must be taken into account that in the liquid-liquid extraction it comprises a mixing step (contact), followed by a phase separation step, so it is important to consider the selection of solvents in both steps, because while vigorous mixing is favorable to the transfer of the metabolite from one solvent to another, it can also deteriorate the ease of phase separation, forming emulsions (Berk, 2018).

Alcohols such as methanol and ethanol are universal solvents due to their ability to extract polar and non-polar compounds, with methanol being the solvent with a wider extraction range compared to ethanol (Zhang *et al.,* 2018).

8.5.1.2 Solid-liquid extraction

In solid-liquid extraction the solvent penetrates the solid matrix and the metabolite (solute) diffuses out of the solid matrix thus allowing the recovery of this in the liquid phase. In this extraction there are several factors, such as particle size, type of solvent, temperature, and extraction time that affect the efficiency of this methodology. In the case of particle size, generally, the finer the particle size, the more efficient the extraction due to improved solvent penetration and solute diffusion. However, too thin a particle size could lead to excessive absorption

of the solvent into the solid matrix making it difficult to separate it later; On the other hand, high temperatures increase the solubility and diffusion of the compound to be extracted. However, too high temperatures can cause solvent loss, leading to extracts of undesirable impurities and the breakdown of thermolabile components.

Alcohols such as methanol and ethanol are universal solvents due to their ability to extract polar and non-polar compounds, with methanol being the solvent with a wider extraction range compared to ethanol (Zhang *et al.* 2018).

Maceration, Soxhlet extraction, percolation, infusion, decoction and ultrasound-assisted extraction are methodologies that can be classified as solid-liquid extractions and of which we will describe below mentioning their characteristics, as well as their advantages and disadvantages.

8.5.1.3 Maceration

This method consists of immersing the raw material in a solvent with a polarity close to the metabolite to be extracted for a certain time (approximately 3 days) at room temperature and with frequent stirring. After extraction, the solvent is removed from the mixture, often by vacuum evaporation, to concentrate the product. The vital point of this method is the choice of solvent, which delineates the classes of compounds recovered from the samples and also allows the use of maceration for the extraction of thermolabile components. It is a simple extraction method, although it presents the disadvantage of a long extraction time and low efficiency (Mazzutti *et al.,* 2020).

8.5.1.4 Soxhlet Extraction

Soxhlet extraction is a comprehensive extraction technique widely applied to analytes that are sufficiently thermally stable. In this extraction, a small amount of dry sample is placed on a thimble, which is placed in a distillation flask which contains the extraction solvent. The vapors of a new solvent, produced in a distillation flask, then pass through the thimble containing the material to be extracted and liquefy in the condenser. When the liquid reaches the overflow level in the thimble, a siphon sucks the solution, and the liquid falls back into the distillation flask, carrying the extracted solutes into the liquid. The separation of the solute from the solvent takes place in the distillation flask. The solute is then left in the flask and the fresh solvent vapors return to the solid bed of the sample material. The advantage of this system is that, instead of passing many portions of hot solvent through the sample, only one batch of solvent is recycled. However, a disadvantage of this procedure is the long extraction times because it is related to high energy consumption and can severely decrease sample performance (Rakhee *et al.,* 2018; Nafiu *et al.,* 2017; Zygler *et al.,* 2012).

8.5.1.5 Infusion

In this method, extraction involves soaking the plant material, either cold or boiling water for a short period of time. The disadvantage of this method is the same as in traditional methods such as soxhlet and maceration, the long extraction times and the large amount of solvent use (Alam *et al.,* 2019; Ngaha Njila *et al.,* 2017).

8.5.1.6 Decoction

Decoction is a method used for the extraction of water-soluble and thermoset compounds. During this methodology, water is added to the plant material and the mixture is subjected to heating for a certain time at a temperature of 100 ºC. Subsequently, it is allowed to cool to room temperature and filtration is carried out to obtain the filtration. This filtrate is concentrated to the extract obtained. The main disadvantage of decoctions is that water is not a good solvent for many of the active components of herbs. This problem is compounded by the relatively short extraction time used in its preparation (usually 5 to 10 minutes) (Ngaha Njila *et al.,* 2017; Bone and Mills, 2013).

8.5.1.7 Percolation

Percolation is a method where plant material is moistened and subsequently packed into a cone (not too soft or too hard) and the solvent is poured over the top of the plant material dripping slowly over several hours. Percolation is more efficient than maceration because it is a continuous process in which the saturated solvent is constantly replaced by new solvent. However, it is a time-consuming process and in this a large consumption of solvents is made (Zhang *et al.,* 2018; Sarker *et al.,* 2006).

8.5.1.8 Ultrasound-assisted extraction

This extraction method is performed using ultrasound radiation provided by an ultrasonic water bath or by other devices, such as probes, sonorreactors, or microplate horns. However, the most available and cheapest is the ultrasonic bath that allows easy simultaneous extraction of several samples. In this methodology the plant material is put in contact with the extraction solvent and then with the ultrasound radiation which is transmitted through a medium causing a disturbance that is repeated periodically and generates cycles of expansion and compression in the molecules of the medium with the formation and collapse of bubbles. The implosion of these bubbles generates changes in temperature and pressure which improve the penetration of the solvent into the matrix. Consequently, the mass transfer of the analytes to the solvent is increased.

This technique minimizes the consumption of solvents and the generation of waste, being therefore safer and more respectful with the environment. Although, it is important to control the experimental conditions to avoid the degradation of analytes that can occur during the extraction procedure (Albero *et al*., 2019; Mussatto, 2015).

8.5.1.9 Supercritical fluid extraction

The supercritical extraction method is a new separation technique developed in recent years. It is used to extract and separate substances using a supercritical fluid as a solvent. In this fluid, both the temperature and the pressure of the supercritical fluid are higher than the critical point, which is why it has favorable transport properties to extract valuable compounds such as its density close to that of a liquid, which generates a high solvation power for extract the compounds of interest. In addition, low viscosity and high diffusion coefficients are other characteristics of a supercritical fluid that confer excellent solvent penetration into the solid matrix and, therefore, high extraction yields (Medina *et al*., 2019).

The most used supercritical fluid is CO2 because the temperature of CO2 is close to room temperature, and it has a low critical pressure which offers the possibility of operating at moderate pressures, generally between 100 and 450 bar. Other supercritical fluids used are hexane, pentane, butane, methanol, nitrous oxide, sulfur hexafluoride, and fluorinated hydrocarbons.

This extraction technique is carried out using a supercritical fluid extraction system, which consists of a mobile phase tank, generally CO2, a pump to pressurize the gas, a container and a co-solvent pump, an oven that contains an extraction container, a controller to maintain high pressure within the system and a flow meter.

The advantage of extraction with supercritical fluids is a faster extraction time compared to convectional methods such as maceration, soxhlet, percolation and decoction. It is a suitable method for thermolabile compounds because it is operated at room temperature; it uses little amount of organic solvent and the selectivity of the supercritical fluid is greater than that of the liquid solvent, since its solvation power can be adjusted by changing temperature and/or pressure. The main disadvantage of supercritical extraction is the cost of operation because this method is more expensive than traditional extraction processes (Ngaha njila *et al.,* 2017; Wang *et al.,* 2016).

8.6 ISOLATION AND IDENTIFICATION TECHNIQUES

Secondary metabolites in a plant are usually found in complex matrices and at low levels, because of this, like extraction, another factor to consider is an isolation and identification protocol for this purpose it is important to know the characteristics of the compounds of interest such as their solubility (hydrophobia or hydrophilicity), the acid-base properties, load, stability and molecular size. These factors are key to choosing the most appropriate method for isolation with a high degree of confidence. However, it is more difficult to design an isolation protocol for a crude extract where the types of compounds present are unknown or have not been previously described (Sarker and Nahar, 2012).

The most commonly used separation techniques to obtain the pure compound from a mixture of compounds in the plant are chromatographic. The International Union of Pure and Applied Chemistry (IUPAC) defines chromatography as a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a defined direction. These techniques have become the most popular and versatile analytical techniques in today's laboratories. One of the key and powerful advantages is that these techniques are extremely flexible and can be adapted to meet analytical needs at stages of development processes. Chromatographic systems have been innovated to solve or minimize many of the problems presented by these techniques. (Aqel, 2018).

Chromatographic techniques can be cataloged in three ways, according to their separation mechanism, based on their stages of development and by the shape of the chromatographic bed. This last classification is the one that will be used because it allows us to catalog all the chromatographic techniques in a more disaggregated way.

8.6.1 FLAT CHROMATOGRAPHY

Flat chromatography methods involve the separation of problem mixtures by using a support matrix, called a stationary phase, which can be a cellulose, alumina or silica gel matrix. Depending on the type of support to be used, flat chromatography can be classified into paper chromatography, when cellulose is used and thin layer chromatography when alumina or silica gel is used.

8.6.2 PAPER CHROMATOGRAPHY

Paper chromatography is the simplest and cheapest technique of chromatographic methodologies. The chromatographic bed of this technique consists of a sheet of paper, that is, cellulose. The stationary phase consists of water adsorbed to the cellulose, as well as to the polymer itself. The sample solution to be separated is applied as a point near one end of the paper. Subsequently, the leaf is immersed in the mobile phase which ascends (or descends, since the descending mode is also possible) in the stationary phase. When the mobile phase has almost reached the other end of the sheet, the paper is removed from the developing tank and dried. If the analytes are not visible because they are not colored, the leaf is treated with a reagent to visualize the spots. (Meyer, 2013).

8.6.3 THIN LAYER CHROMATOGRAPHY

Thin layer chromatography is similar to paper chromatography in terms of methodology development. However, in this technique the stationary phase is composed of an aluminum or glass plate impregnated by an adsorbent that can be silica gel or alumina. The mobile phase can be a solvent or a mix of solvents that will allow chromatographic separation, for this procedure called development, the stationary phase is deposited in a chromatographic chamber along with the mobile phase which rises through the thin layer) by capillary action. As the mobile phase moves up the chromatographic plate, the individual components move at different speeds depending on the intermolecular forces between the components with stationary phase and the components with the mobile phase. These adsorption forces of the compounds increase with the increasing polarity of the functional group, this allows the components of the mixture to separate at different points depending on their attraction relative to the stationary phase or the nature of the solvents that make up the mobile phase. The separation of the components present in the sample is measured with a value called the distribution factor (R*f*)which reflects how the chemicals in the mixture interact with a particular chromatographic system. These retention values can be compared to those of a known sample of the same compound to help confirm its identity. However, another confirmation may also be necessary because other compounds may have similar retention characteristics. One way to obtain additional confirmation is whether the unknown compound and the reference compound have the same retention in various types of chromatographic conditions, such as in different columns or mobile column/phase combinations (Majik *et al.,* 2019; Hage, 2018).

Table 8.2. *Plant metabolites that have been obtained with different extraction methods*

Metabolite Extracted	Plant species	Biotechnology application	Extraction method / yield obtained	References
Paclitaxel	*Taxus chinensis*/ *Taxus Wallichiana*	Medicinal: Antineoplastic	EAM: 99%, EAU: 93.1% ± 4.4%	Lee and Kim, (2019); Ghaffar *et al.* (2019)
Vinblastine	*Catharanthus roseus*	Medicinal: Antineoplastic	FSC: 92%, EAU: 90.9% ± 1.06%	Falcão *et al.* (2017); Yang *et al.* (2011)
Camptothecin	*Nothapodytes nimmoniana*	Medicinal: Antineoplastic	EAU: 78 %, EAM: 95%	Patil and Akamanchi (2017a) Patil and Akamanchi (2017b)
Ergosterol	*Agaricus blazei* Murrill	Medicinal: Antineoplastic	MAE: 25.44 ± 5.1 mg/100 g	Taofiq *et al.* (2019)
Piperine	*Piper nigrum*	Medicinal: Anti tumor Bioinsecticide: Against mosquito Nutraceutical: gastroprotective	EAU: 14.83 g /20 g (74.1%)	Ahmad *et al.* (2019)
Resveratrol	*Polygonum cuspidatum*	Medicinal: anti-inflammatory, antioxidant	ELL: 11.47 mg/g ELL: 0.90 mg/ 100g (73.8%) EAU: 3.82 mg/g,	Wang *et al.* (2019); Wang *et al.* (2013); Kuo *et al.* (2014)
Pilocarpine	*Pilocarpus microphyllus*/ *Pilocarpus jaborandi*	Medicinal: cholinergic agonist	EAU: 18 mg /g, EE: 1.14 µg/mg	Pereira *et al.* (2018); Jun-Ho *et al.* (2013)
Ascorbic acid (vitamin C)	*Myrciaria dubia* / Rough rose Thunb/ *Clinacanthus nutans*	Medicinal: Vitamin	UHPLC-DAD: 1.297 g/100g (92.81%), EAU: 6.38 mg /g EAM: 0.166 mg/g	Cunha-Santos *et al.* (2019); Um *et al.* (2018); Yu *et al.* (2017)
Steviosides	*Stevia rebaudiana*	Food: sweetener	EAU: 68.3 mg/g, EAM: 70.5 mg/g	Yılmaz *et al.* (2020)
Vanillin	*Vanilla Planifolia*	Food: flavoring / flavoring	FSC: 19.6808 g/Kg, ED: 5.3 mg/g EAU: 5.1 mg/g, EAM 6.7 mg/g:	Hernández-Fernández *et al.* (2019); Dong *et al.* (2014)
lycopene	*Solanum lycopersicum*	Food: nutraceutical Antioxidant	UAE: 94.3 mg/kg	Rahimpur and Dinani (2018)
Capsaicin	Capsicum annuum L / *Capsicum frutescens*	Food: flavoring	FSC: 2.10% (p/p) ,EAM: 5.28 mg/g, EAU: 4.01 mg/g	Shah *et al.* (2020); Chuichulcherm *et al.* (2013)
Menthol	*Mentha arvensis* / *Mentha piperita*	Food: flavoring / flavoring	(ED:5.44 mg/g; DPV: 17.0 g/kg)	Cam *et al.* (2019); Batool *et al.* (2018)

Metabolite Extracted	Plant species	Biotechnology application	Extraction method / yield obtained	References
Limonene	*Citrus sinensis* L.	Food: flavoring / flavoring	ESX: 1.20 % p/p ;FSC: 3.3µg / mL	Battista *et al.* (2020); Jokić *et al.* (2019)
Limonene	*Citrus* spp.	Bio insecticide: Against fly	ESL: 1.81 g/g	Ozturk *et al.* (2019)
Geraniol	*Cymbopogon Martini* R.	Bio insecticide: Against fly and mosquito	UAILE: 0.51 g/3g (1.73%)	Thakker *et al.* (2018)
Borneol	*Cinnamomum camphora*	Bio insecticide: Against psocopters	ESX: 4.936mg/g	Fu *et al.* (2020)
Azadirachtin	*Azadirachta indica*	Bio insecticide	EAU: 86.445 mg/g	Farjaminezhad and Garoosi (2020)
Linalol	*Coriandrum sativum*	Bio insecticide: Against weevil	DPV: 13.45g/kg	Zhang *et al.* (2016)
Eugenol	*Syzygium aromaticum*	Bioinsecticide: Repellent activity	FSC: 3.22 ± 0.025 g/kg	Frohlich *et al.* (2019)
Safrole	*Ocotea Odorífera*	Bio insecticide: Cockroach repellent	DPV: 18.01 g/kg	Almeida *et al.* (2018)
Sabineno	*Chamaecyparis obtusa*	Bio insecticide: Against corn weevil	ESL: 11.31 mg/g	Kim *et al.* (2018)
Coumarin	*Mikania laevigata* Schultz	Bio insecticide: Against coleoptera	ESL: 4.5 mg/g	Silva *et al.* (2018)
Luteolin	*Vitex negundo*	Cosmetic: Lightener	EAU: 1.82 ± 0.04 g/kg	Lama *et al.* (2019)
Ferulic acid	*Avena sativaL/Beta vulgaris/ Zea mays*	Cosmetics: Antioxidant	FSC: 53.6 µg / g,ED: 957.4 mg/L, SPM: 8.47 g / kg	Fernández *et al.* (2019); Aarabi *et al.* (2016); Zhao *et al.* (2014)
Esqualeno	Amaranthus sp / Glycine max	Cosmetics: emollient	EAM: 16.456 mg/100 g ,ED: 1.2 g / kg FSC: 3.4 g / kg, ESX: 4.0 g / kg	Lozano *et al.* (2019); Lozano *et al.* (2018); Krulj *et al.* (2016)
alpha-bisabolol	*Eremanthus erythroppapus*	Cosmetics: anti-inflammatory, soothing, healing	EAU: 8.90 g/kg, EFS: 16.53 g/kg	Santos *et al.* (2019); Santos *et al.* (2017)
Gallic acid	*Syzygium cumini/ Avena sativa/ Eucalyptus globulus*	Cosmetics: astringent and antioxidant	EAU: 100.07 mg, FSC: 97.1 mg/100g, EAM: 3.83 mg / g	Mahindrakar and Rathod (2020), Fernández *et al.* (2019); Liu *et al.* (2016)
Theobromine	*Theobroma cacao L*	Cosmetics: antioxidant	FSC: 17.593 mg / g	González *et al.* (2019)

EAU: ultrasound assisted extraction, EAM: microwave assisted extraction, FSC: Super critical fluids, ELL: Liquid-liquid extraction, UHPLC-DAD: Ultra high performance liquid chromatography coupled to photodiode array, ED: Solvent extraction, DPV: steam distillation, ESX: Soxhlet extraction, SPM: membrane separation

8.7 CONCLUSIONS AND REMARKS

According to what is described in this chapter of the book, plants are good biological models to extract different compounds that can be applied in the area of cosmetics, medicine, food, among others. Every day it is necessary to innovate in the techniques of extraction and identification of compounds, since to date many compounds are still far from being detected due to their complex biochemical characteristics. In the future, the incorporation and validation of new methods will allow us to obtain a greater number of compounds from any plant extract or other biological model.

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