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Bio-production d'acide itaconique à partir de biomasse végétale, pour une finalité matériaux

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“La science n’a pas de patrie”

Louis Pasteur

“Despacito y buena letra, que el hacer las cosas bien, importa más que el hacerlas”

Antonio Machado

A mes champignons…

Remerciements

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COMMUNICATIONS LIEES AUX TRAVAUX DE THESE

Publications

Les résultats présentés dans cette thèse font l'objet de 3 articles et d'une revue bibliographique.

Articles publiés :

- Jiménez-Quero A.; Pollet E.; Zhao M.; Marchioni E.; Averous L.; Phalip V. (2016) – « Itaconic and fumaric acid production from biomass hydrolysates by *Aspergillus* strains », *Journal of Microbiology and Biotechnology*, Sep 28;26(9): 1557-1565.
- Jiménez-Quero A.; Pollet E.; Zhao M.; Marchioni E.; Averous L.; Phalip V. (2016) – « Fungal fermentation of lignocellulosic biomass for itaconic and fumaric acid production », *Journal of Microbiology and Biotechnology*, doi: 10.4014/jmb.1607.07057.

Articles que seront soumis ultérieurement :

- Jiménez-Quero A.; Pollet E.; Averous L.; Phalip V. – « Optimization solid state fermentation of lignocellulosic biomass into itaconic and fumaric acid ».

- Jiménez-Quero A.; Prévot F.; Averous L.; Phalip V. – « Building blocks from lignocellulosic resources – Bioproduction of organic acids towards biobased polymers ».

Conférences

Les résultats présentés dans cette thèse ont fait l'objet de différentes communications dans conférences nationales et internationales :

- Jiménez-Quero A.; Pollet E.; Avérous L.; Phalip V. – « Itaconic and fumaric acids production from biomass valorization, towards biobased polymers elaboration », *poster*, 5th International Conference of Biobased and Biodegradable Polymers, Donostia-San Sebastian (Espagne, 6 au 9 Octobre 2015).

- Jiménez-Quero A.; Pollet E.; Avérous L.; Phalip V. – « Biomass valorization to value-added chemicals (Itaconic and Fumaric acids) via microbial fermentation », *communication orale*, Séminaire de Microbiologie de Strasbourg, Strasbourg (France, 3 mars 2016).

- Jiménez-Quero A.; Pollet E.; Avérous L.; Phalip V. – « Value-added chemicals (Itaconic and Fumaric acids) from biomass, towards biobased polymers elaboration », *communication orale et poster*, Forum BioChem, meeting academia-industry, Strasbourg (France, 2 au 3 juin 2016).

- Jiménez-Quero A.; Pollet E.; Avérous L.; Phalip V. – « Itaconic and Fumaric acids, value-added chemicals from lignocellulosic biomass, towards biobased polymers elaboration », *communication orale*, 15th International Symposium on Biopolymers, Madrid (Espagne, 26 au 29 Septembre 2016).

LISTE DES ABREVIATIONS

AFEX	Ammonia Fiber Expansion
ARP	Ammonia Recycle Percolation
Aw	Water activity
AZCL	Azurine-crosslinked
BB	Building Blocks
BDO	Butanediol
CAD	Cis-aconitate decarboxylase
CC	Corn cobs
CDA	Czapek Dox Agar
CoA	Coenzyme A
DA	Dilute Acid
DNS	Dinitrosalicylic Acid
DOE	Department of Energy
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
FA	Fumaric Acid
FAO	Food Agriculture Organization
FDCA	Furandicarboxylic Acid
FH	Fumarase
FTIR	Fourier Transformed Infrared
GA	Glucaric Acid
GBL	γ-butyrolactone
GHG	Greenhouse Gas
HMW	High Molar Weight
HPLC	High-performance liquid chromatography
IA	Itaconic Acid
IAn	Itaconic acid Anhydride
IEA	International Energy Agency
IMI	International Mycological Institute

LA	Lactic Acid
LAB	Lactic Acid Bacteria
LCA	Life Cycle Analysis
LDH	Lactate Dehydrogenase
LHW	Liquid Hot Water
LMW	Low Molar Weight
LP	Lime Pretreatment
LSF	Liquid state Fermentation
MCF	Michigan Cancer Foundation
Mdh	Malate dehydrogenase
MI	Myo-inositol
MIOX	Myo-inositol oxigenase
MSW	Municipal Solid Waste
NRRL	Northern Regional Research Laboratory
OD/g*min	Enzyme activity
PA	Polyamide
PAA	Polyacrylic Acid
PBMS	Poly(butylene 2-methylsuccinate)
PBS	Polybutylene Succinate
PC	Polycarbonate
Pck	Phosphoenolpyruvate carboxykinase
PDA	Dextrose Agar
PDB	Potato Dextrose Broth medium
Pdh	Pyruvate dehydrogenase
PDO	Propanediol
PE	Polyethylene
PEF	Polyethylene furanoate
PET	Polyethylene terephthalate

PGFA	Poly(gadodiamide) Fumaric Acid
PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
PIA	Polyitaconic Acid
PIFB	poly(butylene fumarate-co-butylene itaconate)
Pk	Pyruvate kinase
PLA	Polylactic Acid
PP	Poly(propylene)
PPF	Poly(propylene) Fumarate
PS	Polystyrene
PtsG	Phosphoenolpyruvate-dependent glucose
PU	Polyurethane
PVC	Polyvinyl Chloride
ROP	Ring Opening Polymerization
SE	Steam Explosion
SmF	Submerged Fermentation
SSF	Solid State Fermentation
SuhB	Inositol monophosphatase
TCA	Tricarboxylic Acid
Tg	Glass Transition Temperature
THF	Tetrahydrofuran
TPA	Terephthalic acid
Udh	Urinate dehydrogenase
UMIP	Institute Pasteur
UN	United Nations
UPR	Polyester Resins
WB	Wheat Bran
wt	Weight

LISTE DE TABLEAUX ET FIGURES

Tableaux

Figures

Introduction

Chapitre 1

Chapitre 2

Chapitre 3

Chapitre 4

Conclusion

SOMMAIRE

INTRODUCTION GENERALE

Depuis quelques années le développement durable est une préoccupation majeure dans l'économie mondiale. Les problèmes environnementaux liés à l'utilisation des ressources pétrochimiques, la croissance démographique et l'accumulation des déchets ont conduit les chercheurs et les industriels à envisager l'utilisation des ressources renouvelables pour la production d'énergie et de matériaux. La biomasse est la ressource renouvelable la plus abondante avec une production annuelle de 506 millions de tonnes de carbone. Cependant, une grande partie de cette biomasse est destinée à l'alimentation et donc la stratégie la plus intéressante est l'utilisation de la biomasse lignocellulosique, non alimentaire. Celle-ci est composée de cellulose et d'hémicellulose (polysaccharides) et de lignine (réseau tridimensionnel de composés phénoliques) et est constitutive de la paroi des cellules végétales, du bois et de la paille. Le concept de bioraffinerie, utilisant la biomasse de façon optimale et complète, en particulier comme ressource pour les fermentations par les microorganismes s'est décliné dans différents secteurs comme l'alimentation humaine et animale, les biomolécules, les agromatériaux et les bioénergies (biocarburants, électricité). L'aspect énergétique est le plus avancé (bioéthanol de deuxième génération), mais la biomasse lignocellulosique est également utilisée pour la production de certains synthons (monomères réactifs).

Ce projet de thèse réalisé au sein de l'équipe BioTeam dirigée par le Pr. Luc Avérous à l'Institut de Chimie et Procédés pour l'Energie, l'Environnement et la Santé (ICPEES –UMR 7515– Université de Strasbourg) s'intègre parfaitement dans ce contexte de développement durable. Le projet doctoral a consisté à valoriser des déchets végétaux régionaux (son de blé et rafles de maïs) pour la production d'acide itaconique (Figure I1), un acide organique polymérisable qui peut se substituer à l'acide acrylique, pétro-sourcé. Pour réaliser cette valorisation, différents champignons filamenteux de genre *Aspergillus* ont été utilisés. Ces champignons sont connus pour dégrader les polymères végétaux et produisent différents biomolécules à l'échelle industrielle.

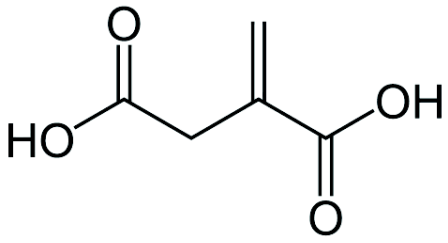


Figure I1: Structure moléculaire de l'acide itaconique.

Le travail de thèse a porté sur le criblage de différentes biomasses et différentes conditions de fermentation pour la production d'acide itaconique. Parallèlement à la bioproduction d'acide itaconique (IA), un autre acide organique, l'acide fumarique (FA) s'est révélé également intéressant du fait de sa production par les champignons filamenteux et de sa capacité à être transformé (synthon d'intérêt industriel).

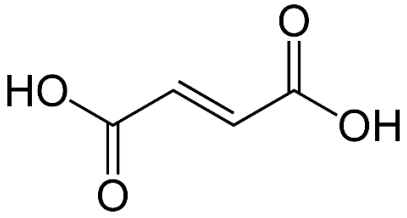


Figure I2: Structure moléculaire de l'acide fumarique.

Une étude bibliographique sur la bioproduction de différents acides organiques à partir de biomasse lignocellulosique est présentée dans le Chapitre 1. Elle est constituée essentiellement d'un article de synthèse bibliographique intitulé « Building blocks from lignocellulosic resources-Bioproduction of organic acids towards biobased polymers». La bibliographie montre l'augmentation des essais de production de certains acides organiques à partir de biomasse végétale, en complément de la production chimique à partir du pétrole. Cependant, l'utilisation des ressources renouvelables est limitée actuellement par l'aspect récalcitrant de la biomasse

lignocellulosique aux traitements biologiques. Des prétraitements sont donc nécessaires pour rendre accessibles les sucres fermentescibles de la biomasse. Cette étude détaille plus particulièrement la production de 4 acides organiques (acide lactique, fumarique, itaconique et glucarique) qui ont un grand potentiel comme synthons pour obtenir des polymères grâce à leurs fonctions réactives (doubles liaisons, fonctions carboxyliques). L'actualité économique dans ce domaine souligne l'importance de la production de ces acides par voie biosourcée (Figure I3).

Figure I3: Représentation schématique du contexte général de ce travail doctoral.

Le Chapitre 2 décrit la production des acides itaconique et fumarique à partir des hydrolysats issus de son de blé et de rafles de maïs. Un criblage a été réalisé avec 3 espèces du genre *Aspergillus* : *A. terreus* (deux souches ont été testées) qui est le producteur au niveau industriel d'acide itaconique; *A. tubingensis*, utilisé industriellement pour produire de l'acide citrique et gluconique ou des enzymes ; et *A. oryzae* très largement utilisé dans l'industrie agro-alimentaire et biotechnologique en Asie pour la production de saké ou de sauce soja, d'enzymes et d'acides organiques. Pour générer les hydrolysats liquides contenant des sucres fermentescibles, les deux biomasses ont subi deux types de prétraitements : un traitement chimique à l'acide sulfurique dilué à 150°C et un traitement biologique par hydrolyse enzymatique. La composition chimique des hydrolysats ainsi que la libération des inhibiteurs (composants phénoliques) ont été analysés pour t'enter d'expliquer le comportement des champignons pendant la fermentation.

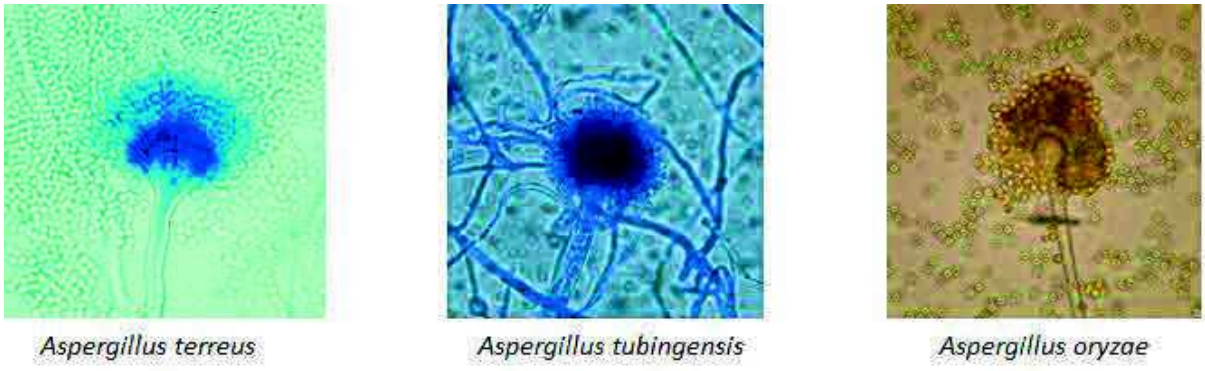


Figure I4: Espèces du genre Aspergillus utilisés pour les travaux de thèse.

Dans le Chapitre 3, la fermentation solide et la fermentation submergée, très utilisées en milieu industriel, sont évaluées sous forme de criblage comme dans le chapitre 2. Ces deux technologies ont été choisies car elles impliquent souvent une réduction du temps total des procédés par rapport à la fermentation liquide qui exige le traitement de la biomasse et ensuite la récupération des sucres libérés. De plus, dans le cas de la fermentation solide, la quantité de déchets générés à l'issue de la fermentation est très faible parce qu'il n'y a presque pas d'eau libre. Cela en fait un procédé intéressant au niveau économique (pas de traitement des déchets). Dans l'objectif de tester une autre alternative utilisé industriellement, les traitements enzymatiques du son de blé et des rafles de maïs ont été réalisés également de façon simultanée à la fermentation.

La fermentation solide s'avérant supérieure pour la production des acides organiques, dans le Chapitre 4 une optimisation des conditions de la conduite de ce type de fermentation a été effectuée. Une étude cinétique a été réalisée afin de de comparer le développement et la production d'acides par *A. terreus* et *A. oryzae* sur les deux biomasses. Une optimisation du pH de fermentation et du taux d'humidité est réalisée. Enfin, pour accomplir une fermentation à plus grande échelle, un prototype de fermenteur en sac de polypropylène a été employé afin de permettre un apport d'oxygène (facteur déterminant pour la production d'acide itaconique par voie fongique).

Au terme de ces volets expérimentaux schématisés par la Figure I5, une conclusion est présentée. L'utilisation de la biomasse lignocellulosique, comme les déchets de l'agriculture, constitue une bonne alternative pour la production d'acides organiques. De plus, dans le contexte de la bioraffinerie, des perspectives sont énoncées pour la co-valorisation d'autres produits issus des fermentations de cette biomasse : enzymes, biomasse fongique par exemple.

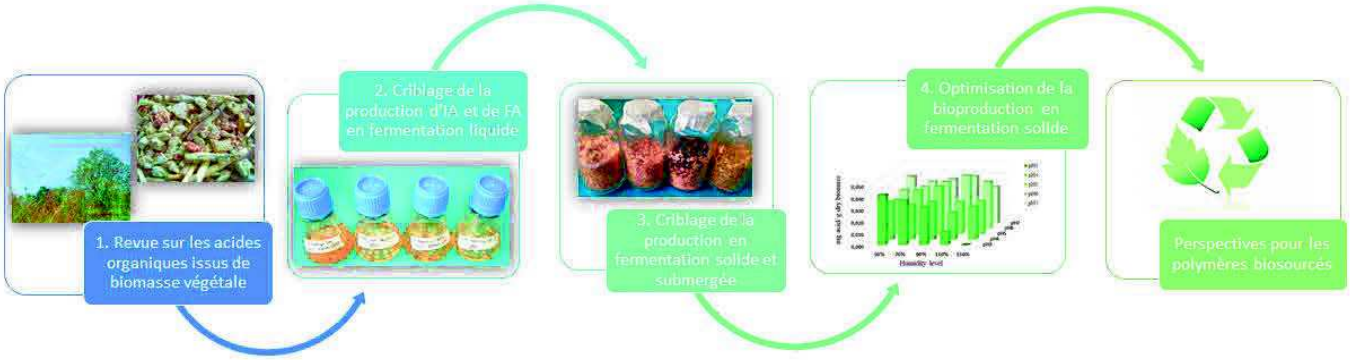
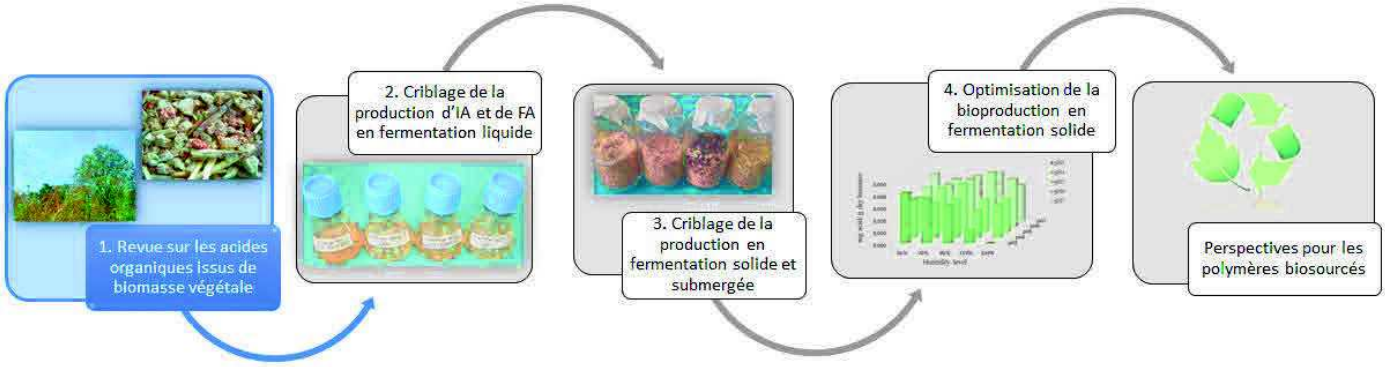


Figure I5: Schéma récapitulatif du travail de thèse.

Chapitre 1. Bioproduction de monomères et polymères issus de biomasse lignocellulosique
Etat de l'art



Chapitre 1

Introduction

La synthèse bibliographique présentée dans ce chapitre est consacrée à l'étude de la production polymères de façon biosourcée. Elle se présente sous la forme d'une revue intitulée « Building blocks from lignocellulosic resources - Bioproduction of organic acids towards biobased polymers ».

Dans un premier temps est exposée la problématique de l'utilisation de biomasse lignocellulosique pour la production des différents types de monomères polymérisables et de polymères. La principale difficulté est liée au caractère très résistant aux traitements biologiques – on parle de récalcitrante - de la biomasse. C'est pourquoi il est décrit dans cette revue les principaux prétraitements de de la biomasse lignocellulosique afin de rendre les sucres disponibles. Ensuite, il est discuté l'importance des acides organiques dans la chimie actuelle en tant que molécules plateformes. La production et la bioproduction de quatre de ces acides (acide lactique, fumarique, itaconique et glucarique) parmi les plus prometteurs sont décrites et les polymères qui en sont issus également. Enfin des perspectives sont énoncées, en soulignant notamment le caractère extrêmement prometteur de la biologie synthétique qui pourrait permettre de produire des polymères par voie entièrement biologique et en une seule étape.

Building blocks from lignocellulosic resources – Bioproduction of organic acids towards biobased polymers

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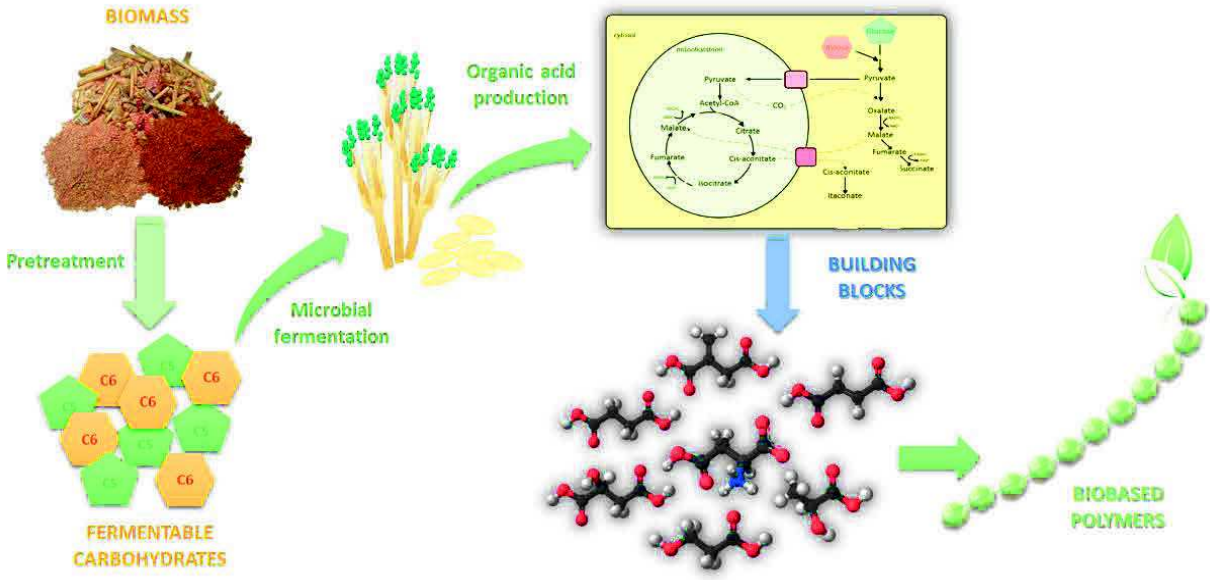

Abstract

In a period when a global awareness took place to prepare for the post-oil age, many scientific fields were stimulated to design products and processes that are able to compete with those from fossil resources. The production of organic acids from the most abundant renewable resource on Earth i.e. the lignocellulosic biomass, is for instance one of these fields. However, this resource is not directly and easily usable since the biomass can be recalcitrant to microorganisms and enzyme treatment. As a consequence, prior to bioproduction by the metabolic pathways of microorganisms, the biomass has to be pretreated to render its richness available. The benefits and drawbacks of the most used pretreatments methods are evaluated in this review. The US Department of Energy provided a list of "the top value biobased (from biomass) chemical" in 2004. This list has been updated more recently. In these lists we can find organic acid which are building blocks with reference of their ability to generate many value-added products thanks to their reactive groups. The objective is that biobased products to take for all or part of their counterpart fossil-sourced but also to generate new molecules not easily accessible by the current chemistry. It is particularly remarkable that the bioproduction processes of the organic acids have now reached very different stages of development from the proof of concept to the industrial development and commercialization. Among these main building blocks, four diacids were studied with more attention in this review: lactic acid (C3), fumaric acid (C4), itaconic acid (C5) and glucaric acid (C6). The reasons for this focus are both the economic forecasts extremely favorable and their capacity to polymerize to yield promising biobased polymers for a large range of applications such as packaging, construction, automotive, electronic, medical and cosmetics.

Keywords: building block, biobased polymer, lignocellulosic biomass, organic acid.

1. Introduction

Green chemistry has been defined as “a domain which efficiently utilizes (preferably renewable) raw materials, eliminates waste and avoid the use of toxic and/or hazardous solvents and reagents in the manufacture and application of chemicals products” (Sheldon, 2014). In connection with the concept of green chemistry, a biorefinery is defined as a facility regrouping processes and equipment to transform the biomass to value added products, biofuel and chemicals. There is a strong analogy to the petroleum refinery (Cherubini, 2010; Sauer et al., 2008). In 2007, the conference IEA (International Energy Agency) released a short definition of this notion: “Biorefining is the sustainable processing of biomass into a spectrum of marketable products and energy” (Van Ree and Annevelink, 2007).

The use of fossil substitutes can be a strong answer to a great number of nowadays issues, with the reduction of emissions of greenhouse gases and limiting the dependence of the economy to volatile prices (Gallezot, 2012; John et al., 2007). In the past few years, the significant increase of scientific papers shows the interest in biomass as raw material for the production of biobased materials with equivalent properties to the fossil ones (Fernando et al., 2006; Lipinsky, 1981). This emerging concept uses the biomass richness to produce diverse chemicals, materials and energy outputs. First, grains, sugar cane and vegetable oils were used, but compete directly with food, with a significant impact on food prices, according to The United Nations (UN) and the Food Agriculture Organization (FAO) (Sheldon, 2014). By contrast, the second generation of biorefinery should enable the development of bio-fuels and platform chemicals using the lignocellulosic biomass as raw material. Lignocellulosic biomass is the most abundant renewable resources on earth (FitzPatrick et al., 2010) and is not in competition with food resources and it is often very cheap. Lignocellulosic biomasses are divided in four categories: (i) annual and perennial dry energy grasses, (ii) forest woody feedstock, (iii) municipal solid waste (MSW) and (iv) agricultural residues (Hadar, 2013).

Lignocellulosic biomass is composed of three main polymers: cellulose, hemicellulose and lignin. These polymers form a tridimensional organization called the cellular wall of plant, which is a very resistant structure (Himmel et al., 2007). Each lignocellulosic biomass is unique regarding the structural composition (Table 1.1).

Table 1.1. Composition of different lignocellulosic biomass (Cao et al., 1997; Miron et al., 2001; Sun and Cheng, 2002)

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwoods stems	40-55	24-40	18-25
Softwoods stems	45-50	25-35	25-35
Corn cobs	30-45	28-36	6-17
Wheat bran	12-15	25-36	3-11
Sugarcane bagasse	36-42	35-43	20-22
Switch grass	45	31	12

The goal of this review is to describe the main ways based on lignocellulosic biomass to produce organic acids by bioproduction, towards biobased polymers. The global process is based on two main steps: (i) a pretreatment of the biomass and (ii) the bioproduction of organic acids by fermentation. The first step often consists in the hydrolysis of polymeric sugars of the lignocellulosic material. For this purpose, many pretreatments involving chemical, physical, physico-chemical and biological approaches have been scrutinized depending the type of biomass and its following use. The choice of the pretreatment can be crucial key for a successful fermentation (Hendriks and Zeeman, 2008; Kumar et al., 2008). The most used pretreatments of lignocellulosic biomass such as steam explosion, liquid hot water, dilute acid, lime pretreatment and ammonia recycle percolation (ARP) / ammonia fiber expansion (AFEX) will be described below. The second step of the process is the fermentation using the liberated sugars by microorganisms to yield an interesting variety of metabolites with various industrial functionalities. In this context, the U.S. Department of Energy has elaborated in 2004 a rapport describing the top added-valued chemicals issue from biomass (Werpy et al., 2004). These chemical platforms are also called building blocks (BB) as they display multiple functional groups enabling them to generate many new or to replace existing end-products (generally issue from fossil resources). BB are able to polymerize, and representing an alternative to petrochemical polymers. More recently this list has been updated with others proposals (Bozell and Petersen, 2010). More particularly, the bioproduction of lactic acid (C3), succinic acid (C4), itaconic acid (C5) and glucaric acid (C6) will be discussed latter as promising BB, especially for the production of biobased polymers.

2. Pretreatment for lignocellulosic biomass conversion

2.1. Lignocellulosic biomass: composition and strategies

The global production of biomass is estimated around 10^{11} tons per year (60% terrestrial and 40% aquatic) (Mauser et al., 2015; Sheldon et al., 2007). The lignocellulosic biomass issue from agriculture and forestry is currently considered as a great source of carbohydrates (Anwar et al., 2014; Naik et al., 2010) able to replace the fossil sources to produce biofuels, chemical platform molecules and derivatives. Many strategies to use lignocellulosic biomass with high yields have been explored in numerous papers.

The lignocellulosic biomass essentially comprises the plant cell wall composed of cellulose, hemicellulose and lignin forming a tridimensional organization through different associations (Cosgrove, 2005). Each component plays a specific role in the plant cell wall. Cellulose is a linear polysaccharide of glucose: a chain of D-glucopyranosyl subunits, linked by β-1,4 glycosidic bonds (Agbor et al., 2011; Gross and Kalra, 2002). The cellulose is organized as a major crystalline (well organized) and an amorphous (not well-organized) structures. Six chains of cellulose packed thanks to hydrogen bonds form the microfibrils which are in turn associated by 6 to form macrofibrils (Thomas et al., 2013). Such a multi-fibers structure is very complex and rigid. This is one of the reasons why cellulose is hard to dissociate and is generally resistant to chemical and biological treatments (McCann and Carpita, 2015). Consequently, cellulose is difficult to decompose into its monomer glucose. Hemicellulose is a polysaccharide composed by pentoses like xylose and hexoses like mannose and glucose. Hemicellulose's structure is amorphous and branched. The hemicellulose ensures the connection between cellulose and lignin. It is responsible for the cohesion of the lignocellulosic structure and provide its high rigidity (Laureano-Perez et al., 2005). Furthermore, chemicals bonds exist between hemicellulose, cellulose and lignin (Boudet et al., 2003). The third component of plant cell wall is lignin which is the most recalcitrant element (Abdel-Hamid et al., 2013). This is an amorphous polymer composed of phenylpropane units (p-coumaryl, coniferyl, sinapyl alcohol).

Agreeing to plant cell wall complexity and its recalcitrance to biological treatments, biomass pretreatments are required and are abundantly documented. The goal of pretreatments is

to create an access to all carbohydrates present in the lignocellulosic biomass (Hendriks and Zeeman, 2008) (Figure 1.1). This step is crucial in the concept of biorefinery mentioned above since the cost of fermentation essentially depends of the cost of the pretreatment process (Kumar et al., 2009).

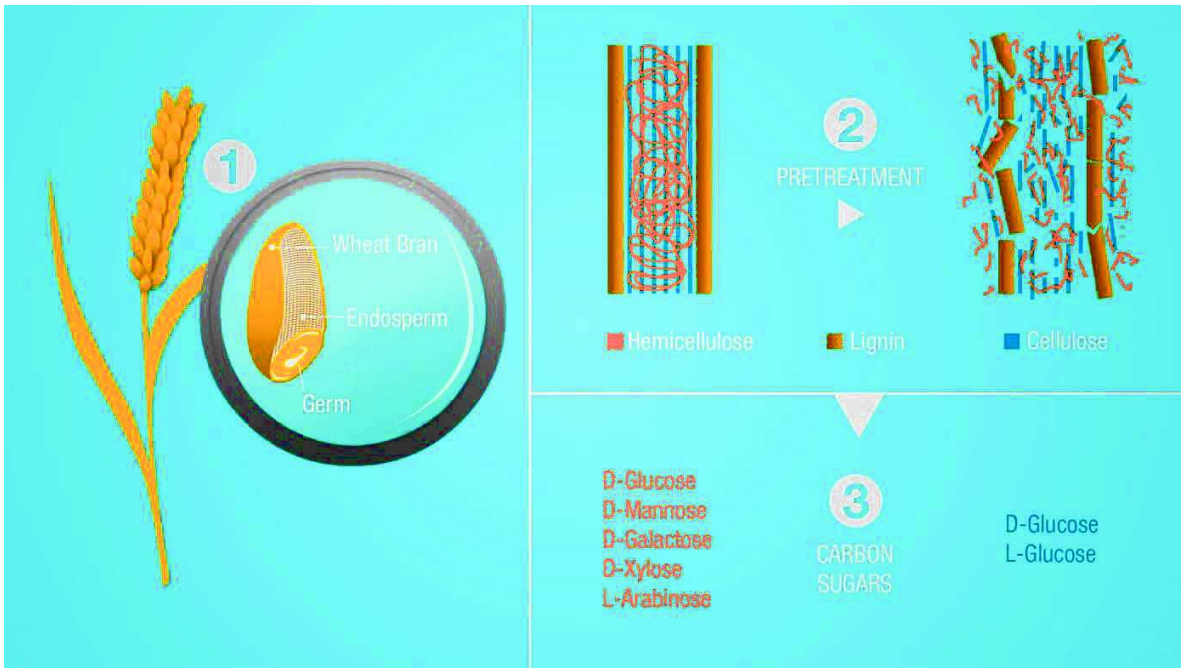

Figure 1.1. Illustration of lignocellulosic pretreatment (based on the wheat bran example).

2.2. Goal of the pretreatment

The usefulness of lignocellulosic material has been established many years ago for the production of the 2nd generation of biofuel in particular. These processes based on waste raw material (corn stover, wheat straw, bagasse…) were directly inspired of the production of biofuel from 1st generation, generally based on starch, which the use became less attractive due to the increase of worldwide food demand (Clark, 2007). Then, the non-food lignocellulosic biomass represents a good choice of starting material, also because of its high sugar content (55% to 75% of carbohydrate by dry weight). Nevertheless, lignin represents one of the major impediment for enzymatic hydrolysis. Furthermore, contrarily to starch, cellulose fibers are highly ordered and tightly packed together with highly crystalline structures, render them water insoluble and resistant

to depolymerization (Mosier et al., 2005a). Hemicellulose represents an additional source of carbohydrates, essentially of pentoses.

The main goal of pretreatment is to prepare the materials for fermentation (Hendriks and Zeeman, 2008). Pretreatment should enhance the digestibility of the lignocellulose to increase the yields of released fermentable sugars from cellulose and hemicellulose (Figure 1.2). The pretreatment should also decrease the cellulose crystallinity, increase the accessible surface area and decrease the protection of cellulose by lignin and hemicellulose (Sun and Cheng, 2002). Besides, the pretreatment should, (i) avoid full destructuration of cellulose and hemicellulose, (ii) enhance the liberation of monomer sugars or their access, and (iii) avoid the formation of inhibitors for the growth of microorganism during the fermentation (Menon and Rao, 2012; Wyman et al., 2005; Zha et al., 2014). It should be cost-effective and follow a green process i.e. minimizing the energy demand, the loss of raw material and the use of chemicals. Figure 1.2 below summarized the main factors to access of a convenient pretreatment.

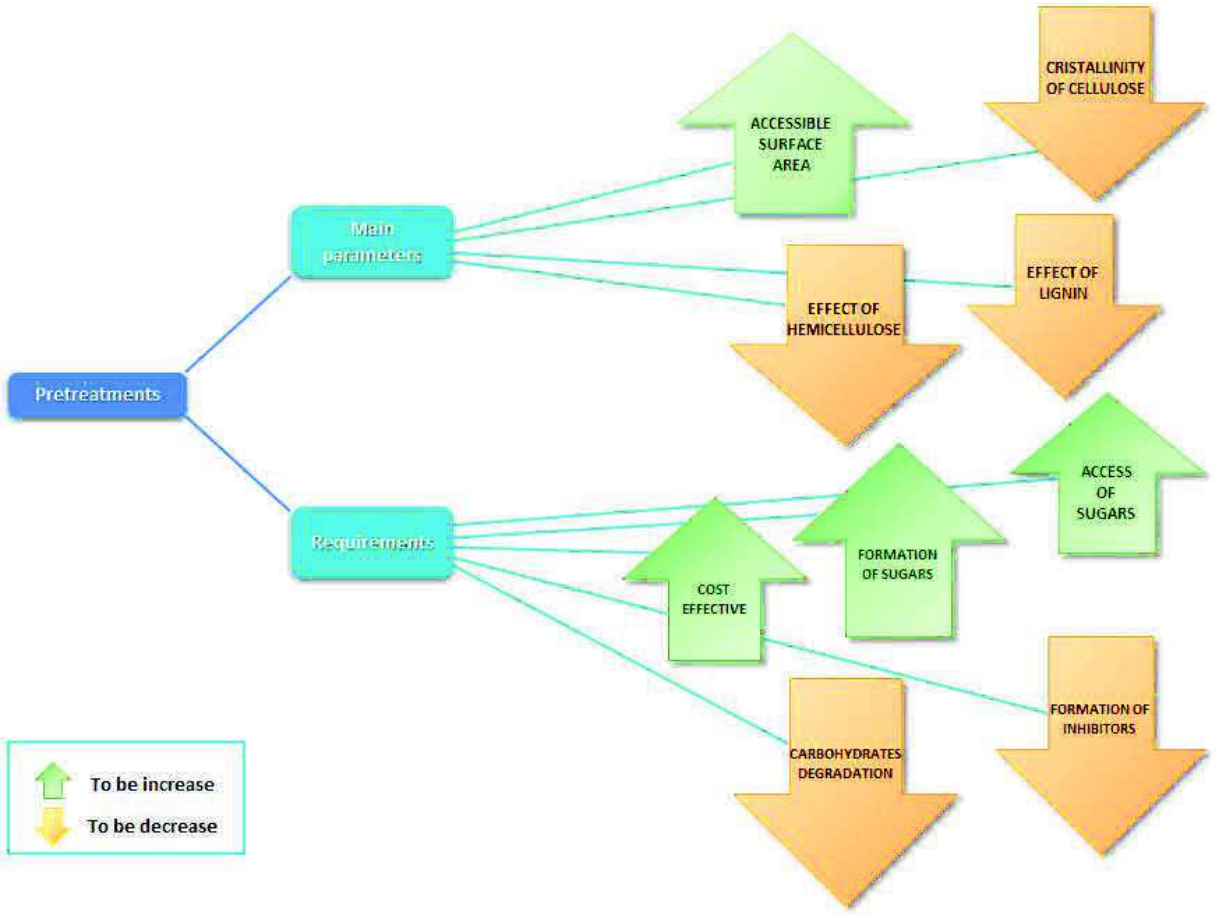

Figure1.2. Main parameters and requirements of lignocellulosic pretreatments.

2.3. Description of some pretreatments

There are four main categories of pretreatments: physical, chemical, physico-chemical and biological.

Physical pretreatments concern essentially size reduction of the particles of lignocellulosic material thanks to milling, grinding or chipping (Taherzadeh and Karimi, 2008). These pretreatments induce the increasing of the specific area and the size of pores, with a concomitant decrease of cellulose crystallinity. Physical pretreatments are generally associated with other pretreatments and represent a first step to enhance biomass digestibility (Karimi et al., 2013; Zheng and Rehmann, 2014). Chemical pretreatments are divided into three categories: acid

(dilute/concentrate), alkali (sodium hydroxide, lime) and oxidizing (hydrogen peroxide, ozonolysis) treatments (Chaturvedi and Verma, 2013). They provoke an enhancement of surface area and porosity by swelling of the biomass. The physico-chemical pretreatments are divided into two categories: hydrothermal (liquid hot water, steam explosion) and a second one using chemicals (acid, base) (Maurya et al., 2015). They allow equally a partial degradation of hemicellulose and lignin transformation (Badiei et al., 2014).

Biological pretreatments constituted as very large topic and will not be discussed in detail in this review since they were reviewed recently (Narayanaswamy et al., 2013). The biological pretreatment of lignocellulosic biomass is usually performed using cellulolytic and hemicellulolytic microorganisms as well as their enzymes. Filamentous fungi isolated from the soil, living plants or lignocellulosic waste material are preferentially employed to degrade lignin, the most recalcitrant polymer in biomass (Saritha et al., 2012). White-rot (as *Pycnoporus cinnabarinus*, (Levasseur et al., 2014)) and brown-rot fungi (*Coniophora puteana*, (Ray et al., 2010)) could be used as well as soft-rot fungi and bacteria, but at a lesser extent. These have shown high delignification efficiency on various lignocellulosic biomasses (Shi et al., 2008; Wan and Li, 2011). These processes are mild and environmentally friendly. Furthermore, they received industrial consideration since they are inexpensive and did not release toxic components.

In this review the attention is focused in six pretreatments selected according to economic criteria of cost efficiency (Mosier et al., 2005a): steam explosion (SE), liquid hot water (LHW), dilute acid (DA), lime pretreatment (LP), ammonia fiber explosion (expansion) (AFEX) and ammonia recycle percolation (ARP). These pretreatments allow the increase of accessible surface area, decrystallize cellulose (especially the ammonia pretreatments), remove hemicellulose, and remove lignin (partially for LHW and DA). The lignin structure is altered in any case (Mussatto, 2016a). These six pretreatments are listed in the Table 1.2, and described below.

Table 1.2. Lignocellulosic biomass pretreatments.

Pretreatment/ Category	Chemicals	Process conditions	Recommended biomass	Advantages	Disadvantages	Applications	References
SE (Hydrothermal Physico-chemical)	None or SO_2	160-260°C (0.69-4.83 MPa). 30s-15 min	Hardwoods / Agricultural residues / Less effective for softwoods	Low energy requirement No recycling or environmental cost Limited use of chemicals	Potential inhibitory compounds	Large scale plant of ethanol in Ottawa NREL pilot plant in Golden SEKAB pilot plant in Sweden Commercial SE in the Masonite process (production of fiber-board)	(Ballesteros et al., 2006; Barbanera et al., 2015; Cotana et al., 2015; Vandenbossche et al., 2014)
LHW (Hydrothermal Physico-chemical)	None	160-230°C 10-30 min	Corn fiber	No chemicals	Large volume of water involved	Industrial processes (e.g. Pulp industries) Tested in lab-scale	(Kim et al., 2009; Ko et al., 2015; Zhuang et al., 2016)
DA (Chemical)	H_2SO_4	160-220°C 1-30 min	Corn residues /woody and herbaceous crops /Softwoods chips	Achieves high reaction rates Use equally as an entire treatment of lignocellulosic biomass	Potential inhibitory compounds Relative high cost Corrosion of the process equipment	Commercially to produce furfural / NREL developed a technique for large-scale application in two stage to enhance digestibility of biomass with high lignin contents	(Lloyd and Wyman, 2005; Mussatto, 2016b; Rocha et al., 2015; Timung et al., 2015)
LP (Hydrothermal Physico-chemical)	CaO (w/w O_2)	25-160°C 120 min – weeks	Hardwoods with a combination of alkali and oxygen pretreatment	Low cost and safety reagent Low energy requirement Removes lignin	Ineffective with biomass contents high percent of lignin Large volume of water involved	Lab-scale	(Chang and Holtzapple, 2000; Chang et al., 1997, 1998; Kim and Holtzapple, 2005)
AFEX (Alkaline Physico-chemical)	NH_4OH	40-180°C 10-60 min	Herbaceous plants /Agricultural residues / MSW Hardwoods for ARP	Allows the pretreated biomass to be directly used No inhibitory compounds Removes Lignin	Idem as AFEX	Lab-scale	(Balan et al., 2009; Bals et al., 2011; Chundawat et al., 2007; Xu and Huang, 2014; Zhao et al., 2014)
ARP (Alkaline Physico-chemical)	NH_3/NH_4OH	160-180°C 15-90 min					

2.3.1. Steam explosion

This physico-chemical process is also known as autohydrolysis, and was extensively studied (Barbanera et al., 2015; Sørensen et al., 2008; Vandenbossche et al., 2014; Wyman, 1996). The lignocellulosic biomass is set in a recipient and steam was applied without any added chemicals, under high pressure and temperature (0.69-4.83 MPa, 160°C-260°C) during 30 sec to 20 min. After that, the pressure is suddenly reduced and the biomass undergoes a quick depressurization yielding hemicellulose solubilization. Several factors affect the efficiency of the SE such as temperature, residence time, particle size, and moisture. This pretreatment is currently applied industrially to hydrolyze hemicellulose by the so called Masonite process. The original Masonite process typically did not employ chemicals, although later work incorporated chemical treatments and was referred as "steam explosion pulping " (Boehm, 1930; Kokta et al., 1993).

Steam pretreatment typically uses acids, such as sulphuric acid, to enhance the solubilization of hemicellulose. Several studies described this treatment applied to different biomasses (Ballesteros et al., 2006; Cotana et al., 2015; Jurado et al., 2009). The efficiency of the subsequent enzymatic hydrolysis have been enhanced after SE treatment comparing with a non-pretreated biomass (Alvira et al., 2011; Ruiz et al., 2008). Some variations were tested. Increasing temperatures (from 185°C to 215°C) enhance the enzymatic hydrolysis but the overall yield of glucose become lower due to cellulose degradation (López-Linares et al., 2015). The addition of H_2SO_4 or SO_2 (1% w/w) could enhance the enzymatic hydrolysis maximizing sugar recovery and could also decrease the occurrence of inhibitors (Ballesteros et al., 2011; García-Aparicio et al., 2006). Steam explosion is more efficient for hardwoods and agricultural residues but presents less interest for softwood biomass. SE is one of the most cost-effective and widely used pretreatment for wheat straw (Sun et al., 2005; Talebnia et al., 2010).

2.3.2. Liquid Hot Water (LHW)

As SE, LHW is a hydrothermal pretreatment. This process is applied since several decades in pulp industries and uses pressure to keep water in a liquid state at high temperature (Kim et al., 2009; Timung et al., 2015; Zhuang et al., 2016). Cellulose is thus hydrated, increasing the accessibility for hydrolytic enzymes whereas hemicellulose and part of lignin are removed (Ko et al., 2015). Compared to steam pretreatment, LHW permits higher pentose recovery. If pH is

maintained in the range 4-7, LHW could prevent the formation of inhibitory degradation products even if the formation of monosaccharide is slightly minimized (Mosier et al., 2005b). A higher cellulose conversion after enzymatic hydrolysis was obtained with a LHW pretreatment of corn stover (Kim et al., 2016). LWH could be performed in batch or in flow through manner. Flow through systems yielded better results to remove hemicellulose and to alter the lignin structure (Yang and Wyman, 2004). However, the use of a fixed-bed reactor combining the advantages of the batch and the continuous-flow reactor seems to be the best strategy (Wörmeyer et al., 2011).

2.3.3. Dilute acid pretreatment

Several acids could be used but sulfuric acid is most usual. Other strong acids are also reported such as nitric acid and chlorhydric acid (Taherzadeh and Karimi, 2008). Two kind of acid pretreatments are developed: at low temperature and high concentration, called concentrated-acid pretreatment and another one at high temperature with low acid concentration, called dilute-acid pretreatment (Li et al., 2010; Timung et al., 2015). The concentrated-acid pretreatment require costly investment for the subsequent neutralization process, therefore the commercial interest is limited (Karimi and Taherzadeh, 2016; Karimi et al., 2013). Since the 80s, dilute-acid pretreatment has been considered as more attractive to prepare the lignocellulosic biomass, and some reviews discussed this pretreatment (Hendriks and Zeeman, 2008; Mussatto, 2016b; Zhang et al., 2013). When performed at 140-190°C and with 0.1% to 1% H_2SO_4, hemicellulose is solubilize almost entirely, lignin is altered, increasing the cellulose accessibility (Bower et al., 2008; Lloyd and Wyman, 2005). The main drawback is the potential formation of inhibitors which could be principally carboxylic acids, furans and phenolic compounds (Zha et al., 2014).

2.3.4. Lime pretreatment

The lime pretreatment (calcium hydroxide) is the most developed alkali treatment of biomasses (Dredge et al., 2011; Ishfaq et al., 2015; Kim and Holtzapple, 2005). In addition to lime, other reactants could be used as sodium, potassium, or ammonium hydroxide. Lime is considered as a low cost, safe and recyclable reagent (Chang et al., 1997, 1998). The treatment is generally performed at temperatures between 25°C and 160°C, from 120 min to several weeks (Kim and Holtzapple, 2005; Sierra et al., 2009). The digestibility of the pretreated biomass by the enzymes increased after treatment as indicated by an increase of total sugar yield (Chang and Holtzapple,

2000; Hendriks and Zeeman, 2008). In case of very recalcitrant biomass, the combination of the alkali pretreatment and oxygen treatment allows an increase in digestibility of lignocellulosic material. For instance, oxidative lime pretreatment has shown impressive results on poplar wood, a high-lignin content biomass (Chang et al., 2001).

2.3.5. Ammonia recycle percolation (ARP) and Ammonia fiber expansion (AFEX)

AFEX and ARP are others alkaline pretreatments classified into the physical chemical pretreatments. The processes are based on liquid ammonia treatment. Ammonia, is cheap (almost 4 times less than H_2SO_4) and recyclable (Chundawat et al., 2010). During this processes, cellulose and hemicellulose are preserved avoiding the formation of inhibitors (Bals et al., 2011). Furthermore, the pretreated biomass could be used directly for enzymatic hydrolysis without neutralization (Balan et al., 2009). A solubilization of 15-20% of the lignin is observed (Zhao et al., 2014). AFEX is based on temperatures below 90°C. In the opposite, ARP is performed at higher temperatures: 160-180°C. Both were shown to be efficient for herbaceous plants, agricultural residues and municipal solid waste (MSW) (Lau et al., 2009; Taherzadeh and Karimi, 2008). ARP is preferred for hardwood biomass. During AFEX pretreatment, biomass and ammonia are mixed together in a closed system before pressure setting (generally 3MPa, at 60-90°C during 10-60 min). The mass ratio of ammonia/ dry biomass are from 1:1 to 1:2 kg/kg. The reaction reached the desire temperature and after 5-10 min, the pressure is rapidly released to create “explosive” treatment such as in the SE procedure. As a result, enzymatic hydrolysis could be performed and almost complete conversion of cellulose and hemicellulose to fermentable sugars was observed (Chundawat et al., 2010). This was exemplified with corn stover (Teymouri et al., 2005) and with different other biomasses (Gollapalli et al., 2002; Kim et al., 2008).

ARP operates on the same principle than the AFEX, unless ammonia is percolated through a packed bed reactor with a flow through mode. The operating conditions are generally a temperature between 150°C and 180°C, a concentration of aqueous ammonia between 5% and 15%, a flow rate of 1-5 ml/min and a residence time between 10 min and 90 min (Chundawat et al., 2011). According to Fourier transformed infrared (FTIR) analysis and lignin staining, a study of ARP pretreatment of corn stover showed a reduction in lignin content of 70-85% and high hemicellulose solubilization: 40 to 60% (Kim et al., 2003).

3. Building block

A large majority (see above) of the chemicals used today originate from fossil resources. However, in the last years, the production of chemicals from renewable resources, such as lignocellulosic biomass, cellulose fibers and cellulose derivatives, has increased progressively (Lucia et al., 2006; Ragauskas et al., 2006). In 2012, the global production of bio-based chemicals and polymers is estimated to be around 50 million tons (de Jong et al., 2012). The current market represents $10-15 billion, and is growing by nearly 10 per cent every year thanks to the emerging sector like bioplastics (Shukla, 2016). However, fossil resources still provide more than 85% of feedstocks for chemicals manufacture but bio-based chemicals production is increasing in volume and diversity (Carbonell et al., 2012; Jang et al., 2012). Commercial prospects for biobased chemicals depends of a number of factors, including feedstock costs, availability of technology and environmental impacts as greenhouse gas (GHG) emission, land use … (Clark, 2007).The WorldWide Fund for Nature reports that by 2030, the use of biobased chemicals could prevent 660 million tons of carbon dioxide reaching the atmosphere each year (de Jong et al., 2012).

As defined by the US Department of Energy, a building block is a molecule with multiple functional groups that possess the potential to be transformed into new families of useful molecules (Werpy et al., 2004). All building block chemicals could be further converted into a wide spectrum of platforms molecules through chemical processes, such as reduction, oxidation, dehydration, hydrogenolysis and direct polymerization (Lipinsky, 1981; Sheldon et al., 2007). These platforms chemicals could be used as precursors for the production of a large variety of chemicals and materials (solvents, fibers, antifreeze, and polymers) (Bozell, 2008; Jang et al., 2012; Xu et al., 2008; Yang et al., 2013). The most common building blocks used in polymerization are carboxylic acids (oxalic, malonic, succinic, glucaric, lactic, fumaric, itaconic, adipic or acetic acids), diols (ethylene glycol, butanodiols, propanediol, pentanols or buanediols), diamines (cadaverine, ethylenediamine or putrescine) and aldehydes (formaldehyde) (Belgacem and Gandini, 2011; Holmberg et al., 2014; Lackinger and Heckl, 2009).

The raw materials used to produce building blocks are more and more based on lignocellulosic biomass to avoid competition with food for production of building blocks (Clark, 2007; Naik et al., 2010). Biomasses are transformed by chemical conversion or microbial

bioconversions. The choice between these possibilities is based on complete sustainability assessment, availability of resources and logistics of resource transportation through the evaluation of the life cycle analysis (LCA) (Corma et al., 2007). The domains which describe the researches involving chemical or biotechnological routes (based on microorganisms or enzymes) for the production of chemicals and materials from biomass are termed Green Chemistry and White Biotechnology (formerly Industrial Biotechnology), respectively (FitzPatrick et al., 2010; Pandey et al., 2015).

However, production of chemicals by biorefinery faces the primary challenge of renewable carbon conversion into chemicals, which could need new technology and process (McKendry, 2002; Menon and Rao, 2012). Furthermore, this production is challenged by a large number of more or less identified by-products formed during processes (Bozell and Petersen, 2010). Besides, for the development of some end-products, new processes can be also needed.

Building blocks can be distinguished by the prevailing strategy for their development towards their market (Table 1.3). Strategies can be classified as:

i. Direct substitution of a petrochemical compound. This strategy implies that a bulk chemical which is presently produced from petrochemical resources would be substituted by an identical chemical, produced from biomass thanks to biotechnology. The advantage of this strategy is that the markets for these products already exist but, on the other hand, the bio-based products have to compete against optimized and often, low cost production based on cheap fossil resources (Hatti-Kaul et al., 2007). One of the best example of this strategy is for instance, the production of biobased ethylene from ethanol produced from sugar cane.

ii. Introduction of bio-based building blocks with new chemical structures. In this case, biorefinery provides new building blocks usable (a) to yield the same kind of products (functional competition) than refinery but also (b) to open a new panel of chemicals. By providing new market opportunities, bio-based compounds could become more competitive (Agbor et al., 2011; Corma et al., 2007). However, this second strategy is much financially more risky compared to the first strategy due to necessity of developing (a) new technologies and processes, and (b) new markets.

Table 1.3. Strategies for biomass market entry.

	Direct substitution	**New bio-based building block**	
		Functional competition	**Novel product**
Characteristics	Can replace an existing petrochemical	Compete with an existing petrochemical but with different characteristics	New properties for existing functionalities or new applications
Examples	2,5 Furandicarboxylic acid can replace terephthalic acid for polyesters production (e.g., PEF to replace PET)	Reduction of levulinic acid form 1,4-pentanediol used in new polymers	Polylactic acid (PLA) from glucose fermentation
Advantages	Existing market (knowledge of cost structure and reduction in market risk)	Potential market is expanded (advantages of replacement and novel product) Product swing strategies can be employed to reduce market risk	No competitive petrochemical routes New market opportunities Most effective use of biomass properties
Disadvantages	Cost competition between biobased and petrochemical based sources.	Select where to focus research and development	Market not clearly defined High capital risk Long time to commercialization

In some cases, the products obtained from biomass acquire additional functionalities, leading to higher value products (e.g., chiral products or biodegradable plastic) (Xu et al., 2008). In specific niche markets, the green image of these products makes higher their prices. However, the development of new products is still difficult due to the competition with conventional products obtained in high yields and low costs (Krzyżaniak et al., 2014).

The advances in biofuels industries will play an important role in bio-based chemicals development since similar methodologies and selection criteria should also be applicable to the identification and utilization of new biobased products.

3.1. Top value biobased chemicals

In 2004, the U.S Department of Energy (DOE) published a report of "Top Value Added Chemicals from Biomass" intended to examine value-added products from all biomass components and identify the potential building blocks that could be produced from sugars via biological or chemical conversions. This list has been recently updated (Bozell and Petersen, 2010).

The identification of these top value-added chemicals from biomass should support the technology and economy of fuels and power's production, and integrate the common challenges and barriers of associated production technology (Cherubini, 2010; Ragauskas et al., 2006). To obtain a final list, 300 building block candidates were examined. The screening criteria included estimated processing costs, estimated selling prices, the technical complexity of the best available processing pathway and the market potential. To integrate the final list, a given building block should then fulfill several requirements such as displaying large potential of conversion in derivatives, being C1-C6 monomers, been able to be produced from starch or lignocellulosic biomass, and not be already a super-commodity chemical (produced in a large scale to satisfy global demands). The initial DOE list has been re-analyzed and revisited, to give a new updated list (Table 1.4) (Bozell and Petersen, 2010).

Table 1.4. Top 15 biobased chemicals from carbohydrates (based on 2004 DOE's list and 2010 revisited list)

Top Chemical opportunities from carbohydrates
Succinic acid, Fumaric acid, Malic acid
Furans (2,5 furan dicarboxylic acid)
3-Hydroxypropionic acid
Aspartic acid
Glucaric acid
Glutamic acid
Itaconic acid
Levulinic acid
Lactic acid
3-hydroxybutyrolactone
Glycerol
Sorbitol
Xylitol/arabinitol
Ethanol
Biohydrocarbons

The production of building blocks and their derivatives occur generally in a two-step process. First the building blocks are produced from plant feedstock via fermentation using fungi, yeast or bacteria, or by enzymatic and chemical transformations (Erickson et al., 2012; Lee et al., 2011a; Straathof, 2014). Secondly, the building block are transformed in derivatives or polymers. The most common pathways for conversion are chemical reduction, oxidation, dehydration, bond cleavage and direct polymerization (Corma et al., 2007; Lee et al., 2011b). However, some biological transformations do not need an intermediate building block to produce an end product. For example, 1,3-propanediol is directly polymerized by successive biological transformations (Liu

et al., 2008). Chemical transformations generally operate with less conversion specificity (Werpy et al., 2004). Current industrial biological conversions concern citric acid, lactic acid or ethanol driven by high production's capacity, selectivity and low by-products formation (Abdel-Rahman et al., 2011; Lin and Tanaka, 2006; Singh Dhillon et al., 2011).

Additional researches are performed to identify, characterize and even to modify the enzymes or the living organisms and processes to use better the renewable resources. Therefore, genetic engineering appears as a fundamental tool in this field (Kern et al., 2007; Liu and Jarboe, 2012). In addition, these cells or the enzymes can be used immobilized to improve productivity (Franssen et al., 2013).

High selectivity and high potential production of building blocks are good arguments in favor to biological transformation instead of chemical conversion, however, there are some drawbacks as the sterility required in microbial processes that implies high energy demand and the separation of products from fermentation broth is sometimes challenging (Dodds and Gross, 2007), (Sauer et al., 2008). Consequently, many improvements are still necessary to develop cost-effective processes.

3.2. Organic acid biobased building-block top value

Organic acids market is one of the biggest market for chemistry. It reached 6.55 $billion in 2015 according to RnR Market Research, 2016. Wide range of intermediates could be generated from them as polymers, coatings, lubricating oils, pharmaceuticals, cosmetics, solvents, diesel fuel oxygenates, and some other applications. The main top value biobased organic acids are presented in Figure 1.3.

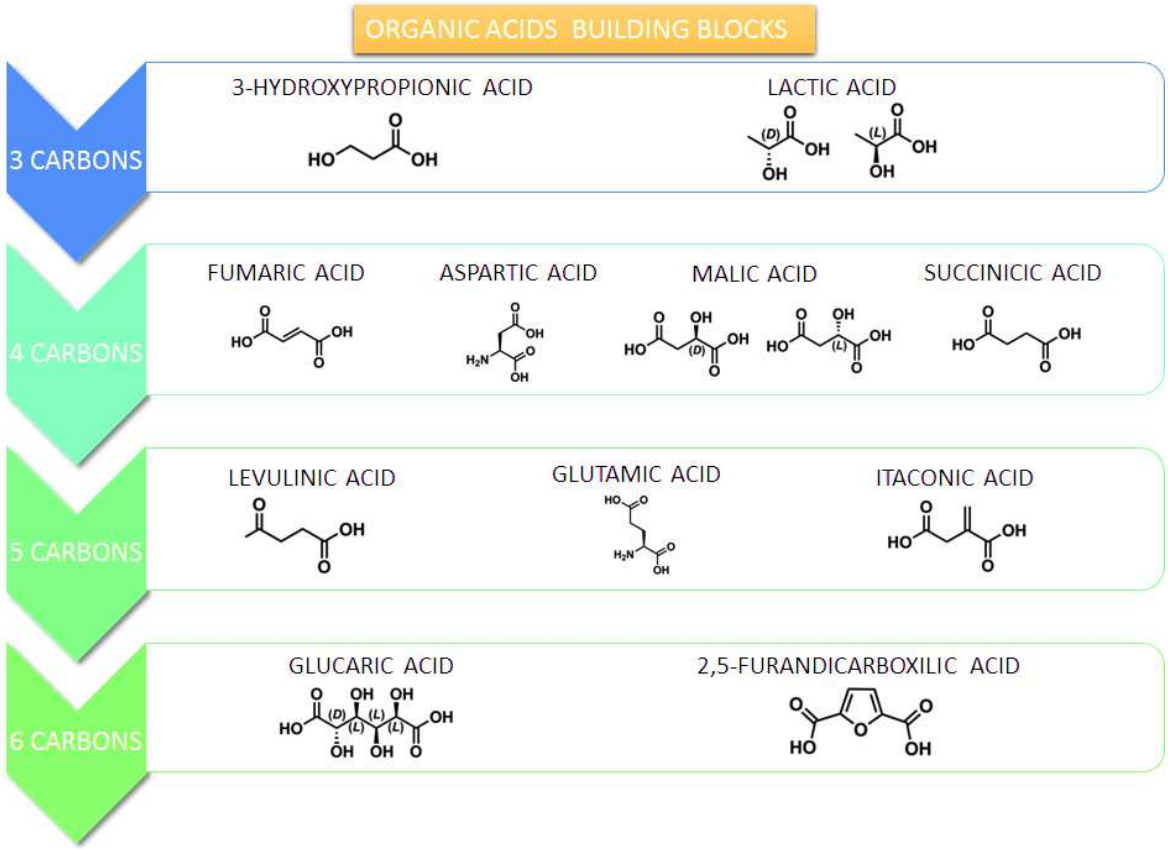

Figure 1.3. Top Organic Acid Building Blocks.

The functional carboxylic groups are particularly interesting for the combination of donor and acceptor characters to form bonds (e.g. esterification, amidification). With the chemical with low molar mass (solvent, additives), the production of polymers is one the main outlets of these organic acids (Bozell, 2008). Microbial production is one of the greener way to produce these building block. For example, itaconic and malic acids are synthetized by fungus species (*Aspergillus* genus), fumaric acid by others fungi, and lactic acid is produced by bacteria species (*Lactobacillus*) and by the fungus *Rhizopus* (López-Garzón and Straathof, 2014; Tkacz and Lange, 2012). BioAmber, Myriant, Genomatica, BASF, DSM Cargill, Novozymes, Natureworks LLC, Dow Chemicals, and Metabolix are the major companies involved in organic acids production (Lückstädt et al., 2014).

To be competitive, organic acids production has to be cost-effective. To achieve this requirement, some parameters have to be considered such as the use of inexpensive substrates, the improvement of fermentation pathways for instance by lowering end-products inhibition (Tsao, 1999). Strain selection and bioprocess engineering are not strategies leading to sufficient yields yet and consequently, metabolic engineering and systems biology were used to enhance microbial building blocks production recently (Chen et al., 2013). Metabolic engineering can help to alleviate many hindrances for the bioproduction of these building blocks with the elimination of competing pathways or by-products formation for the reorientation of metabolism towards the desired pathway. New chemicals can also be formed using the synthetic biology (Gaspar et al., 2013; Lee et al., 2011a; Park et al., 2008).

As mentioned earlier, an interesting approach for polymer applications is the direct synthesis of the polymer by fermentation by wild or genetic modified microorganisms without subsequent chemical processes. One step microbial production of polymers allows the control of the proportion of metabolic intermediaries and of course reduces the steps number from renewable resources to end-products (Chen et al., 2011). As a consequence, it will also bring down costs and avoid additional processes involving environmentally dangerous intermediates. As an example, polylactic acid can be produce by one step microbial fermentation by combining synthetic biology with metabolic engineering in *E. coli* (Jung and Lee, 2011). The heterologous enzyme propionate CoA transferase and PHA synthase were cloned in this bacterium and subsequently, the fluxes of carbon metabolism are redirected to produce PLA from glucose.

3.3. Current production of organic acids form renewables resources

Lactic and 3-hydroxypropionic acids are C3 building blocks useful for the production of biodegradable polyesters, such as PLA and poly(3-hydroxypropionate). Currently, lactic acid is produced by fermentation of carbohydrates by various microorganisms (Gaspar et al., 2013; Wee et al., 2006). The global production volume in 2013 was 800.000 tons (RnR Market Research, 2014). Current 3-hydroxypropionic acid production is conducted by chemical synthesis routes, including the oxidation of 1,3-propanediol, the oxidation of 3-hydroxypropionaldehyde, and the hydration of acrylic acid. The biological transformation of glycerol by *Klebsiella pneumonia* is however a promising route, and some research in metabolic engineering was developed (Ashok et

al., 2011). The use of glycerol could be sustainable process because glycerol is a major byproduct of the oleochemistry and biofuel production.

The industrial production of C4 building block malic, succinic, fumaric and aspartic acids are mostly dependent on petrochemical synthesis routes. The four components can be synthesized by biotransformation, with wild or genetically modified producer (Xu et al., 2012). C4 acids can be converted into 1,4-butanediol (BDO) that can be further converted into numerous chemicals, including plastics, polymers and resins (Goldberg et al., 2006). Bio-based succinic acid, after lactic acid, is the most developed chemical for industrial production (Figure 1.4), with 47 ktons in 2013 to increase to 92 ktons by 2020 (Grand View Research, 2014a; Shmorhun, 2015). Global fumaric acid (FA) market was 225.2 ktons in 2012. This market is currently increasing because FA is used as a potential substitute for synthetic organic acids such as citric acid and tartaric acid for food applications (Hexa Research, 2015). Recent reports have indicated a 6% augmentation of FA market over the period 2014-2020 (Grand View Research, 2015a; Research and Markets, 2015). However, to date, its bio-based production is weakly developed.

C5 acids, glutamic and itaconic, are industrially produced by fermentation in majority in China. Itaconic acid (IA) is attracting as a renewable chemical, because it could be a substitute for acrylic and methacrylic acid for the production of polymers. IA, although widely sourced from petrochemical feedstock, could be produced from renewable resources and is therefore viewed as an ideal bio-based platform for the chemicals industry (Steiger et al., 2013). The worldwide production capacity of itaconic acid is expected to be about 50 ktons per year, facing a demand of about 30 ktons (Global Industry Analystis, Inc, 2016). The total world production of monosodium L-glutamate by fermentation is estimated to be around 2 million tons/y (2 billion kg/y) (Sano, 2009) with the Japanese group Ajinomoto being the largest producer. As for IA, efforts were focused on low cost fermentation routes by enhancing the use of renewable resources (Drupnar, 2013). Global levulinic acid market demand is expected to reach 3.8 ktons by 2020. It is formed by acid treatment of starch or lignocellulosic resources and DuPont, Segetis and Biofine have already developed patented technologies for its production through renewable sources (Grand View Research, 2014b).

Glucaric acid, a C6 compound, is produced from glucose by nitric acid oxidation or catalytic oxidation. Significant industrial and academic efforts around the world are directed for producing glucose from biomass resources. A synthetic organism, based on *E.coli* was constructed and produces 50% more of glucaric acid at commercial scale (Moon et al., 2010).

Another C6, 2,5 furandicarboxylic acid (FDCA) faces a great demand and the market is expected to reach 500 ktons by 2020, with the potential to replace some aromatic chemicals like phthalic acid in polyesters (PET) (Grand View Research, 2014b). Avantium is currently developing biobased routes using commercially available carbohydrates (first generation sugar and starch corps) to produce FDCA (Grand View Research, 2014c). In the future it should be improved by using second generation, from non-food feedstock.

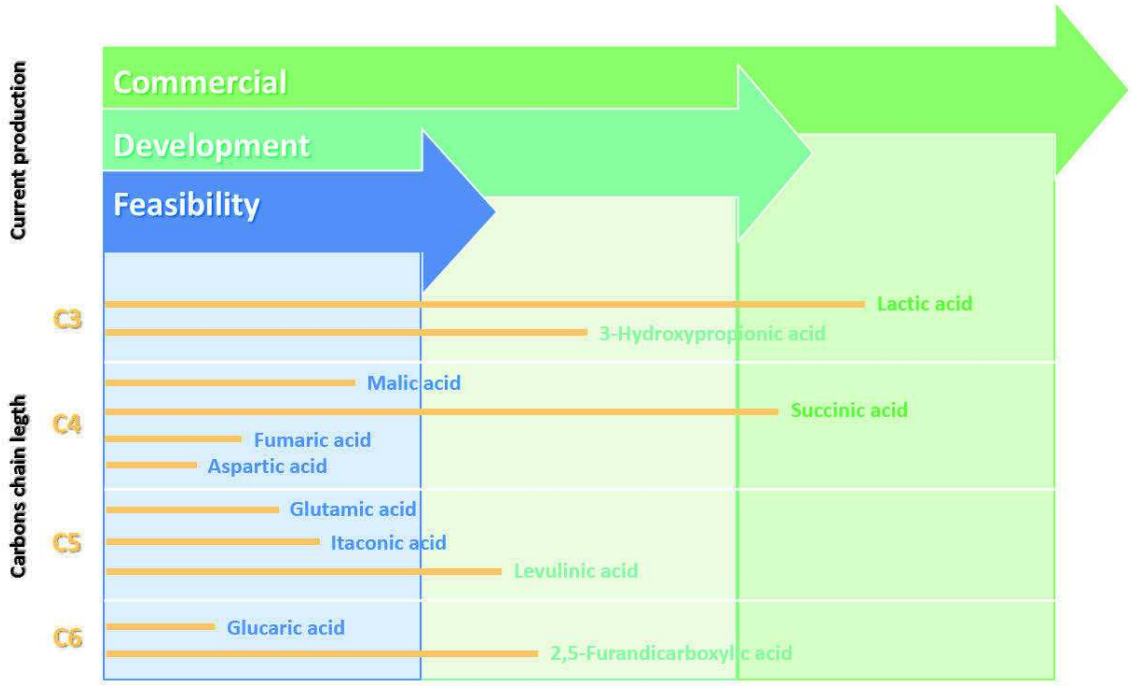

Figure 1.4. Current organic acid production from lignocellulosic resource.

4. C3-C6 building block.

This part is more particularly focused on 4 representative organic diacids: lactic acid (C3), fumaric acid (C4), itaconic acid (C5) and glucaric acid (C6). These building blocks were predicted to display a huge production expansion for the next decade (van Dam et al., 2005). They are already produced via fermentative pathways using glucose thanks to wild or engineered strains to reach the high production yields.

Fermentations are based on two groups of low cost and raw materials: (i) those containing mono- and disaccharides and (ii) those containing polymeric substrates. The main substrates on the first group are molasses, whey or date juice (Alvarez et al., 2010; Kotzamanidis et al.; Koutinas et al., 2016; Nancib et al., 2015). The polymeric substrates are divided in two main categories: the starchy and lignocellulosic materials biomasses. The latter induces a significant decrease of the costs compared with the use of refined sugars (and yeast extract) (Anwar et al., 2014; Keijsers et al., 2013). Thanks to the pretreatment of the lignocellulosic biomasses, large amounts of fermentable sugars derived from natural polymers (cellulose, hemicellulose) are available. The characteristics of a "good" substrate for the production of organic acids, were the described as "available abundantly throughout the year, renewable, cheap, produce less or no by-products, stereo specific, produced at high rates with low level of contaminants, with no competition with food" (Brar et al., 2013; Pandey et al., 2015).

4.1. Lactic acid

4.1.1. Production of lactic acid

Lactic acid (LA) is one of the best known and most promising building blocks. Lactic acid (2-hydroxypropanoic acid) was discover in 1780 by Carl Wilhelm Scheele during a study of sour milk (Benninga, 1990). Its industrial production was obtained by fermentation in 1881 (Castillo Martinez et al., 2013). Lactic acid possesses two distinct enantiomeric forms, L(+)-lactic acid and D(-)-lactic acid. A large range of value-added products is potentially derived from lactic acid due to the hydroxyl and carboxyl groups. For the first time in 1932, a cyclic dimer lactide was used to undergo of ring opening polymerization (ROP) enable to form polylactic acid (PLA) with a high

molar mass (Carothers et al., 1932). This approach is now the most usual way to produce this polymer in large volume.

LA is used in many sectors such as food, packaging, chemicals, medicals, cosmetics and pharmaceutical industries (Abdel-Rahman et al., 2013; Castro-Aguirre et al., 2016; Torino et al., 2015). The global lactic acid market was estimated to be 714.2 kilo tons in 2013, which is expected to reach 1,960.1 kilo tons by 2020, growing of 15.5% per year from 2014 to 2020. In terms of revenue, the market was valued at $ 1,285 million in 2013, which is expected to reach $ 4,312 million by 2020 (+ 18.3% per year) (Grand View Research, 2014e). The major manufacturers of biobased lactic acid are NatureWorks LLC (USA), Purac (The Netherlands), Galactic (Belgium), and other minor companies essentially Chinese ones such as Musashino Chemical Co. Ltd (RnR Market Research, 2014).

LA can be produced by chemical synthesis or by fermentation. The most current chemical synthesis is performed in a liquid phase and under high pressure (Narayanan et al., 2004). Lactonitrile is first produced thanks to the reaction between acetaldehyde and hydrogen cyanide in presence of a base. After recovery and purification, lactonitrile is hydrolyzed with hydrochloric acid or sulfuric acid to lactic acid (Ghaffar et al., 2014). Lactic acid can also be produced by fermentation thanks to several microorganisms, especially bacteria and fungi (Abdel-Rahman et al., 2013). Currently, fermentation is the preferred industrial pathway and it is a promising evolution towards green chemistry. 90% yield of lactic acid based on the glucose consumption is obtained in the current commercial fermentation (Moresi and Parente, 2014).

Fermentations routes allow a rapid production with high yields to obtain a selective production of one of the stereoisomers of lactic acid or a racemic mixture (Salminen and Wright, 2004). Bacteria are currently preferred. Lactic acid bacteria (LAB), *Bacillus* strains, *Escherichia coli* and *Corynebacterium glutamicum* are the main producers groups (Abdel-Rahman et al., 2013). Depending on the producer, the fermentation conditions can be variable, with a pH range varying from 3.5 to 10 and a temperature range between 5 to 45°C (Castillo Martinez et al., 2013). Lactic acid is directly produced from pyruvate by the activity of the lactate dehydrogenase (LDH) of many microorganisms. This microorganisms identified as producers are essentially bacterium from genera *Carnobacterium, Lactobacillus, Lactococcus, Enterococcus, Leuconostoc, Oenococcus,*

Steptococcus, Tetragenococcus, Vagococcus, Pediococcus and *Weisella* (Wackett, 2016). These lactic acid bacteria have complex requirement in nutrients and have diverse biological pathway to ferment sugars such as homo-fermentation or hetero-acid fermentation (Hofvendahl and Hahn-Hägerdal, 2000). They use two different pathways depending on the kind of consumed sugar. The first one, called Embden-Meyerhof pathway (Figure 1.5) uses hexoses with a lactic acid theoretical yield of 1.0 g/g (2.0 mol/mol) and the pentoses with a yield of 1.0 g/g (1.67 mol/mol) (Sonomoto and Yokota, 2011). The homo-fermentatives LAB are *Lactococcus, Streptococcus, Pediococcus, Enterococcus* and some *Lactobacillus*. They produce lactic acid as the main product. The second pathway is called pentose phosphate pathway and consumes hexoses with a lactic acid theoretical yield of 0.5 g/g (1.0 mol/mol) and pentoses (0.6 g/g; 1.0 mol/mol). This route is used by *Leuconostoc*, *Oenococcus* and some *Lactobacillus* species.

Figure 1.5. Simplified lactic acid metabolic pathway (Embden-Meyerhof pathway). Abbreviations are: Ldh, lactate dehydrogenase; Pk, pyruvate kinase.

Embden-Meyerhof pathway is currently preferred due to its high selectivity and is already used for LA industrial production (Abdel-Rahman et al., 2013). However, the main issue now is to use secondary generation with low-cost resources as lignocellulosic materials. As mentioned previously, filamentous fungi, especially *Rhizopus oryzae* can produce lactate (Koutinas et al., 2007; Maas et al., 2006; Magnuson and Lasure, 2004). Fungi possess the advantage to grow without prior saccharification inducing low-cost processes. *Rhizopus oryzae* is particularly interesting for its production of optically pure L-lactic acid. Nevertheless, the limit of this kind of production is the coproduction of undesirable byproducts such as ethanol and fumaric acid (Vink et al., 2010). Some researchers have explored the field of metabolic engineering starting from the *E. coli* host in order to enhance the productivity and the yield of the LA production from lignocellulosic resources (Chang et al., 1999; Zhou et al., 2003). Several similar studies have been conducted with glucose (Zhu et al., 2007), xylose (Dien et al., 2002; Lu et al., 2016), sucrose (Lunelli et al., 2010; Mazumdar et al., 2013; Reddy et al., 2016; Zhou et al., 2006). LA

bioproduction from lignocellulosic biomasses as corncobs, wheat bran, sugarcane bagasse, is well documented in the literature during the last few years (Table 1.5). The increasing demand for biobased poly-lactic acid (PLA) enhance the interest for the production of LA from renewable resources (Ghaffar et al., 2014; Zhang et al., 2016b). However, the use of lignocellulosic biomass can liberate some inhibitors for microorganisms (van der Pol et al., 2016; Zhang et al., 2016a). Therefore, the choice of the pretreatment and type of fermentation must be adequate for each couple biomass-microorganism.

4.1.2. Lactic acid as a platform molecule for different chemicals

A large range of value-added products could be formed from LA thanks to hydroxyl and carboxyl groups (Gao et al., 2013). These are lactate ester, 1,2-propanediol (PDO), pyruvic acid, acrylic acid and lactide (Figure 1.6). Lactate ester is obtained by esterification of lactic acid and is usually used in food, pharmaceutical and cosmetic sectors (Aparicio and Alcalde, 2009). PDO is currently product thanks to propylene oxide obtained by petrochemical resources. However biobased PDO can be also produced by a catalytic reduction of LA (Niu and Guo, 2015). Pyruvic acid is currently produced by chemical and biological processes, but biobased pyruvic acid could be also obtained by dehydrogenation of lactic acid, as acrylic acid, one of the most important industrial chemical derivatives (Dusselier et al., 2013). Indeed acrylic acid and its derivatives are used as raw products for the production of acrylate polymers.

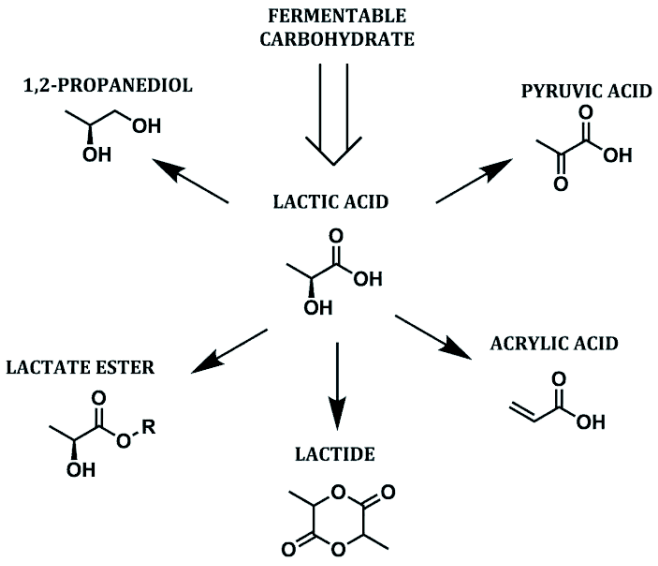

Figure 1.6. Lactic acid as a platform for different molecules

4.2.Fumaric acid

4.2.1. Production of fumaric acid

As shown earlier (Table 1.4), fumaric acid (FA) was classified in the top building blocks (Werpy et al., 2004). It is also called (*E*)-2-butenedioic acid. It belongs to the C4 dicarboxylic acid family and is an isomeric unsaturated dicarboxylic acid. Thanks to its functional groups FA is an excellent building block for polymerization and esterification. FA is widely used in food industry as additive for acidity regulation and as substitute of tartaric acid and citric acid. The attraction of pharmaceutical industry in FA is increasing because of recent studies as therapeutic drugs against psoriasis (Kokelj et al., 2009). Currently, fumaric acid is produced chemically from petroleum derivative, but as others organic acids, the current industrial trends impulse his biotechnological production (Roa Engel et al., 2008). The annual worldwide demand of FA was 225 kilo tons in 2012 but market size is expected to reach 346 kilo tons by 2020 (QYResDeep Research Reports, 2016; Research and Markets, 2015). However, a marginal part of commercial FA is currently biobased. As an example, Myriant is developing a biobased FA for the production of unsaturated polyester resins (UPR) (Smitthipong et al., 2014).

The chemical route for FA production is based on catalytic isomerization of petroleum-derived maleic acid with a high yield conversion (up to 90 %) (Yang et al., 2011). The maleic acid (less stable than FA) is produced from maleic anhydride via oxidation of hydrocarbons as benzene or butane (Lohbeck et al., 2000). The use of FA as substitute of maleic anhydride as precursor of UPR boosts the researches on FA production via biotechnological route in order to reduce the production prices. These studies are oriented on different strategies to enhance the yields and the productivity of fumarate production, mainly through an improvement of the substrate consumption. Avoiding the production of byproducts is another challenge since purification is the most expensive step in biobased FA production (Xu et al., 2012). Thanks to the recent progresses in biotechnology with the improvement of carbohydrates conversion and purification, FA should rapidly become an economically attractive biobased acid.

The literature cites filamentous Mucoralean fungi, particularly from *Rhizopus*, *Mucor, Cunninghamella, and Circinella* genera, as FA producers. Among these strains, Rhizopus species (*nigricans*, *arrhizus* and *oryzae*) were the best-producing ones (Roa Engel et al., 2008). Recent

work identified also some *Aspergillus* species as producers (Jiménez-Quero et al., 2016). Furthermore, in last years the research were focused on metabolic engineering to optimize the industrial-scale production of FA using *Saccharomyces cerevisiae* or *Escherichia coli* species (Shah et al., 2016; Song and Lee, 2015). Frequently fumarate production is accompanied by others organic acids as lactic, formic or malic, so others studies were conducted to reduce these by-products formation (Thakker et al., 2015; Wang et al., 2013).

The production of fumarate depends on the tricarboxylic (TCA), especially through the reversible conversion to malate, catalyzed by fumarase as illustrated in Figure 1.7. As intermediate of TCA pathway in mitochondria, fumarate is used for the biosynthesis of cellular components, so it is not accumulated during active cell growth (Roa Engel et al., 2008). However, the cytoplasm reductive TCA pathway provides biological accumulation of FA thanks to the carboxylation of pyruvate and subsequent oxaloacetate dehydrogenation. Then, fumarase catalyzes the final malate hydration to fumarate with a theoretical yield of 2 mol/mol of glucose (Mondala, 2015). The yield principally depends on the conditions of fermentation and of the microorganism employed, leading to more or less byproducts. The major drawback of this fermentation is due to NADH regeneration. The secretion pathway of fumaric acid in filamentous fungi are not yet fully elucidated. However, in yeasts it was suggested that FA was transported by an accumulative dicarboxylate proton symporter (Saayman et al.).

In order to develop the biotechnological production of FA, the type of fermentation and the substrate used are important factors affecting the cost of processes. Pure sugars as glucose are the preferred carbon source for organic acid production by fungi, but economically biomass seems to be the better alternative even if the yields are relatively lower as shown in Table 1.5. Initially, studies used starchy materials as fermentation carbon source, but as described above for LA, the lignocellulosic biomass is the current objet of many works for biobased FA production.

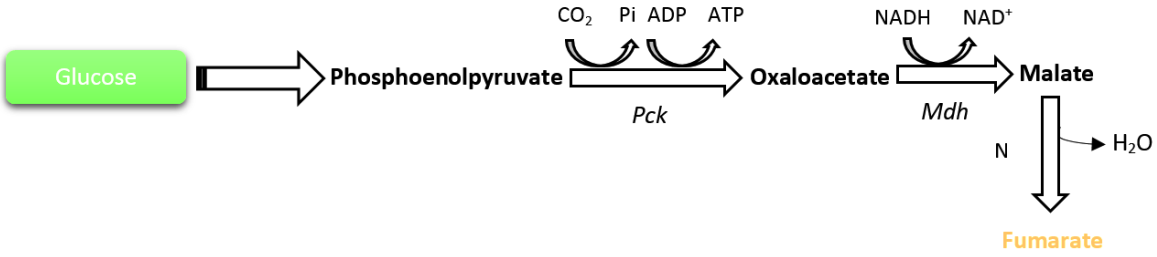

Figure 1.7. Simplified fumaric acid metabolic pathway. Abbreviations are: FH, fumarase; Mdh, malate dehydrogenase; Pck, phosphoenolpyruvate carboxykinase.

4.2.2. Fumaric acid as platform molecule for different chemicals

As described earlier, FA is an excellent chemical platform for the production of UPR, as a direct substitute for maleic anhydride which has a global market size of $ 2.3 billion in 2015 (Grand View Research, 2016; Wojcieszak et al., 2015). Moreover FA has great interest as a chemical platform for the production of succinic acid, a chemical intermediate for 1,4-butanediol (BDO), γ-butyrolactone (GBL) and tetrahydrofuran (THF) as shown in Figure 1.8. Globally, succinic acid with its derivatives represent a huge market of bulk chemical representing about $ 170 billion in 2014 (Bechthold et al., 2008; Delhomme et al., 2009; Wise Guy Reports, 2015). BDO, GBL and THF are produced by hydrogenation/reduction of succinic acid and could be used as solvents or in fibers production. Succinic acid possesses a second family of derivative produced via reductive amination called pyrrolidone, which can be used as green solvent, and with water soluble polymers. Also FA can be converted into L-malic acid, a chemical with a large market (64 kilo tons in 2012) in liquid and powdered beverages (Grand View Research, 2015b; Lee et al., 2011c).

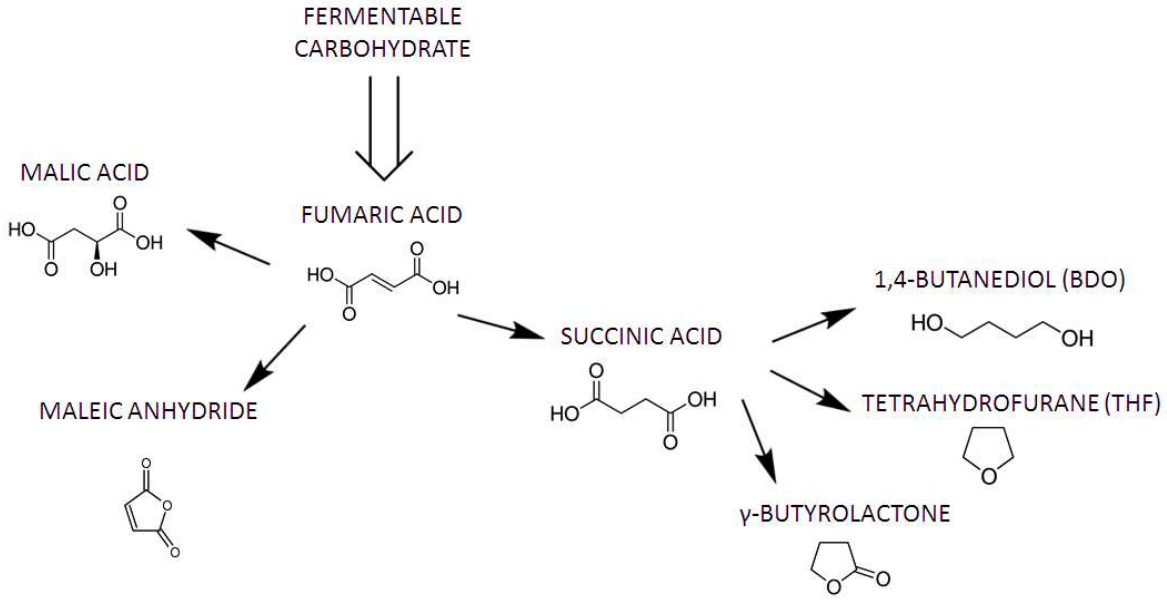

Figure 1.8. Fumaric acid molecules platform.

4.3. Itaconic acid

4.3.1. Production of itaconic acid

Also called methylene succinic acid, itaconic acid (IA) has been claimed to be one of the most promising building blocks obtainable from biomass (Table 1.4). IA was discovered in 1837 as a product of pyrolytic distillation of citric acid (Willke and Vorlop, 2001). Almost a century later, IA was isolated from the fungal strain *Aspergillus itaconicus* (Steiger et al., 2013). IA can substitute methacrylic acid, which is mainly obtained from fossil chemicals, and used for diverse applications in polymer materials (Le Nôtre et al., 2014). IA presents a market which should reach $ 127 million in 2014 with an expected two-fold increase by 2023 (Transparency Market Research, 2015). The annual production is rather imprecise and should reach between 40 000 tons to 80 000 tons per year (Brar et al., 2016). An estimation of future production is 400 kilo tons per year in 2020 if IA is successfully incorporated into the methyl methacrylate production (Medway and Sperry, 2014).

Nowadays, IA can be chemically synthetized via the distillation of citric acid but the main part is biotechnologically produced with the mold *A. terreus* (Bentley and Thiessen, 1957;

Bonnarme et al., 1995; Okabe et al., 2009). In addition, several other producers were reported such as *Ustilago maydis*, *Candida sp*. or *Pseudozyma antartica* (Levinson et al., 2006; Tabuchi et al., 1981; Voll et al., 2012). Recently, IA was identified also as a product of macrophages where it eventually exhibits antibacterial function (Strelko et al., 2011). Some microorganisms could be modified to achieve higher production (Yahiro et al., 1995). The metabolic pathway for IA involves the TCA cycle and it was elucidated in the 90's (Bonnarme et al., 1995; Jaklitsch et al., 1991). The corresponding pathway use aconitase and cis-aconitate decarboxylase (CAD) as shown in Figure 1.9. However, the transport from the cytoplasm to the culture medium is still not fully understood.

The theoretical IA yields from glucose is 1 mol/mol. As this value is not attained, many attempts were made in order to enhance its production yield. The usual process for IA production was batch fermentation of *A. terreus* with glucose (Bentley and Thiessen, 1957; Rychtera and Wase, 1981; Vassilev et al., 1992). Although the IA biotechnological production is already established in relatively large scale, current processes with this fungus still need optimization (Gyamerah, 1995; Klement and Büchs, 2013). Recently, great progresses has been made providing new insights into the relevant pathways, which should open the possibility for optimization of the current producer *A. terreus* and even the design of new microbial production platforms (Jiménez-Quero et al., 2016; Steiger et al., 2013) (Blumhoff et al., 2013; Harder et al., 2016; Li et al., 2012; Vuoristo et al., 2015). However, recently IA production has drastically changed due to new and green constraints. Therefore, new biotechnological methodologies involving fermentation processes and technologies based on cheap substrates as carbon source are currently under investigation and development (El-Iman and Du, 2014; Zhang et al., 2009). Different lignocellulosic biomasses were tested for IA production by fungal fermentation (Table 1.5). In some cases, IA production is associated with other interesting biomolecule as enzymes (Kocabas et al., 2014).

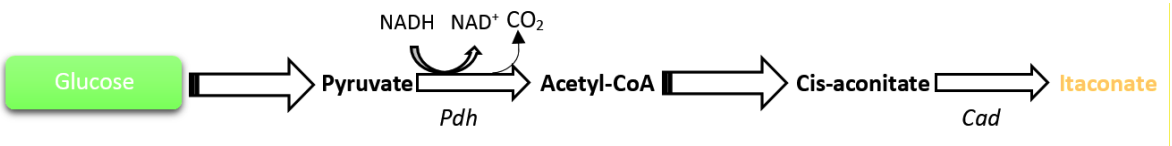

Figure 1.9. Simplified itaconic acid metabolic pathway. Abbreviations are: Acetyl-CoA, acetyl coenzyme A; Cad, cis-aconitate decarboxylase; Pdh, pyruvate dehydrogenase.

4.3.2.Itaconic acid platform molecules

From IA, a large rank of derivative chemicals such as 2-Methyl-1,4-butanediol, 3-methyltetrahydrofuran, 3-&4- γ-butyrolactone, methacrylic acid and the pyrrolidones can be obtained (Figure 1.10). They are produced by hydrogenation/reduction of IA (Xian, 2015). Methacrylic acid can obtained from itaconic acid by decarboxylation and by esterification methyl methacrylate can be also obtained (Le Nôtre et al., 2014). IA possesses a third family of derivatives called pyrrolidone with potential use as solvent or as polymer precursor (Qi et al., 2016).

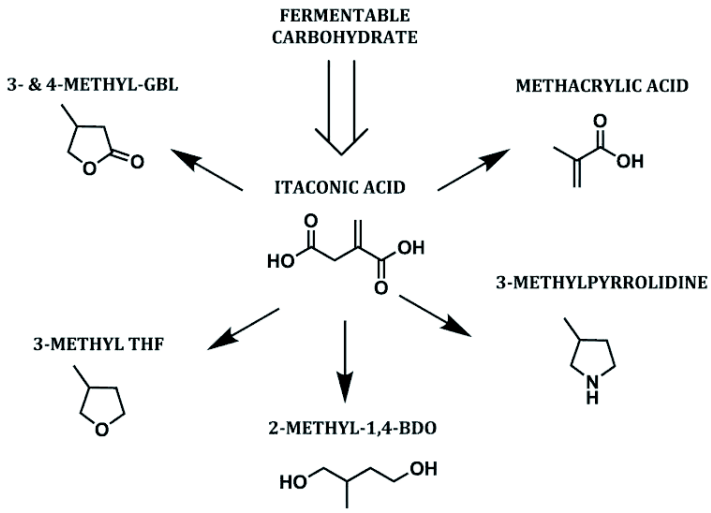

Figure 1.10. Itaconic acid molecule platform.

4.4.Glucaric acid

4.4.1.Production of glucaric acid

Glucaric acid (GA) also called saccharic acid, was identified as a valuable molecule to develop a large range of products (Werpy et al., 2004). It belongs to the group of aldaric acids, a group of sugar acids, for which the terminal hydroxyl groups of the sugars have been replaced by carboxylic acids functions. It is found in fruits, vegetables and mammals (Walaszek et al., 1996, 1997). Glucaric acid is useful in several markets such as environmental protection and remediation, building materials, health and hygiene (Mehtiö et al., 2015). The current glucaric acid market is

relatively small in accordance with its high selling price. Indeed, the current cost of glucaric acid varies from $24 to $200 per kg. Rivertop Renewable, the main producer of biobased glucarate, has recently raised $26 million from Cargill in order to develop a large-scale glucaric acid industrial production thanks to glucose's corn starch fermentation (Lane, 2014). They expect that this strategy could significantly decrease the price and consequently opens the market. Rennovia also announced that they will concentrate on the production of bio-based glucaric and adipic acid (Hagemeyer and Volpe, 2014).

Glucaric acid can be formed chemically by nitric acid oxidation of starch or by catalytic oxidation of starch with bleach (Roy Goswami et al., 2016). Glucaric acid possesses the singularity of having no known biological pathway. Indeed, no microorganism producing it was isolated to date. Consequently, researchers have constructed biosynthetic pathways in suitable host organisms in order to reach a biological production of glucaric acid (Moon et al., 2009; Reizman et al., 2015). Recently, a direct pathway was assembled in *E.coli* by introducing *myo*-inositol-1-phosphate synthase from *Saccharomyces cerevisiae*, *myo*-inositol oxygenase from mouse, and urinate dehydrogenase from *Pseudomonas syringae* (Figure 1.11) (Moon et al., 2009). In order to improve this production, an enzyme scaffold was designed to form a complex containing the three activities (Moon et al., 2010). Recent works (Table 1.5) focused in the production of glucaric acid from biomass, but the research is scarce compared with other organic acids probably due to the lack of natural producers (Wang et al., 2015b).

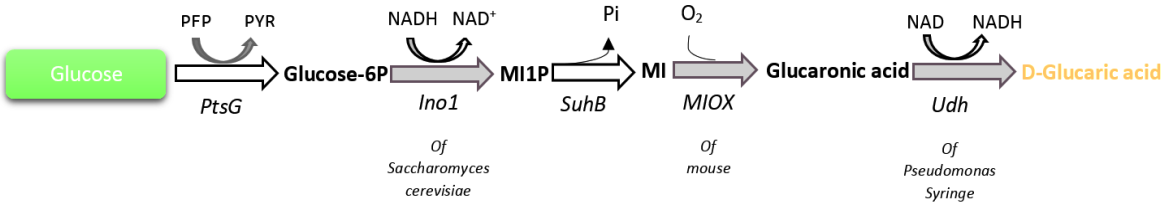

Figure 1.11. Simplified glucaric acid synthetic metabolic pathway (adapted from (Moon et al., 2009)). Abbreviations are: PtsG, phosphoenolpyruvate-dependent glucose; MI, myo-inositol; MIOX, MI oxygenase; SuhB, inositol monophosphatase; Udh, urinate dehydrogenase.

4.4.2.Glucaric acid as chemical platform for new molecules.

From GA different chemical derivatives can be obtained. Several lactones are obtained by dehydration: Glucaro-γ-lactone, Glucaro-δ-lactone, Glucarodilactone and adipic acid (Figure 1.12). Lactones could be potentially used for solvents and for polymers (Alonso et al., 2013; Ashton, 2013). As well, adipic acid is a monomer for polyurethane and for nylon (Han et al., 2013).

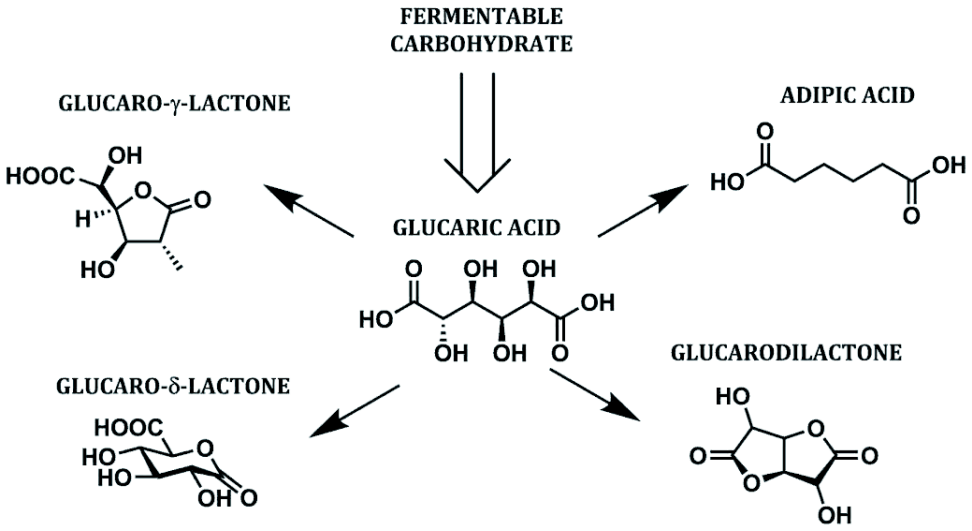

Figure 1.12. Glucaric acid molecules platform.

Table 1.5. Organic acid Building Blocks production from lignocellulosic biomass.

Organic acid	Substrates	Microorganisms	Fermentation	Yield	Productivity	References
Lactic Acid	Corn cobs	*Rhizopus oryzae*	Simultaneous saccharification and fermentation	0.3 g/g **	-	(Ruengruglikit and Hang, 2003)
		Rhizopus sp. MK-96-1196	Liquid state fermentation	0.61g/g *	-	(Miura et al., 2004)
		Rhizopus sp. MK-96-1196	Simultaneous saccharification and fermentation	0.24g/g **	-	(Miura et al., 2004)
	Sugarcane bagasse	*Lactobacillus delbrueckii*	Simultaneous saccharification and fermentation	0.83 g/g **	0.93 g l^{-1} h^{-1}	(Adsul et al., 2007)
		Rhizopus oryzae	Solid state fermentation (inert support)	0.76g/g *	1.43 g l^{-1} h^{-1}	(Soccol et al., 1994)
	Wheat bran	*Lactobacillus amylophilus* GV6	Solid state fermentation (inert support)	0.36 g/g **	-	(Naveena et al., 2005)
		Lactobacillus sp. RKY2	Liquid state fermentation	0.95 g/g **	3.1 g l^{-1} h^{-1}	(Yun et al., 2004)
	Cassava peel waste	*Rhizopus oligosporus and Lactobacillus plantarum*	Solid state fermentation	0.50 g/g **	-	(Nwokoro, 2014)
	Poplar	*Lactobacillus brevis and Lactobacillus plantarum*	Liquid state fermentation	0.8 g/g **	-	(Zhang and Vadlani, 2015)
Fumaric Acid	Corn cobs	*Aspergillus terreus*	Liquid state fermentation	0.002 g/g **	-	(Jiménez-Quero et al., 2016)
	Corn straw	*Rhizopus oryzae*	Liquid state fermentation	0,35 g/g *	0,33 g l^{-1} h^{-1}	(Xu et al., 2010)
	Apple peel waste	*Rhizopus oryzae*	Submerged fermentation	0,052 g/g **	0.035 g l^{-1} h^{-1}	(Das et al., 2015)
	Wheat bran	*Rhizopus oryzae*	Liquid state fermentation	0.4 g/g **	-	(Wang et al., 2015a)
	Cassava bagasse	*Rhizopus formosa*	Liquid state fermentation	0,14 g/g**	-	(Carta et al., 1999)
Itaconic Acid	Corn cobs	*Aspergillus oryzae*	Liquid state fermentation	0.07mg/g **	-	(Jiménez-Quero et al., 2016)
	Sugarcane bagasse	*Aspergillus terreus*	Liquid state fermentation	0.18 mg/g *		(Kocabas et al., 2014)
		Aspergillus niger	Solid state fermentation	0.008mg/g **	-	(Paranthaman et al., 2014)
	Sugarcane pressmud	*Aspergillus terreus*	Solid state fermentation (inert support)	0,0036 mg/g **	0.0003 g kg^{-1} h^{-1}	(Tsai et al., 2001)
Glucaric Acid	Birch wood	*Escherichia coli*	Liquid state fermentation			(Lee et al., 2016)

*g/g-sugar;**g/g-substrate

5. Towards biobased polymers

Bio-based plastics (bioplastics) are derived from renewable raw materials (Chua et al.). There is no consensus definition for the bio-based plastics since bio-based polymers consist wholly or partly of renewable resources. The biobased content is mainly determined through the C_{14} content. Biobased or renewable content of a product is then the amount of biobased carbon in the material or product as fraction weight (mass) or percent weight (mass) of the total organic carbon in the material or product. ASTM has set a method standard to determine the level of biobased or renewable material in a resin (i.e., ASTM D6866 – Standard Test Methods for Determining the Biobased Content of Solid, Liquid, and Gaseous Samples Using Radiocarbon Analysis). Although, no minimum threshold of renewable origin is specified to use the term “biobased” (Niaounakis, 2013), the European committee for normalization recommends a proportion between 40% and 100% of renewable raw material (Lapointe, 2012). 5.7 million tons of biobased polymers were produced in 2014, representing only 2% of global polymers production. However, the global demands will continue to increase exponentially. Recently Published Market Data planed a growth of biobased polymers production up to over 17 million tons in 2020 (Aeschelmann and Carus, 2015). Biobased polyethylene terephthalate (PET) and polyethylene (PE) are the most developed polymers from renewable resources, followed inter alia by polylactic acid (PLA) and polyhydroxyalkanoates (PHA). The most important area for bioplastic production is Asia, which is expected to control 80% of the production in 2020. The most developed industries using biobased plastic are rigid and flexible packaging, automotive and transports, consumer goods and textile (Allied Market Research, 2015).

The history of bio-based plastics is relatively long and has shown many twists. The first synthetic thermoplastic was invented in 1860 by John Wesley Hyatt, produced from collodion wool (cellulose nitrate), nitric acid, sulfuric acid and camphor and named "celluloid" (Rustemeyer, 2004). Latter, a polymer from casein and formaldehyde was also elaborated and marketed as Galatith (Krische and Spitteler, 1900). Henry Ford who was mindful of the valorization of the agricultural surpluses has used plastics from soy for many automobile components (steering wheels, interior trim, and dashboard panels). In 1941, a prototype of « plastic car » has been constructed (Bial, 2007). In 1926, at the Pasteur Institute, Maurice Lemoigne discovered that the

Gram-positive bacterium *Bacillus megaterium* produced an intracellular bioplastic: polyhydroxybutyrate (PHB). PHBs are now recognized as one of the most commonly derived forms of polyhydroxyalkanoate (PHA) (Girdhar et al., 2013). Nevertheless, after 1945 all these materials were rapidly supplanted because of the competition with new synthetic plastics from fossil resource displaying higher properties. Consequently, in the past, the researches on bio-based plastics production were abandoned and these first promising results were not fully exploited. In the last few years, the scientific approaches and the global context have changed drastically, the need for biobased materials is seen as a strong necessity for the future on agreement with a societal perspective. The challenges we face today could be summed up in a single doctrine: sustainable development meaning "Development that meets the needs of the present without compromising the ability of future generations to meet their own needs" (Burndtland, 1987). Issues are on three different levels: social, environmental and economic. Dwindling some oil fractions was one of the first driver (Madbouly et al., 2015). Besides, greenhouse gasses and preservation of the environment can be another driver (Philp et al., 2013). The development of new macromolecular architectures based in part on chemical structure extracted from biomass is also key point (John and Thomas, 2008). One of the best example of fully biobased plastic is polyamide 11 (PA11), obtained from modified a fatty acid from castor oil: ricinoleic acid (Lligadas et al., 2013).

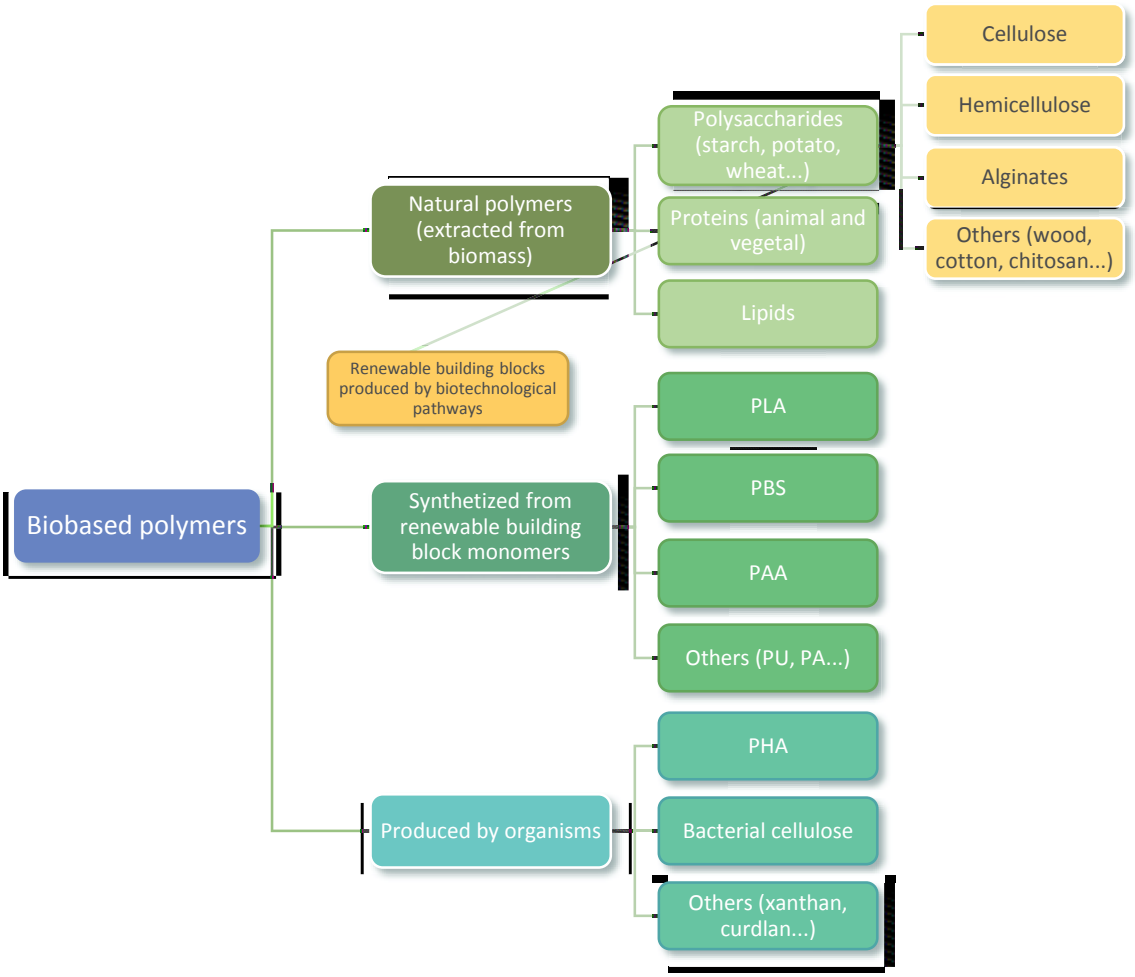

Figure 1.13. Biobased polymers (based on (Avérous and Pollet, 2012)).

Bio-based polymers could be classified into three categories as shown in Figure 1.13. The first category concerns the biomacromolecules extracted directly from biomass also called "natural polymers", they abounds in the wood, leaves, seeds, fruits … (Shen et al., 2010). Among these, polysaccharides as cellulose, hemicellulose, alginates, chitin, and chitosan are considered as abundant and low-cost feedstock (Corma et al., 2007; Yao and Tang, 2013). The second category concerns the biomacromolecules produced directly by micro-organisms such as PHAs (Koller et al., 2010, 2016). The third category of biobased polymers are elaborated from renewable building blocks (BB). These BB are produced from biomass by biological pathways (Kamm and Kamm,

2004). Around 17 x 10^{10} metric tons biomass are produced a year and only 3.5% are valorized (Kamm, 2007). This review is mainly focused on this category.

5.1.Polylactic acid (PLA)

PLA is one of the pioneer of polymers from biobased building blocks from renewable resources, so it is highly developed and used in several applications (Murariu and Dubois, 2016). In 1932, ring opening polymerization (Figure 1.14) and dimerization of polycondensated lactic acid into lactide were reported (Carothers et al., 1932). The produced PLA displayed a high molar mass comparatively to the one obtained by direct polymerization (Amgoune et al., 2005; Inkinen et al., 2011).

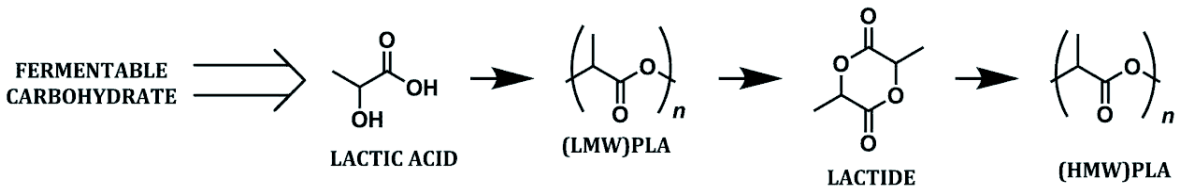

Figure 1.14. Renewable lactic acid polymerization to PLA. Low molar weight (LMW) polylactic acid and high molar weight (HMW) polylactic acid.

The PLA researches have started to increase drastically, in 1960's, due the use of PLA-based-materials in medical applications (Schindler and Harper, 1976). Presently, there are many companies around the world producing PLA thanks to large industrial applications of lactic acid, lactide and PLA (Lim et al., 2008; Nampoothiri et al., 2010; Södergård and Stolt, 2002). In 2003, Nature Works LLC has developed a low cost continuous process for PLA production and they are the major supplier of PLA under the brand Ingeo® (150 000 tons / years in 2014) (Vink et al., 2007). Ingeo® is currently produced from glucose derived from corn and use less than 0.04% of the annual global corn and therefore have no impact on food prices or supply (Vink and Davies, 2015). To better follow the green chemistry approach, recent works developed a process including cellulosic as raw material for PLA production (Shen and Xia, 2006). The strong market demand has contributed to the development of many other companies in this field such as Synbra, dedicated to PLA for technical applications. Biofoam® (Synbra) is produced by PURAC lactic acid (from

tapioca starch/sugarcane) and is suitable for the replacement of expanded polystyrene (Groot and Borén, 2010; Schut, 2008). Corbion still produce PLA from sugar cane, but the company is involved in various fundamental researches and development programs to use cellulose as sustainable feedstock (Eagle, 2016). This company launched recently diverse biobased PLA resins for extrusion, thermoforming, injection molding and fiber spinning (Romanik, 2016).

Due to the chirality of the lactic acid (L or D), various PLA could be obtained with variable properties depending on the D/L ratio (Garlotta et al., 2003). Usually, PLA displays some interesting properties such as good transparency, glossy appearance, and processability. PLA possesses odor and flavor barrier properties (Lim et al., 2008). PLA can show similar mechanical properties than conventional polymers but are not competitive in term of thermal properties and stability due to strong decrease of properties at temperatures higher than Tg (Tg=60°C) (Garlotta, 2001).

These different properties render PLA to replace conventional fossil-based polymers (PET, PS, PC) especially on packaging (Auras et al., 2004; Nampoothiri et al., 2010). PLA is also used in textile and fiber applications (Gupta et al., 2007). In the recent years, some researchers developed PLA membrane for use in automotive and chemical industry (Harmsen et al., 2014). As discussed below, thanks to its biocompatibility and its biodegradability, PLA displays many applications in medical field such as resorbable sutures, prosthetic devices or for regenerating tissue (Kalia and Avérous, 2016; Lasprilla et al., 2012; Merloz et al., 1995).

5.2.Fumaric acid (FA) derived polymers

Thanks to its chemical properties described above, FA has a great potential as a starting chemical for polymerization and esterification reactions, especially in the production of unsaturated polyester resins (Otsu and Matsumoto, 1988). FA could be a better option for the polymer industry among other carboxylic acids or their as a nontoxic chemical (Roa Engel et al., 2008). However, the larger industrial trend for FA for polymer synthesis is as succinic acid precursor, which is currently used as biobased monomer for polybutylene succinate (PBS) and several polyamides (Xu and Guo, 2010). PBS is currently produced by direct esterification of succinic acid with 1,4-butanediol (BDO) both precursors that could be produced from renewable fumaric acid (Figure 1.15) (Jacquel et al., 2011). PBS is an aliphatic polyester with some comparable properties as polypropylene, but is much more easily degradable. Then, it represents a

great environmental alternative to common plastics (Kalia and Avérous, 2016; Tokiwa et al., 2009). The PBS production represents around 40 000 tons / year (Deep research Reports, 2013). This production will increase in near future due to the expansion plans made by several companies, as Mitsubishi Chemical Company and PTT (Petroleum Authority of Thailand) (Business Standard, 2014). Another interesting opportunity for FA is as precursor of terephthalic acid (TPA), the

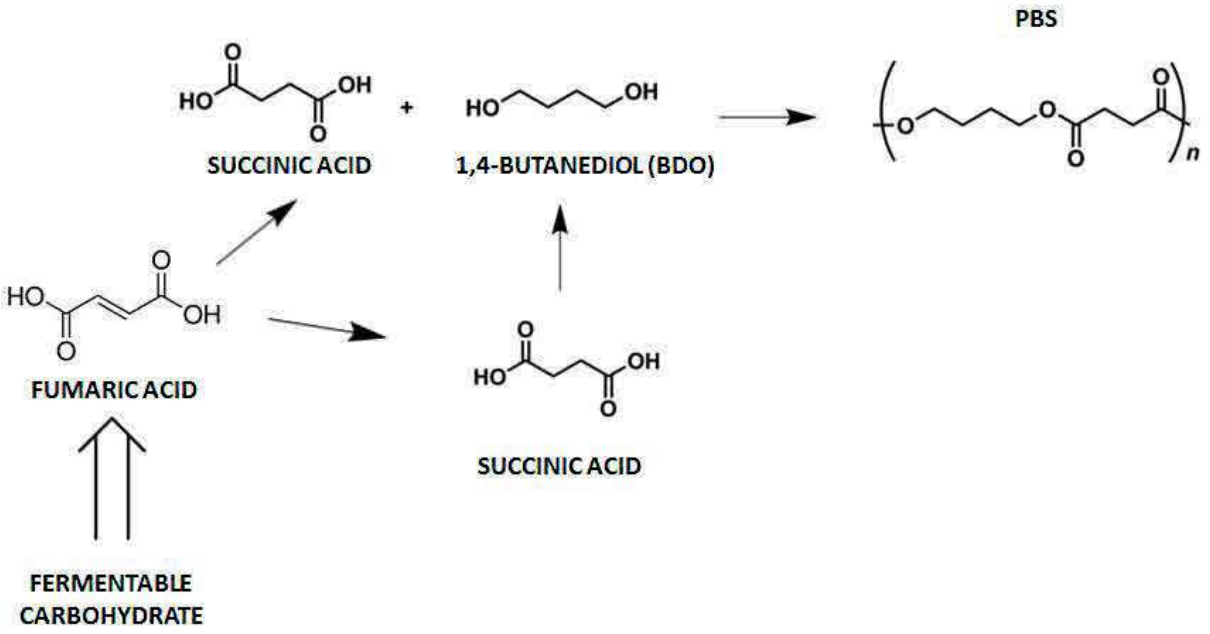

Figure 1.15. Renewable succinic acid and BDO polymerization to PBS from biobased fumaric acid.

principal monomer for the production of PET (Tachibana et al., 2015). The wide market of PET makes its biobased production one of the most promising feature for bioplastic industry.

Recent studies were dedicated on medical properties of derived polymers from FA, as poly(propylene fumarate) (PPF) or Poly(gadodiamide fumaric acid) (PGFA) (Goodfriend et al., 2015; Kasper et al., 2009). PPF is a linear polyester with the ability to form a biocompatible and biodegradable network. PPF could be injected as solution for the *in situ* formation of rigid polymer. Due to these properties, PPF-based polymers have large biomedical applications, such as creation of implants, scaffolds for tissue engineering or bioactive factor delivery systems (Fisher et al., 2002; Hacker et al., 2009; Ueda et al., 2007). PGFA is a derived of PPF synthetized from diethyl

fumarate and propylene glycol, with gadodiamide. The main application of PGFA is as resorbable radiopaque polymer injected for medical imaging techniques such as X-ray or magnetic resonance imagining (Ulery et al., 2011).

5.3.Polyitaconic acid (PIA) and corresponding copolymers

Itaconic acid is an emerging chemical platform but the challenge with IA was to resolve its polymerization process with high conversion yields. The most known polymer of itaconic acid is polyitaconic acid (PIA) which represents a new polymer opportunity (Werpy et al., 2004). It was first described by Sheperd and Tate in 1960's (Marvel and Sheperd, 1959; Tate, 1967).PIA is a water soluble polymer with a wide range of applications as superabsorbent, co-builders in detergent, anti-scaling detergent, and dispersants for minerals in coatings (Nuss and Gardner, 2012; Willke and Vorlop, 2001). Furthermore, itaconic acid could be easily incorporated into existing materials and could become a substitute for fossil-based acrylic or methacrylic acid (Harmsen et al., 2014; Le Nôtre et al., 2014). In the recent years, Itaconix Corporation, with the help of the University of New Hampshire, became the world leader in polymers from IA (De Guzman, 2009). PIA is currently produced by IA from fermentation of glucose from corn or rice. However, the most recent researches were focused on its direct production from low cost raw materials as lignocellulosic biomass to be competitive with polyacrylic acid (Brar et al., 2016; Transparency Market Research, 2015).

Several examples of IA-based polymers from renewable resources are currently described in the literature. A polyester based on isosorbide (derived from glucose), itaconic and succinic acid was produced by polycondensation (Goerz and Ritter, 2013). Thanks to dimethyl itaconate, the copolyesters were crosslinked and the final material presented shape memory effect with remarkable applications for medical or building industry, for example (Guo et al., 2011). Other interesting polymer is a thermostable degradable polyamides synthetized by a polycondensation of biobased itaconic acids salts with aromatic diamines (Ali et al., 2013). This itaconic acid-derived polyamides presents a high molar mass and higher thermal and mechanic properties than conventional bio-based plastics as PLA or more conventional polyamides. Furthermore, the polyamide become soluble in water under ultra-violet light irradiation. The biodegradability make this biobased polyamide an important polymer especially for reducing waste and ensure the

sustainability for instance by preservation of life marine creatures. Another example of aliphatic polyester based on renewable itaconic acid is the poly(butylene 2-methylsuccinate) (PBMS), a new polymer presenting an excellent thermal stability (Wu et al., 2014)

Itaconic acid anhydride (IAn) is also used as renewable monomer for bio-based polyesters synthesis (Okuda et al., 2012; Yamaguchi et al., 2014). The final polyesters possess the vinylidene double bonds from IAn and could be use as macromonomers, telechelics or crosslinking agents. The most recent researches focused deeply on medical uses, in drug release and as bioactive compound in anticancer therapy (Bera et al., 2015; Taşdelen et al., 2005). A novel copolyesters formed from fumaric, itaconic and butanediol formed by transesterification and polycondensation (Figure 1.16) was recently tested for its anticancer properties (Gowsika et al., 2014).

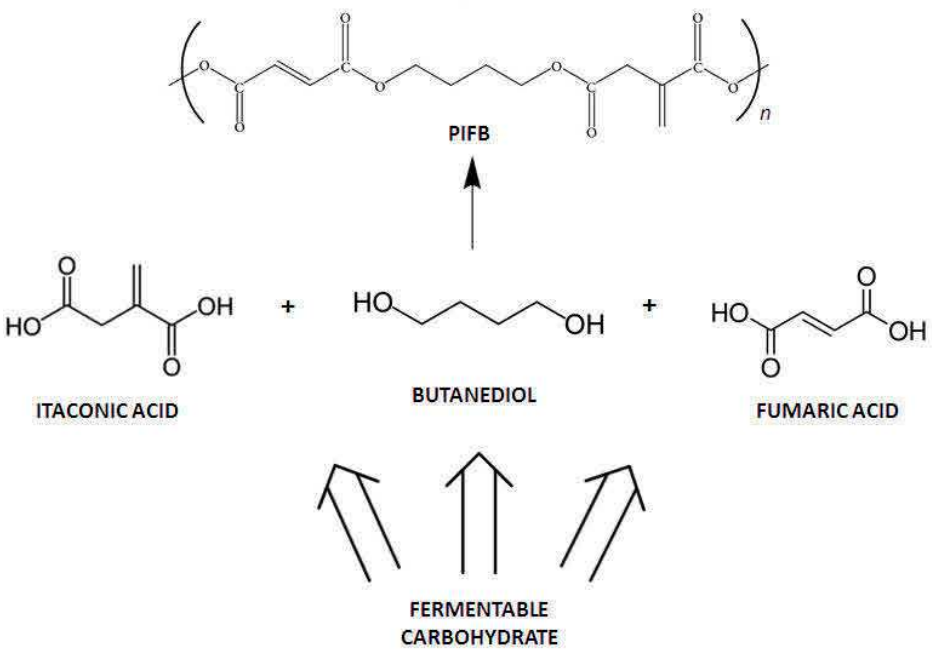

Figure 1.16. Derived polymer from itaconic and fumaric acid (based on Gowsika et al., 2014).

5.4.Other Polyamides (PA)

Polyamides can be naturally produced, such as wool or silk, or artificially synthesized via polycondensation or ring-opening polymerization (Harmsen et al., 2014). Main PA properties are oils and solvent resistance, low friction and creep, toughness, high durability, good processability, stability at elevated temperatures, fatigue and abrasion resistance (Herzog et al., 2000). PA has various applications mainly for fibers production for e.g., clothing, carpets, tire reinforcement...(Deopura et al., 2008). It also possesses numerous applications in the medical field due its good biocompatibility as bone support and scaffold for tissue culture (Risbud and Bhonde, 2001; Wang et al., 2007). The most common polyamides in industry are polyamide 6, also known as poly(hexano-6-lactam) or polycaprolactam, polyamide 6,6 and polyamide 4,6. Currently, these PA are synthetized from fossil monomers, PA-6 by ring-opening polymerization of caprolactam, and PA-6,6 and 4,6 by polycondensation containing equal parts of amine and carboxylic acid (Deopura et al., 2008). However, these monomers could also be produce from renewable resources, as showed above for carboxylic acids (Serrano-Ruiz et al., 2010). With a global market of roughly $20.5 billion in 2013 and an expected growth to reach $30 billion by 2020 the production of fully biobased PA would be extremely attractive (Acmite Market Intelligence, 2014).

PA 6,6 is formed by polycondensation of adipic acid and hexamethylenediamine. Then the corresponding production could be partially biobased with adipic acid obtained from renewable glucaric acid (Figure 1.17a). Nevertheless, PA 4,6, obtained by polycondensation of adipic acid and 1,4-butanediamine could be fully biobased (Palmer, 2002). Adipic acid could be reach by reduction of renewable glucaric acid. Renewable 1,4-butanediamine could be obtained via a chemical conversion of renewable succinic acid (Figure 1.17b). PA 4,6 has higher crystallinity and chemical resistance than polyamide 6 or 6,6 and better retention of properties at elevated temperatures, reportedly thanks to the "highly symmetrical" polymer chain. PA 4,6 is a polymer adapted for extruded materials as film, wire or cable. This polymer, produced principally by DSM and named Stanyl, can find some technical functions on automobile, electrical and electronic applications (DSM Engineering Plastics, 2015). PA 4,10 is produced by a polycondensation of 1,4-butadiamine and succinic acid and could be partially or fully biobased (Figure 1.17c). Adipic acid and 1,4-butadiamine could be produced by renewable ways as mentioned above. Furthermore, succinic acid is already produced from castor oil and a pathway to reach renewable 1,6-

hexanediamine with *E.coli* have been already studied (Adkins et al., 2012a). Biobased PA are already produced thanks to the renewable sebacic acid. EMS-Grivory develops different biobased PA with high biobased content. PA10,10 is produce with a bio content up to 99% and PA 6,10 with a bio content up to 62%. Recent researches focused on direct microbial production of renewable polymers by metabolic engineering (Adkins et al., 2012b; Kind et al., 2014; Schneider and Wendisch, 2011). Some companies such as Rhodia and Avantium have signed a collaboration to jointly develop new biobased polyamide (Chai, 2012).

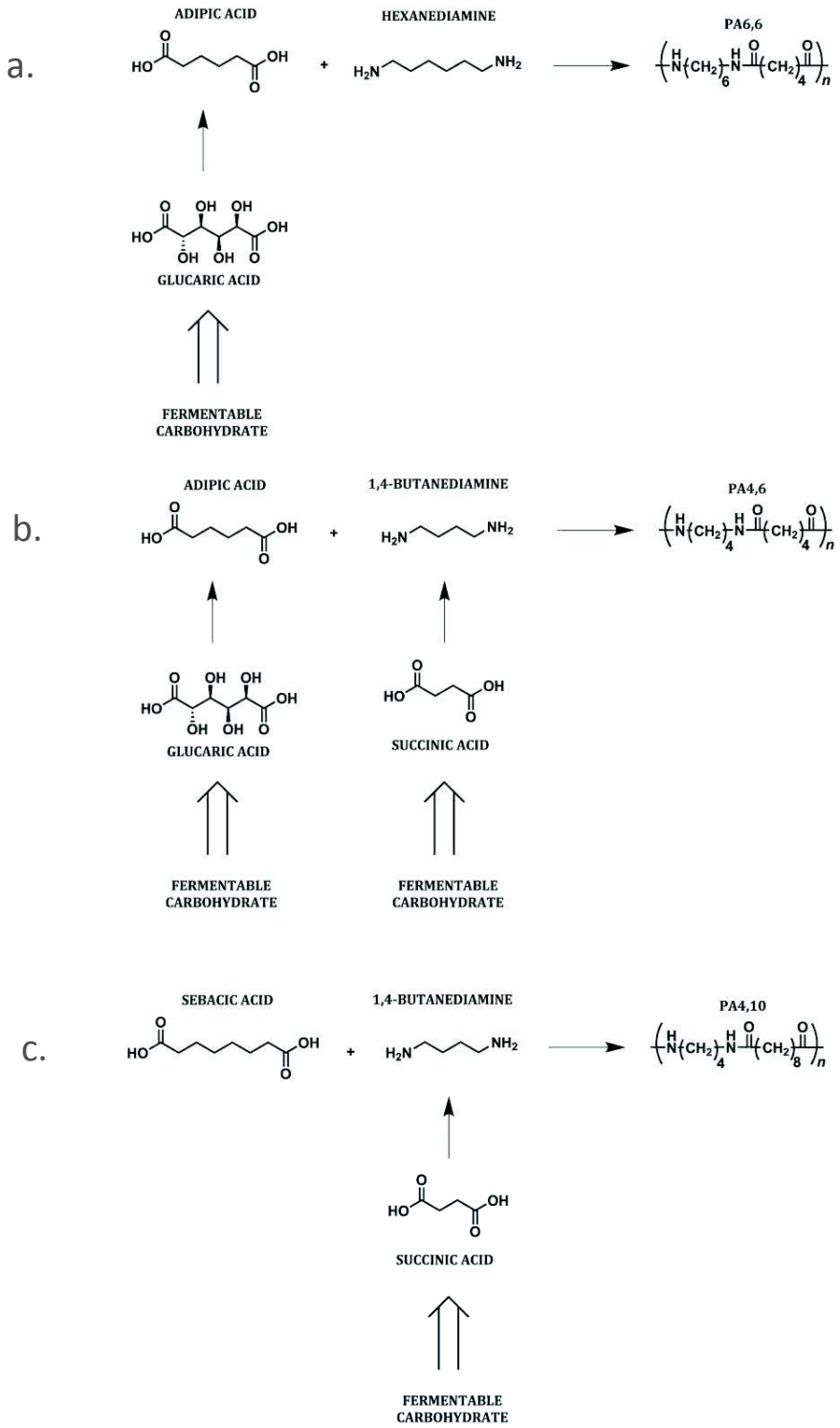

Figure 1.17. Renewable production of polyamides from biobased glucaric and succinic acids.

6. Conclusions and perspectives

The synthesis of organic acids and their polymers based on renewable resources is yet less developed than fossil-based counterparts. Furthermore, for many reasons the bioproduction of each organic acids has reach diverse stages of development (research, pilot scale or industrialization) depending of economic and technical concerns. Due to the great interest raised by bio-sourced molecules, many of current academic and industrial researches are focused on pretreatment improvement, rational strain choice and/or genetic modification, and in scale-up processes.

An attractive industrial alternative for lignocellulosic biomass deconstruction is the ionic liquid pretreatment. This treatment could be used for a wide range of feedstocks which is great advantage compared with other pretreatments of biomass (Brandt et al., 2013). Ionic liquid treatment allow a better de-crystallization of the cellulose portion and a simultaneous disruption of the lignin and hemicellulose network to facilitate the enzymatic saccharification. Moreover, the possibility of recover the different parts of lignocellulose separately ensure a higher valorization of biomasses following the biorefinery concepts. Furthermore, the impact on the downstream processes is reduced because less fermentation inhibitors are liberated. Finally, the short time of pretreatment and the low cost of ionic solutions and co-solvents make this pretreatment idyllic for the industrial bioproduction of organic acids (Wang et al., 2014).

Among other perspectives linked to the elaboration of bio-based polymers from building blocks which were recently opened, we must cite the stupendous progresses of molecular biology and metabolic engineering leading to synthetic biology. The basic principle is to use a microorganism scaffold but replacing the original metabolism by a metabolism thought by the researcher and fully dedicated to a single purpose, here building blocks or directly polymer production. As pointed out by (Cameron et al., 2014), this panel of techniques is currently changing biotechnology and medicine. As often, *E. coli* is one of the preferred hosts. The combination of two papers is particularly interesting here. An *E. coli* strain was modified by the addition of two heterologous engineered genes supposed to make able the bacteria to produced PLA directly by fermentation (i.e. without any external polymerization process) (Yang et al., 2010). Unfortunately, due to the complex metabolite fluxes, the yield was quiet low. Then, the authors studied the metabolic limitations and decided to delete in particular three genes allowing a metabolic

reorientation resulting in a PLA yield up to 11% of glucose (Jung et al., 2010). Similar strategies with *E. coli* and other microorganisms to produce directly several biopolymers were reviewed recently (Chung et al., 2015). It could be safely predicted that synthetic biology will be used in the forthcoming years to produce more and more biobased polymers in one-step processes.

Regarding scale up, the solutions will depend on the up-stream processes. If synthetic biology revealed sufficiently efficient in the near future, the current industrial plans could be used for organic acids or polymers bioproduction at large scale. Otherwise, more research efforts will be required in reactors design to optimize every unit operation of the whole process from biomass to end-products.

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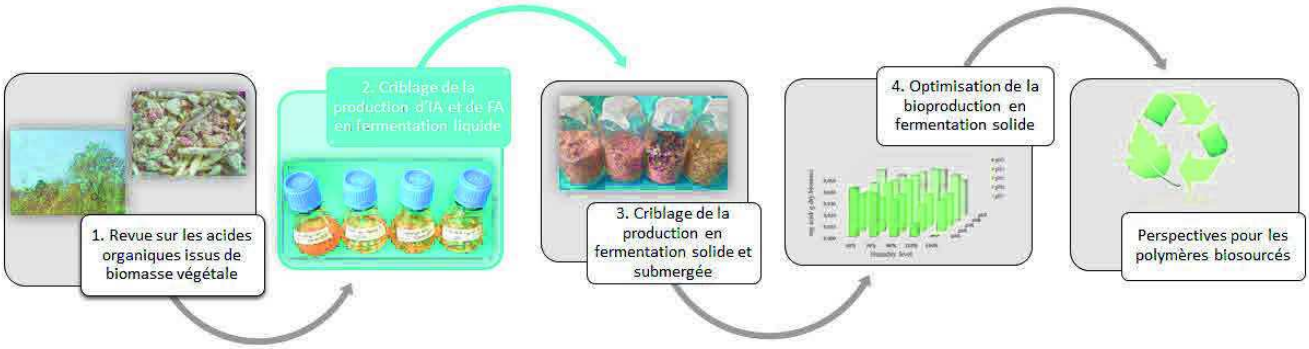
Chapitre 2. Criblage de la production d'acides en fermentation liquide



Chapitre 2

Introduction

L'étude bibliographique montre que des progrès ont été réalisés dans les laboratoires académiques et industriels pour la production durable d'acides organiques. Le contexte global issu de l'intégration des considérations environnementales dans l'économie est un force motrice importante pour que cette évolution se poursuivre.

Le travail de thèse se place résolument dans le contexte du développement durable pour la production d'acide itaconique et fumarique par voie fermentaire.

Le chapitre 2 explicite d'abord le choix des souches de champignons filamenteux du genre *Aspergillus* utilisées tout au long de cette étude. Ensuite, les traitements du son de blé et des rafles de maïs sont détaillés. Les milieux utilisés pour les fermentations sont des hydrolysats liquides issus de ces traitements (fermentation en milieu liquide: FML). Les compositions de ces hydrolysats sont analysés afin d'appréhender leur éventuelle correspondance avec les besoins nutritionnels des souches. Le criblage de différentes conditions de fermentation en milieu liquide avec ces hydrolysats acides et enzymatiques est ensuite décrit.

Ce chapitre est présenté sous forme d'une publication « Itaconic and fumaric acid production from biomass hydrolysates by *Aspergillus* strains » par Amparo Jiménez-Quero, Eric Pollet, Minjie Zhao, Eric Marchioni, Luc Averous et Vincent Phalip (2016). Acceptée pour publication dans *Journal of Microbiology and Biotechnology*.

Itaconic and fumaric acid production from biomass hydrolysates by *Aspergillus* strains

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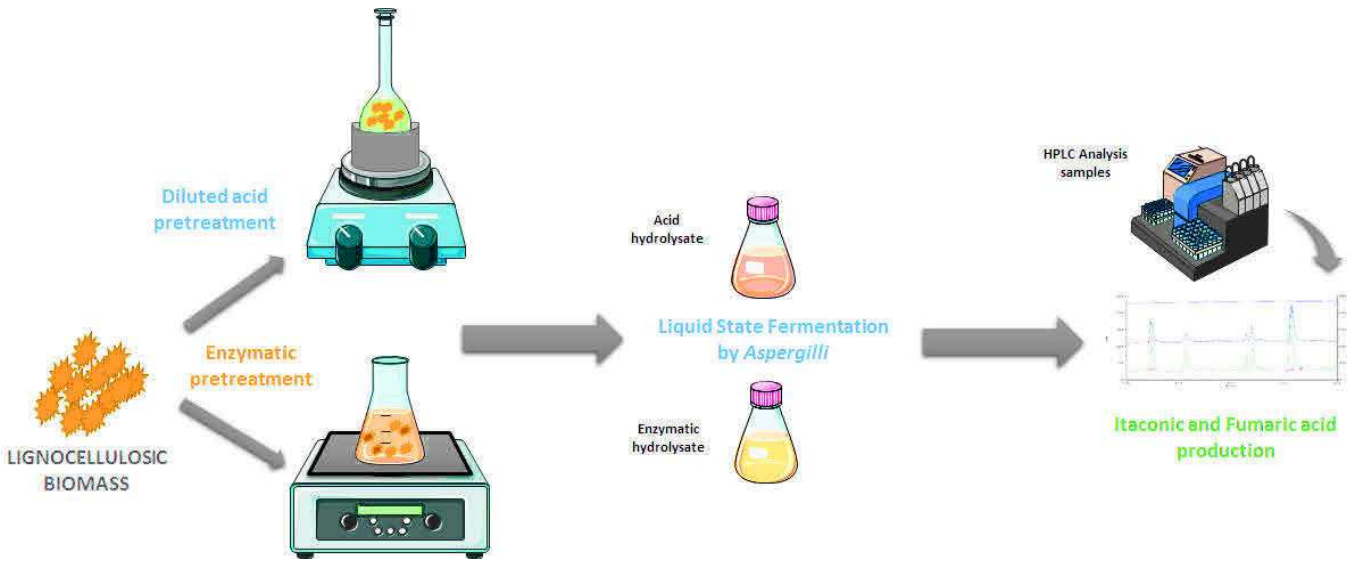

Abstract

Itaconic acid (IA) is a dicarboxylic acid included in the U.S Department of Energy's (DOE) list (2004) of the most promising chemical platforms derived from sugars. IA is produced industrially using liquid state fermentation (LSF) by *Aspergillus terreus* with glucose as the carbon source. To utilize IA production in renewable resource-based biorefinery, the present study investigates the use of lignocellulosic biomass as a carbon source for LSF. We also investigated the production of fumaric acid (FA), which is also in the DOE's list. FA is a primary metabolite, whereas IA is a secondary metabolite and requires the enzyme cis-aconitate decarboxylase (CAD) for its production. Two lignocellulosic biomasses (wheat bran and corn cobs) were tested for fungal fermentation. Liquid hydrolysates obtained after acid or enzymatic treatment were used in LSF. We show that each treatment resulted in different concentrations of sugars, metals or inhibitors. Furthermore, different acid yields (IA and FA) were obtained depending on which of the 4 *Aspergillus* strains tested were employed. The maximum FA yield was obtained when *A. terreus* was used for LSF of corn cobs hydrolysate (1.9% total glucose); and an IA yield of 0.14% was obtained by LSF of corn cob hydrolysates by *A. oryzae*.

Keywords: lignocellulosic biomass, liquid state fermentation, itaconic, fumaric, biomass valorization

1. Introduction

The reduction of fossil resource reserves is currently driving research for viable, renewable and environmentally friendly alternatives to replace conventional resources as raw material for the production of a large range of chemicals. In this context, the biorefinery concept can be defined as the utilization of plant biomass for simultaneous production of different goods, such as food, feed, materials, energy and chemicals, with added value. This process should be economically and ecologically sustainable (Menon and Rao, 2012). To also achieve social sustainability, it is important to use inedible parts of plants, such as lignocellulose, which is a low value byproduct of agriculture or forestry and which represents a non-competitive food resource. This wasted biomass is the most abundant carbon feedstock on Earth (Kamm, 2007). Lignocellulosic biomass is a recalcitrant material that often requires pretreatment to denature its compact structure and liberate and separate its main components (cellulose, hemicellulose and lignin) (Kumar et al., 2009). The use of this waste material is an opportunity to develop new, greener processes. In 2004, the U.S. Department of Energy (DOE) studied 300 molecules produced from biomass with industrial and strategic interests and published a final list of 12 molecules with great potential to act as building-block chemicals (Werpy et al., 2004). During the last decade, different proposals have been submitted to utilize these biobased chemicals and the technologies required for their production (Bozell and Petersen, 2010).

Among these molecules, organic acids represent an interesting group. Most are bioproducts from the metabolic pathways of microorganisms, and their functional chemical groups make them useful platform chemicals for the industry. Itaconic (IA) and fumaric acid (FA) are two biobased organic acids, which were included in the DOE's 2004 list (Werpy et al., 2004). Both are intermediates of the oxidative phase of the TCA cycle in cells, but FA is also involved in CO_2

fixation during the reductive phase of the TCA cycle (Goldberg et al., 2006). The metabolic pathway for IA and FA synthesis is illustrated in Figure 2.1. The production of these acids is modulated by the concentration of glucose and the need for energy (in form of ATP or GTP) and reducing power (in form of $NADH_2$ or $FADH_2$). IA and FA are unsaturated dicarboxylic acids formed by five and four carbons, respectively (Figure 2.1). The presence of a double bond and two carboxyl groups allows them to polymerize into high molar mass compounds (Lutz and Börner, 2008; Veličković et al., 2008). FA is also a valuable intermediate in the preparation of edible products, such as L-malic acid and L-aspartic acid, with increasing usefulness in the production of sweeteners, beverages and other health foods. The worldwide demand for fumaric acid and its derivatives are projected to grow annually at rate of 5.8% from 2013 to 2018 (Xu et al., 2012). IA has a broad application spectrum in the industrial production of resins and is used as a building block for acrylic plastics, acrylate latexes, super-absorbents, and anti-scaling agents (Klement and Büchs, 2013).

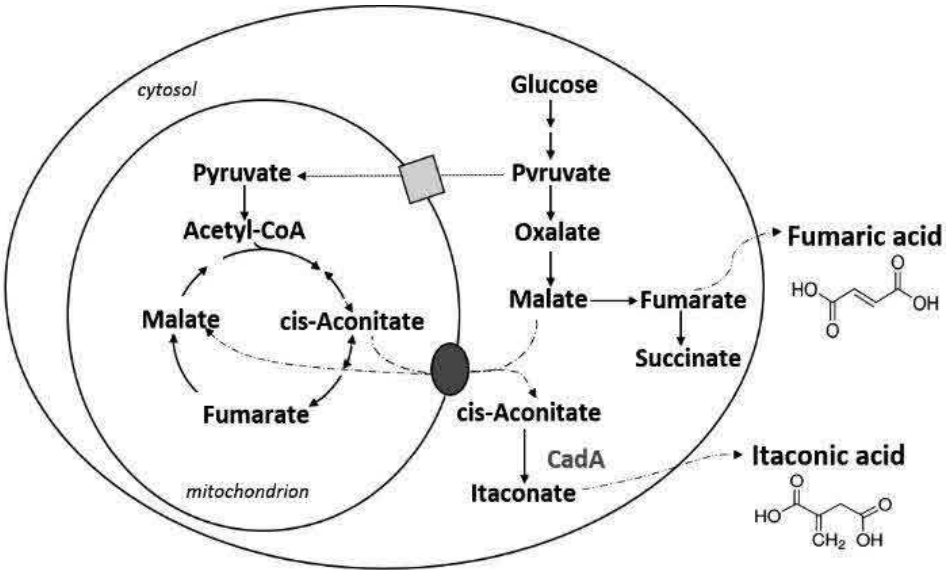

Figure 2.1. Simplified metabolic pathway of itaconate and fumarate production.

1.

These organic acids can be produced by filamentous fungi at high concentrations (Magnuson and Lasure, 2004). Thanks to their metabolic versatility, *Aspergillus* species are used in biotechnology for the production of a variety of products, such as organic acids, pharmaceuticals, and enzymes. Since 1960, the production of IA has been achieved by fermentation of liquid sugar-containing media by *Aspergillus terreus*. The strain NRRL1960 achieves production yields of 80–90 g/L (Kuenz et al., 2012). In recent decades, investigations have focused on the production of IA from renewable biomass sources, at first from starchy materials such as cornstarch, molasses or grains. Nonetheless, these primary biomasses are expensive and used in the food industry. Consequently, lignocellulosic feedstocks are more attractive for biorefinery (Lucia et al., 2006). In contrast, the industrial production of FA from biomass is still in its early stages and is not competitive with chemical synthesis from petrochemical feedstock. However, *Rhizopus* species were shown to be the best producers of FA using renewable biomass as an energy source (Xu et al., 2010). However, some research efforts are still necessary to produce IA and FA from waste biomass with sufficiently high yields to be competitive with current industrial processes (Mondala, 2015).

The aim of this study was to determine the abilities of 4 *Aspergillus* strains (*A. terreus* 826, *A. terreus* 62071, *A. tubingensis* and *A. oryzae*) to produce IA and FA from liquid state fermentation of two lignocellulosic biomasses (wheat bran and corn cobs). Two different biomass treatments (acid and enzymatic) were tested.

2. Materials and methods

2.1. Microorganisms

A. terreus (DSM 826 and DSM 62071) was provided by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany) Culture Collection. The strains were

revived on potato dextrose broth medium (PDB) for 5-6 days at 25°C. *A. oryzae* (UMIP 1042.72) was provided by the Fungal Culture Collection of the Pasteur Institute (France). *A. tubingensis* (IMI 500512) was isolated in our laboratory from agricultural residues and identified by CABI Bioscience (United Kingdom). The microorganisms were maintained on potato dextrose agar (PDA) and Czapek dox agar (CDA). The spore suspensions were harvested from 5/6 days old PDA and CDA plates with 0.2% (v/v) Tween-80. The spores were counted using a Malassez counting chamber and stored at -20 ± 1°C.

2.2. Substrate and pretreatment

Two agro-industrial waste biomasses, wheat bran and corn cobs (Comptoir Agricole, France) were used as substrates to evaluate their suitability for the production of IA and FA but also for the production of enzyme cocktails. The lignocellulosic material was crushed (SX 100, Resch) to obtain particles that were 0.5-1 mm in size. Moreover, the liquid extracts that resulted from these biomasses after pretreatment (2.2.1 and 2.2.2) were used in a liquid state fermentation (LSF). The physico-chemical characterizations of the extracts are provided in Table 2.1. The chemical analyses were performed by SOCOR (France) and CSIC (Spain).

2.2.1. Dilute acid pretreatment

Wheat bran and corn cob slurries were made with 1% dilute H_2SO_4 (42 g dry solid substrate in 230 ml dilute acid, 18.26% w/v), preheated at 150°C for 25 min and cooled down to room temperature according to previously described methods (Lenihan et al., 2010). The liquid was collected by centrifugation (8000 g for 30 min) and then adjusted to the desired pH with 5 M NaOH and finally filter-sterilized with a 0.22-µm filter.

2.2.2. Enzymatic pretreatment

Both biomasses were treated with a liquid enzymatic cocktail of *A. tubingensis* and *A. oryzae* produced by solid state fermentation of wheat bran. These cocktails displayed endo-xylanase, amylase and cellulase activities (data not shown). The hydrolysates were obtained as follows: 60 ml of the enzymatic cocktail was added to 5 g of dry biomass in 125-mL baffled Erlenmeyer flasks, and the flasks were incubated at 37°C with 80 rpm shaking. Sugar formation was estimated at regular intervals for 216 hours. The hydrolysates were sterilized by filtration after a 9-day incubation period before they were used for fermentation.

2.3. Liquid state fermentation (LSF)

Fifty millimeters of the liquid hydrolysates (acid pretreatment and enzymatic pretreatment) were poured into 125-mL baffled Erlenmeyer flasks. The hydrolysates were inoculated with spore suspensions at 10^5 spores/mL. The flasks were incubated on a rotatory shaker (Infors Multitron, Switzerland) for 7 days at 33°C with 120 rpm shaking. The hydrolysates from wheat bran and corn cobs were set at two different pH values for fermentation (pH 3-4 and pH 6-7). Unless specified otherwise, these fermentation conditions were maintained throughout the study. All experiments were conducted in duplicate. Samples of 500 µL were taken at regular intervals, filtered with a 0.2-µm membrane and stocked at -20°C for HPLC analysis and additional studies. Diluted hydrolysates from acid and enzymatic pretreatments were also used in LSF. These diluted solutions were tested with and without the addition of glucose and/or metals up to the level of the optimized medium (used as a positive control).

2.3.1. LSF of optimized culture medium

The optimized IA production media contained 180 g glucose, 0.1 g KH_2PO_4, 3 g NH_4NO_3, 1 g $MgSO_4 \cdot 7H_2O$, 5 g $CaCl_2 \cdot 2H_2O$, 0.00167 g $FeCl_3 \cdot 6H_2O$, 0.008 g $ZnSO_4 \cdot 7H_2O$, and 0.015 g

$CuSO_4 \cdot 7H_2O$ per liter of media (Kuenz et al., 2012). The pH was adjusted to 3.1 with 1 M H_2SO_4 before addition of the $CaCl_2$ solution. The solutions were autoclaved for 20 min at 121°C and 1.4 bars/min. The $FeCl_3$ solution was not autoclaved but was filter sterilized. The fermentations were performed in 500-mL baffled Erlenmeyer flasks with a working volume of 100 mL. The experiments were conducted on a rotatory incubator shaker (Infors Multitron, Switzerland) at 33°C with 120 rpm shaking and an initial spore concentration of 10^5 spores/mL. The conditions for sampling and analysis were the same as those used for LSF. The fermentations were conducted in duplicate.

2.4. Analytical procedures

2.4.1. Sugar assays

The reducing sugars present in the different hydrolysates were determined by the DNS method with spectrophotometric measurements at 550 nm (Miller, 1959). Glucose was measured by a colorimetric method measured at 420 nm (Okuda et al., 1977).

2.4.2. Acid assays

A chromatographic system, made of a 616 pump, a 2996 photodiode array detector operating in a range of 200 to 450 nm, and a 717 Plus autosampler (Waters, France) controlled with Empower 2 software (Waters) was used to analyze the samples. Chromatographic separation was achieved on a hypercarb porous graphitic carbon LC column (150 x 3.0 mm i.d., 3 µm, Thermo Scientific, USA) at 70°C. The mobile phase consisted of water:formic acid (99.9:0.1 v/v, phase A) and acetonitrile:formic acid (99.9:0.1 v/v, phase B) at a flow rate of 0.55 mL/min. Elution was performed with a gradient as follows: 0% B (0-5 min), 0-27% B (5-18 min), and 27-27% B (18-26 min). Finally, phase B was decreased to its initial concentration (0%) in 1 min, and the column was re-equilibrated for 14 min. The injection volume was 20 µL. For the analysis of samples from LSF

on optimized culture medium, the temperature was 60 °C and the gradient was as follows: 10-34% B (0-8 min), 34-10% B (8-9 min) and 10% B (9-23 min) to re-equilibrate the column. The columns were calibrated using commercial IA and FA with a 99.9% purities (Sigma-Aldrich, USA) and a UV measurement at 205 nm. Each sample was supplemented with 10 ppm IA or FA to confirm the acid production.

2.4.3. Phenolic compound assays

The pretreatment, which is useful for increasing the yield of available sugars, can also release metabolism inhibitors (Hendriks and Zeeman, 2008; Jönsson et al., 2013). The specific inhibitors released from the lignin portion after treatment were measured to compare the acid and enzyme treatments. These compounds with an aromatic ring liberated from the lignin solubilization can be detected between 280-320 nm by photometry (Le Digabel and Avérous, 2006). The acid and enzymatic hydrolysates were filtered with 0.22-µm filters before their respective absorbances were measured with an Ultrospec 7000 spectrophotometer (GE Healthcare, United Kingdom).

Table 2.1. Hydrolysates composition after acid and enzymatic biomass treatments.

Constituents	Acid hydrolysates		Enzymatic hydrolysate *A. tubingensis*		Enzymatic hydrolysate *A. oryzae*	
	Wb	CC	Wb	Cc	Wb	Cc
Sugars (g/L)						
Reducing sugars	19.8	6.9	24.1	17.3	36.5	27.2
Glucose	3.2	4.2	8.4	4.7	12.9	7.7
Total nitrogen (% dry wt)	0.34	0.25	0.79	0.48	2.87	1.53
Metal (mg/L)						
Calcium	121.9	241.9	3.6	1.2	18.2	21.8
Iron	10.1	19.5	4.7	4.4	4.5	1.5
Magnesium	286.5	33.5	327.8	112.6	516.9	292.8
Manganese	2.5	2.5	8.3	2.8	11.5	5.9
Potassium	1608.5	1083.5	2650.6	2244.9	2749.1	2555.2
Sodium	4892.1	6099.2	2203.8	2211.9	1892.5	1975.9
Zinc	1.5	5.1	5.5	3.7	4.7	2.7
Anions (mg/L)						
Orthophosphates	478.5	107.5	3760.2	3240.3	3970.1	3330.2
Phenolic compounds (mg/L)						
Determined at 280 nm	970.2	945.7	627.8	677.1	904.3	917.7
Determined at 320 nm	573.9	585.9	287.8	368.1	454.4	486.1

3. Results

3.1. LSF in the optimized culture medium

A. terreus is the most frequently used fungus to produce IA in large-scale formats, but it is particularly sensitive to the cultivation conditions. IA production is strongly affected by several components including the type and concentration of carbon source; levels of nitrogen, phosphate, trace minerals, dissolved oxygen and carbon dioxide; pH; and temperature (Kuenz et al., 2012).

In this study, the four *Aspergillus* strains were tested in the optimized medium (2.3.1) to check IA production. This medium was optimized for *A. terreus* DSM 23081 to produce IA with high yield (Kuenz et al., 2012). This condition could be considered as a positive control for the IA production in this study. All of the strains grew quickly in this medium, but IA was only produced by the two *A. terreus* strains (Figure 2.2). FA was not produced by any *Aspergillus* strains under these conditions. The final concentration of IA for *A. terreus* 826 was 33.2 g/L and for *A. terreus* 62071 7.8 g/L. The IA production yield based on the initial total glucose content was 180 mg/g glucose (18%) for *A. terreus* 826.

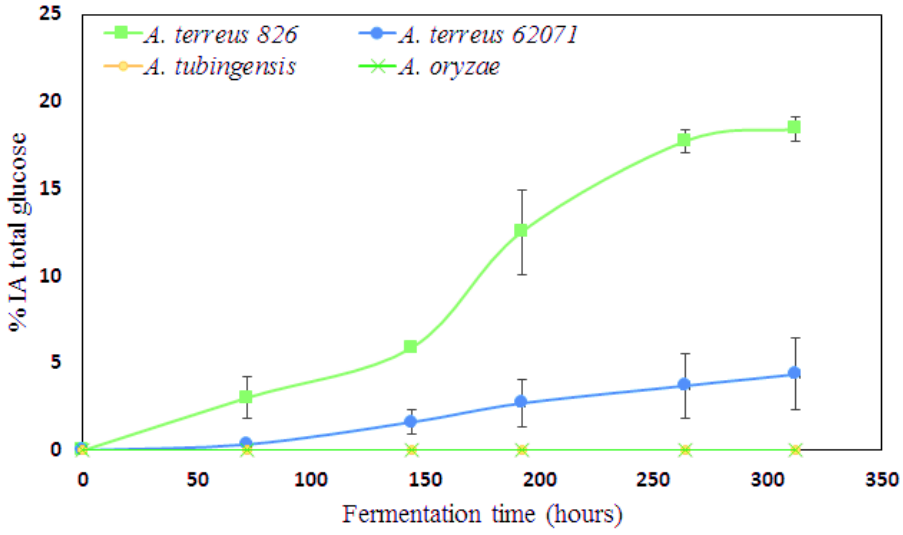

Figure 2.2. IA production by Aspergillus strains in optimized culture medium.

3.2. LSF of acid hydrolysates

The pretreatment releases reducing sugar and glucose from the two biomasses (Table 2.1). In the wheat bran hydrolysate, 3.2 g of glucose/L hydrolysate was released, and in the corn cob hydrolysate, 4.2 g of glucose/L hydrolysate was released. However, IA was not produced during LSF of the hydrolysates from the diluted acid pretreated biomasses.

The *A. terreus* strains produced the highest levels of FA, especially in the corn cob hydrolysate at pH 6 with a yield of approximately 2% of FA from the initial glucose (Figure 2.3). *A. oryzae* produced 8-times less FA, and *A. tubingensis* did not produce FA at all from the corn cob hydrolysate. On the contrary, these 2 strains showed better FA yields from wheat bran hydrolysate at pH 3.

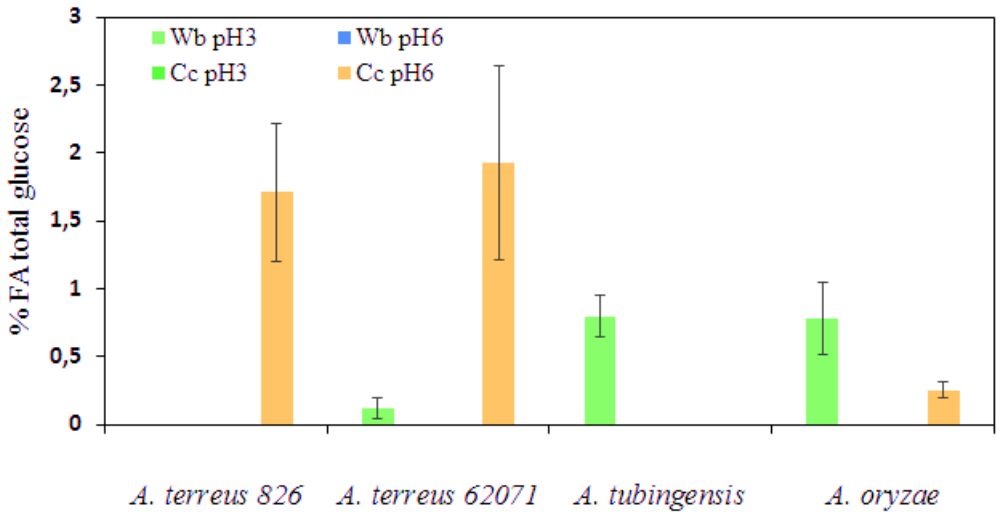

Figure 2.3. Fumaric acid production in liquid state fermentations of acid pretreated biomass hydrolysates.

3.3. Enzymatic treatment of biomasses

3.3.1. Sugar release from biomasses

Fungi enzymatic cocktails are well known to produce a large diversity of cell wall degrading enzymes (Phalip et al., 2012). High cellulase activity is crucial for glucose release from biomasses (Dashtban et al., 2009). Consequently, the *A. tubingensis* enzyme cocktail that displayed the highest cellulase activity (data not shown) was chosen for this study.

The enzymatic hydrolysis was conducted on both the raw and the acid pretreated biomasses. As shown in Figure 2.4, the biological treatment released more reducing sugars and glucose from the raw biomasses. Glucose concentration after 216 hours from the raw wheat bran (11.09 g/L) was 4.6-times greater than that for the corn cobs (2.49 g/L), and the reducing sugars were 1.4-times higher in the raw wheat bran (37.53 g/L vs. 27.12 g/L).

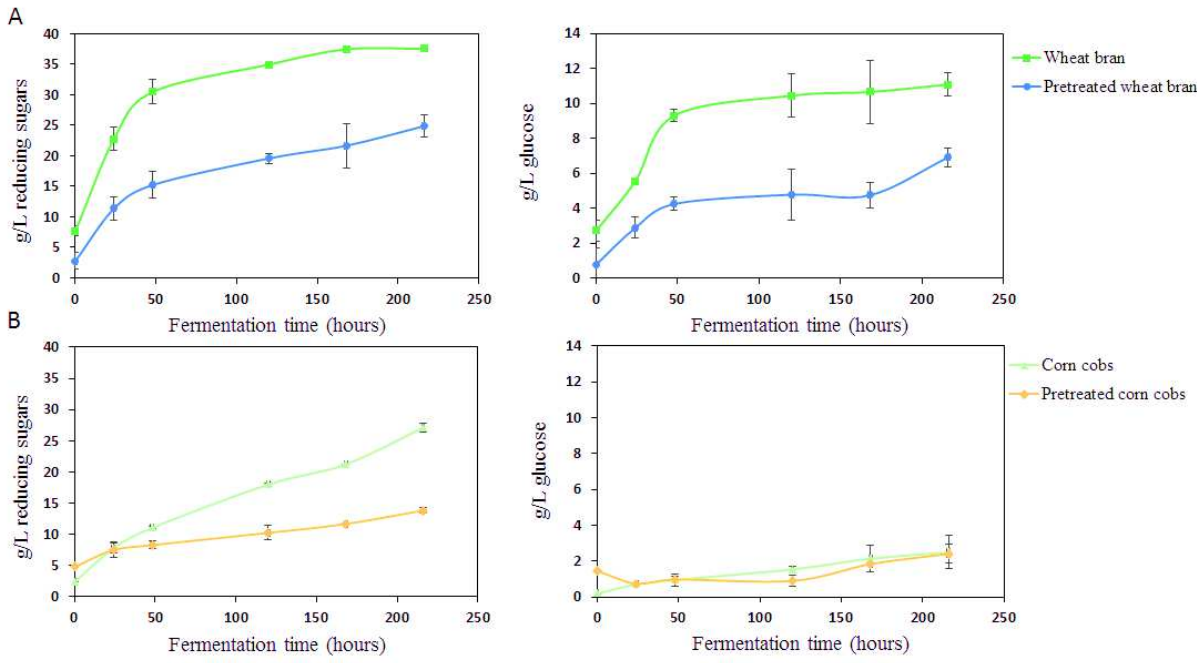

Figure 2.4. Sugars release from biomasses by enzymatic treatment (216h) with A. tubingensis cocktail. A: from wheat bran. B: from corn cobs.

3.3.2. LSF of enzymatic hydrolysates

The use of two fungal cocktails produced liquid hydrolysates from raw lignocellulosic biomasses with their respective sugars concentrations shown in Table 2.1. Almost 2-times more glucose was liberated from the biomasses treated with the *A. oryzae* enzymatic cocktail. The enzymatic hydrolysates were collected as previously described to perform LSF with the four *Aspergillus* strains. The LSF lasted for 168 hours, and the acids produced are shown in Figure 2.5. Whereas FA was produced by the four strains, IA was produced by only three (not by *A. tubingensis*). *A. terreus* 826 and *A. oryzae* produced 0.12% and 0.14% IA from the initial glucose, respectively, from the corn cob hydrolysate produced by treatment with the *A. tubingensis* enzymatic cocktail.

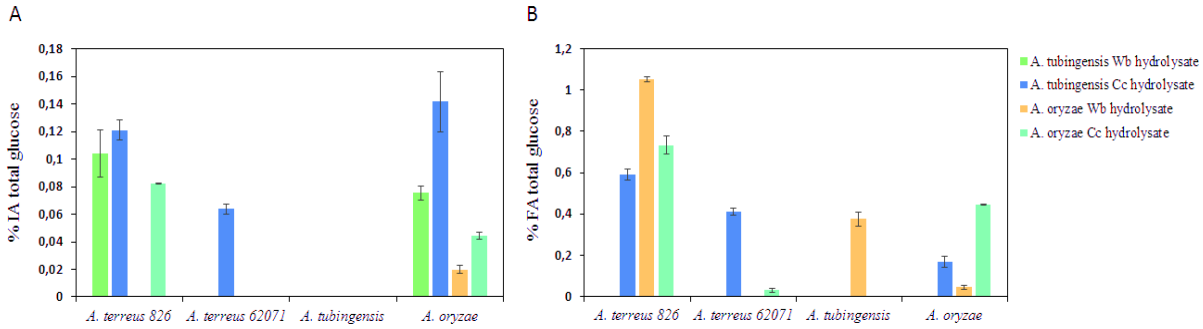

Figure 2.5. Production of IA and FA on the enzymatic hydrolysates. A: IA production and B: FA production.

FA was produced at the highest concentration by *A. terreus* 826 in the enzymatic hydrolysate of raw wheat bran from the *A. oryzae* enzyme cocktail, with a yield of 1.07% from the initial glucose (14.9 g/L).

3.4. LSF of diluted hydrolysates

Based on the comparison between the optimized culture medium (2.3.1.) and analyses shown in Table 2.1, some constituents in the hydrolysates were too concentrated. The more concentrated metals, mostly in the acid hydrolysates, such as calcium and sodium, must be reduced

to approach the appropriate concentrations in the optimized culture medium to facilitate IA production. The dilution of the hydrolysates also reduces the presence of potential inhibitors such as phenol compounds.

Unfortunately, the fermentation of different acids and enzymatic hydrolysates after dilution did not result in increased acid production.

The low acid production following hydrolysate dilution could be due to the reduced sugar concentration. Therefore, glucose was added to reach to the initial glucose concentration of the optimized culture medium (180 g/L). The fermentation was performed for the acid and enzymatic hydrolysates with a 10 times dilution and glucose added. No IA was formed with any of the strains tested. FA was produced from the wheat bran diluted acid hydrolysate by *A. tubingensis* and *A. oryzae* (0.04 and 0.03% total glucose, respectively), and the 4 strains produced FA from the corn cobs diluted acid hydrolysate, with the highest yield of 0.09% total glucose by *A. terreus* 826. In the same way, this strain produced the maximum FA from the corn cobs diluted enzymatic hydrolysate with 1.21% total glucose. As expected, the addition of metals but not glucose did not allow for any acid production.

To complete this experiment, other concentrations of sugars and metals were added to the diluted hydrolysates in order to mimic the optimized culture medium. For the diluted acid hydrolysates with additions, the production yields are shown in Figure 2.6. All of the strains produced FA from the two biomasses, but the corn cobs diluted acid hydrolysate displayed the best IA production (0.018% total glucose). The *A. terreus* strains also produced IA from wheat bran diluted acid hydrolysate, but these yields were 900 times and 130 times lower than those from the corn cob hydrolysates for *A. terreus* 826 and 62071, respectively. The most surprising result was

the IA production by *A. oryzae* (1.6% total glucose) because this strain could not produce IA during LSF of optimized culture media. The introduction of corn cobs hydrolysate into the fermentation medium contribute to fulfill the requirement for *A. oryzae* to produce IA.

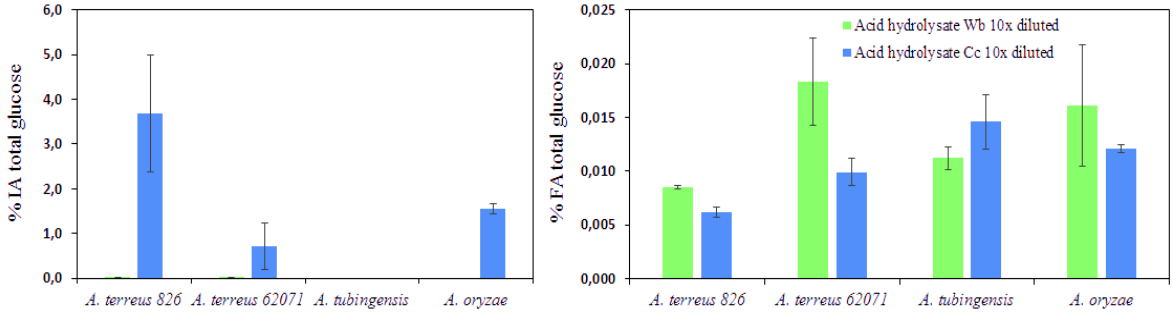

Figure 2.6. IA and FA production from acid hydrolysates 10 times diluted with the addition of glucose and metals.

4. Discussion

In the present study, FA was produced by four different *Aspergillus* strains (*A. terreus 826, A. terreus 62071, A. oryzae* and *A. tubingensis*). In contrast, IA was only produced by *A. terreus* strains and *A. oryzae.* Furthermore, although the production FA was quite general, IA was only produced in a few specific fermentation conditions. These results are quite surprising because a recent study reported that these three species were not found previously as FA producers (Liaud et al., 2014). Moreover, the capacity to produce IA was only showed by *A. terreus* (Liaud et al., 2014), nonetheless, our study proves the IA production by *A. oryzae* also.

IA is produced by cis-aconitate decarboxylase (CAD, Figure 2.1), an enzyme found and characterized in *A. terreus* (Dwiarti et al., 2002). Recently, some studies have focused on cloning this enzyme to produce IA in host microorganisms such as *E. coli* or *A. niger* (Kanamasa et al., 2008; van der Straat et al., 2014). In our work, the production of IA by *A. oryzae* is described for the first time, suggesting the presence of CAD in this species. An examination of the *A. oryzae*

genome revealed a gene-encoding a protein (AO090010000161) displaying 54% identity and 69% similarity with CAD of *A. terreus* (ATET_09971), supporting this hypothesis.

The LSF in optimized culture medium confirmed the ability of *A. terreus* strains to produce IA. *A. terreus* 826 produced 90 g/L of IA in totally controlled bioreactor with 180 g/L of glucose, resulting in a yield of 0.5 g IA/g total glucose (Kuenz et al., 2012). Our experiments were conducted in flasks in an uncontrolled atmosphere. This could explain the lower production yields (0.18 g IA/g total glucose) as pH and aeration control are necessary for a high yields (Gyamerah, 1995b; Hevekerl et al., 2014; Riscaldati et al., 2000). *A. terreus* 62071 displayed a lower production. In contrast, FA was not formed by any strain. This could be because the medium was optimized for IA production by *A. terreus,* and the other species have different requirements.

When the medium was prepared with diluted acid hydrolysates and with addition of glucose and metals (Figure 2.6), the 4 strains produced FA. The addition of WB and CC hydrolysates induced similar FA production. Nevertheless, IA was produced at greater concentrations when diluted acid corn cob hydrolysates were used, even if the metals and phosphates were at the same concentrations. A possible explanation for this is that each hydrolysate has a particular composition that affects the fungal growth and interferes with IA production (Gyamerah, 1995a).

Biomass treatment, which permits sugar release from the polysaccharides chains of lignocellulose, also released some inhibitors of microorganism metabolism and interfered with the fermentation yield (Schmidt et al., 2014; Zha et al., 2014). The performance of biomass hydrolysates as fermentation media vary due to the presence of inhibitory compounds and their concentrations. After acid treatment, the phenolic compounds were more concentrated in the wheat bran hydrolysate than in the corn cob hydrolysate (Table 2.1). Indeed, IA production was impaired

after this treatment, although FA production was unaffected. Unlike IA, FA is a primary metabolite, and as the four strains grow in the acid hydrolysate, it is not surprising that FA was produced.

FA displayed the highest production yield (1.9% total glucose) for *A. terreus* in LSF of corn cob acid hydrolysate. A possible explanation for this is that the corn cob hydrolysate is richer in glucose than is the wheat bran hydrolysate, with 4.2 and 3.2 g/L, respectively.

The enzymatic treatment study showed that raw biomasses are better alternatives than acid treated ones. The comparison of two fungal enzyme cocktails displayed (in 3.3.1.) an unexpectedly greater extent of sugar liberation with the *A. oryzae* enzyme cocktail, which supported the highest FA yield (1.07% total glucose). This cocktail also released higher manganese concentrations from both biomasses. However, a manganese ion concentration less than 3 ppb seems necessary to obtain the greatest IA yield (Karaffa et al., 2015). This requirement is close to fulfilled with the corn cob hydrolysate treated with the *A. tubingensis* enzyme cocktail, and thus the IA production yield was higher under these conditions.

In the same way, the acid treatment of biomasses liberated higher concentrations of sodium, 5 g/L from wheat bran and 6 g/L from corn cobs, compared with the enzymatic hydrolysates (Table 2.1). However, the optimized culture medium (2.3.1) did not contain any sodium source, and the presence of sodium could be deleterious for IA production.

This production of IA could be compared with the production in the optimized medium by *A. terreus* 826, the positive control (3.1). The IA yield production was 18% from the initial glucose, i.e., 160 times the production from the enzymatic corn cob hydrolysate. However, a high sugar concentration is a critical parameter for IA production by *A. terreus*, and the corn cob enzymatic hydrolysate had 50-times less glucose than did the optimized culture medium.

In summary, the use of lignocellulosic biomass with enzymatic treatment brings us closer to potentially renewable production of IA and FA. Furthermore, the identification of *A. oryzae* as an IA and FA producer leaves open the possibility of a simultaneous saccharification and fermentation thanks to the great enzymatic capacity of this fungal strain (de Castro and Sato, 2014; Sandhya et al., 2005). This option is better suited for an industrial process because the treatment time could be reduced, and the cellulosic enzymes formed by the microorganism are also commercial products (Begum and Alimon, 2011). Furthermore, the results obtained could boost future research about the organic acids metabolism and metabolic engineering (gene expression, metabolic fluxes, coenzyme availability…) in this species. The biorefinery concept can become economically and environmentally more interesting by the generation of multiple products with the optimal utilization of renewable resources.

Acknowledgments

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Supplementary data

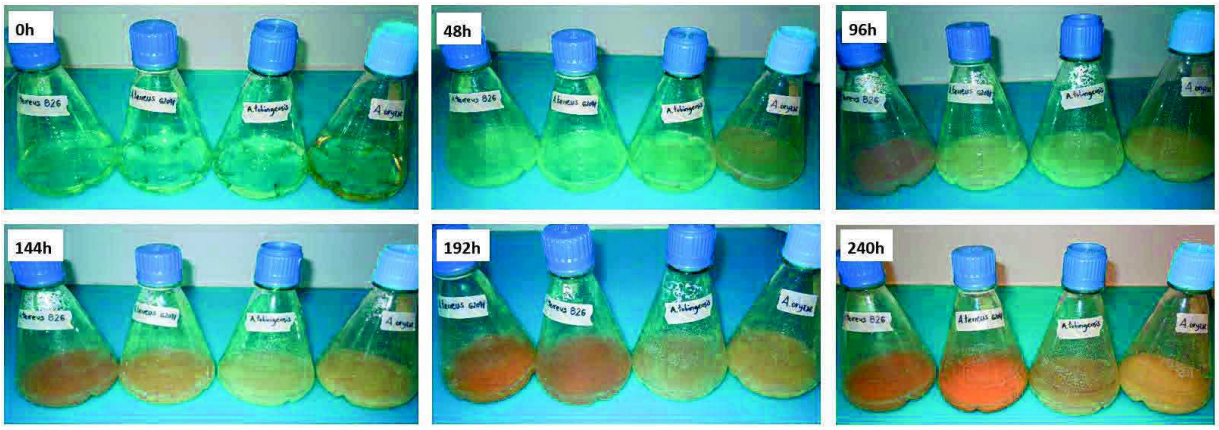
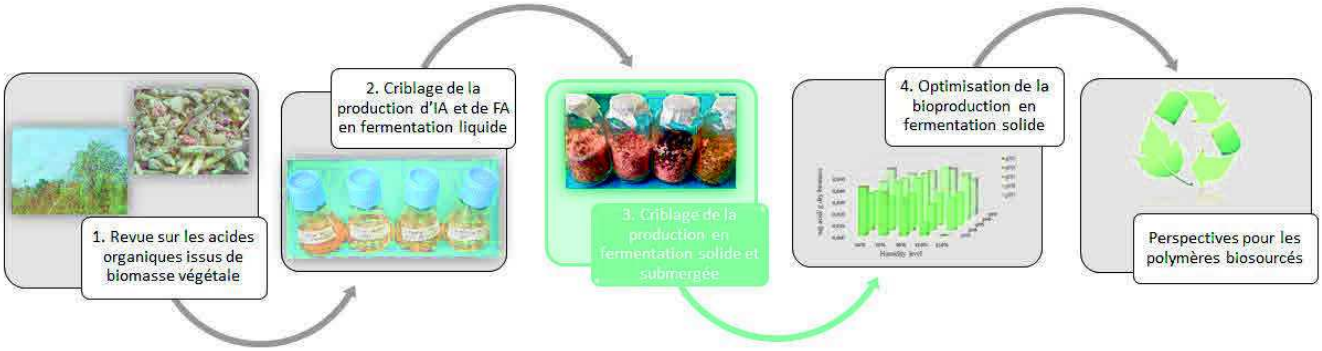


Figure.S2.1 Kinetic fermentation of 4 Aspergillus strains in optimized medium for IA production (Kuenz, 2012). Left to right flasks were inoculated with A. terreus 826, A. terreus 62071, A. tubingensis and A. oryzae.

Chapitre 3. Criblage production d'acides en fermentation solide et submergée



Introduction

Le chapitre 2 a montré la capacité de certaines souches d'*Aspergillus* à produire de l'acide itaconique et de l'acide fumarique en utilisant des hydrolysats liquides de son de blé et de rafles de maïs. Un certain nombre de conclusions peuvent être tirées de ces expériences. La production d'acide fumarique est relativement universelle pour les conditions testées, alors que la production d'acide itaconique est beaucoup plus aléatoire et versatile, sans que l'on ne puisse toujours en expliquer les raisons. C'est en tout cas cohérent avec la nature de métabolite secondaire de l'acide itaconique. Malgré une croissance plus faible, les rafles de maïs sont supérieures au son de blé pour la production d'acide itaconique. Le prétraitement enzymatique est préférable au traitement acide. Enfin, *Aspergillus oryzae* se montre la souche la plus productrice d'acide itaconique alors que d'autres auteurs n'avaient pas identifiée cette souche comme telle - il est vrai en utilisant d'autres conditions fermentaires -.

Dans le chapitre 3, le criblage s'effectue en utilisant les biomasses brutes et prétraitées. Il s'agit donc d'étudier la production des deux acides mais cette fois par des fermentations en milieu solide (c'est-à-dire avec la biomasse en absence d'eau libre: FMS) mais également en fermentation submergés, c'est-à-dire avec la biomasse mais en présence d'eau libre (SmF).

Sur l'ensemble du travail de thèse ce choix d'étudier les hydrolysats (liquide) mais également la biomasse (solide) est très cohérent avec le concept de la bioraffinerie. En effet, l'un des attendus de la bioraffinerie, est l'utilisation la plus complète possible de la richesse de la biomasse. Le second aspect de la bioraffinerie qui est la génération de différents produits avec des valeurs ajoutées différentes est également abordée dans cette étude au travers de la co-génération d'acides organique et d'enzymes.

Ce chapitre est présenté sous forme d'une publication « Fungal fermentation of lignocellulosic biomass for itaconic and fumaric acid production » par Amparo Jiménez-Quero, Eric Pollet, Minjie Zhao, Eric Marchioni, Luc Averous and Vincent Phalip, soumise pour publication dans *Process Biochemistry*.

Fungal fermentation of lignocellulosic biomass for itaconic and fumaric acid production

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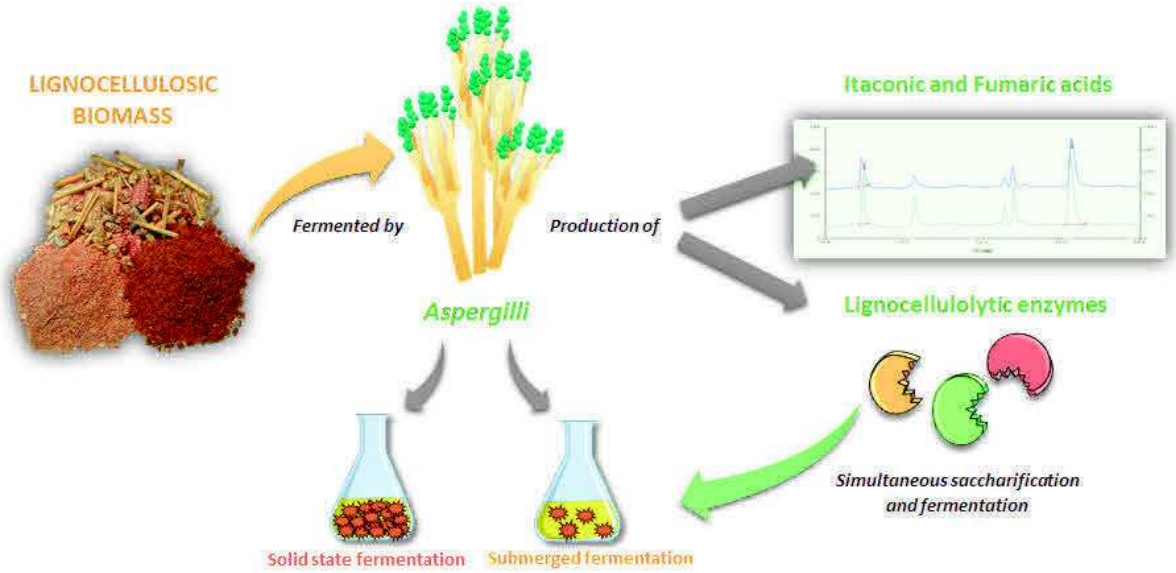

Abstract

The production of high-value chemicals from natural resources as alternative of petroleum-based products is currently expanding in parallel with biorefinery. The use of lignocellulosic biomass as raw material is promising to achieve economic and environmental sustainability. Filamentous fungi, particularly *Aspergillus* species, are already used industrially to produce organic acid as well as many enzymes. The production of lignocellulose degrading enzymes opens the possibility for direct fungal fermentation towards organic acids as itaconic and fumaric acids. These acids have wide-range applications and potentially addressable markets as platform chemicals. However, current technologies for the production of these compounds are mostly based on submerged fermentation. This work showed the capacity of two *Aspergillus* species (*A. terreus* and *A. oryzae*) to yield both acids by solid-state fermentation and simultaneous saccharification and fermentation. FA was optimally produced at by *A. oryzae* in simultaneous saccharification and fermentation (0.54 mg/g wheat bran). The yield of 0.11 mg IA/ g biomass by *A. oryzae* is the highest reported in the literature for simultaneous solid-state fermentation without sugar supplements.

Keywords: lignocellulosic biomass; solid state fermentation; submerged fermentation; *Aspergillus oryzae*

1. Introduction

Itaconic (IA) and fumaric (FA) acids are products of the metabolic pathways of microorganisms and are intermediates of the oxidative portion of the TCA cycle (Goldberg et al., 2006). Owing to their carboxylic functions, these diacids are considered key platform chemicals (or building blocks). For instance, both acids were among the top twelve biomass-derived platform chemicals selected by the U.S. Department of Energy (DOE) with several high-value applications and emerging markets for fine chemicals, pharmaceuticals and materials (Werpy et al., 2004).

IA and FA can be commonly produced by filamentous fungi in high concentrations (Magnuson and Lasure, 2004). These microorganisms stand out for their diverse portfolio of products such as enzymes, organic acids, alcohols, singe-cell oils, proteins (amino acids), biopolymers (chitin/chitosan and glucans), antibiotics, and other bioactive compounds. The genus *Aspergillus* is particularly interesting, with more than 250 species including commercially exploited species (e.g., *A. niger, A. oryzae, A. sojae* and *A. terreus*). *Aspergillus* can be used for solid-state or submerged fermentation. Some fermentation protocols have been established for large-scale industrial processes. Since 1960, the industrial production of IA has been achieved by fermentation with *Aspergillus terreus* in liquid glucose-containing media (> 100 g/L) (Okabe et al., 2009) and (Willke and Vorlop, 2001). The strain NRRL1960 has demonstrated the highest yield: 129 g/L (Hevekerl et al., 2014). In contrast, FA bioproduction is still in its early stages, and the corresponding reported yields (Mondala, 2015) have yet to be competitive compared to equivalent chemical synthesis from petrochemical feedstock.

Currently, research is focused on viable, renewable and environmentally friendly alternatives to replace conventional resources (i.e., fossil fuel derivatives) for the production of chemicals. In this context, the biorefinery concept can be defined as the full integration of incoming biomass for

simultaneous production of different compounds such as food, feed, materials (fibers, papers, etc.), energy and chemicals with added values. Corresponding biomass streams should be economically and ecologically sustainable (Kromus et al., 2005). To also achieve social sustainability, lignocellulose, an inedible, low-value byproduct of the agriculture or forestry industries could be used as a valuable resource (Kawaguchi et al., 2016) (Lucia et al., 2006). This biomass is the most abundant carbon feedstock on Earth (Kamm et al., 2005). However, lignocellulosic biomass is a recalcitrant material that often needs pretreatment to achieve effective disentanglement of its complex multiphase structure, separating the main components (cellulose, hemicellulose and lignin) (Menon and Rao, 2012).

Filamentous fungi can be grown on renewable resources such as lignocellulosic biomass due to their capacity to hydrolyze biopolymers to yield easily assimilable energy sources (de Vries and Visser, 2001). In the last few decades, some investigations have focused on the production of IA from renewable biomass, first from starchy materials such as corn starch, molasses or grains, achieving the production of 0.36 g of IA/g of sago starch (Dwiarti et al., 2007) or 48.70 g/L from Jatropha seed cake hydrolysates (El-Imam et al., 2013). *Rhizopus* species were shown to be the best producers of FA. For instance, FA was produced from corn straw with a yield of 0.35 g of FA/g of consumed sugar (Xu et al., 2010).

Filamentous fungi are particularly adapted to solid state fermentation (SSF) because the substrate also provides a support for the growth of microorganisms via intimate contact between the organism and the biomass (Prévot et al., 2013). This phenomenon is abundantly used for the production and excretion of enzyme cocktails (Pandey, 2001). Furthermore, several operational and technical challenges exist with submerged fermentation processes involving filamentous fungi,

hindering economical and commercial-scale adoption. These drawbacks could possibly be alleviated by the use of SSF technology (Mondala, 2015) (Hölker et al., 2004) (Ferreira et al., 2016).

The aim of this study was to investigate and optimize the use of wheat bran and corn cobs as cheap and common lignocellulose sources for the microbial production of two dicarboxylic acids, IA and FA, respectively. Four strains of *Aspergillus* were employed, and the fermentation strategy comprised solid state (SSF) and submerged fermentation (SmF). Additionally, a simultaneous saccharification and fermentation process was investigated to verify the efficiency of enzyme cocktail production by SmF and SSF.

2. Materials and methods

2.1. Microorganisms

A. oryzae (UMIP 1042.72) was provided by the Fungal Culture Collection of the Pasteur Institute (France). *A. tubingensis* (IMI 500512) was isolated in our laboratory from agricultural residues and identified by CABI Bioscience (United Kingdom). *A. terreus* strains (DSM 826 and DSM 62071) were provided by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany). The strains were revived in potato dextrose broth medium (PDB) for 5-6 days at 25 °C. The microorganisms were then grown and sporulated on potato dextrose agar (PDA). The spore suspensions were harvested from 5-6-day-old PDA plates with 0.2% (v/v) Tween-80. The spores were counted using a Malassez counting chamber and stored at -20 °C.

2.2. Substrates

The agricultural waste biomasses used as carbon sources were wheat bran and corn cobs obtained from Comptoir Agricole (France). The lignocellulosic material was crushed (SX 100, Retsch) to obtain particles 0.5-1 mm in size. The results of the physico-chemical characterizations of these solid substrates are given in Table 3.1. The chemical elementary analyses were performed by SOCOR (France). The water activity (Aw) was measured with 1 g of dry substrate with an Aw meter Fast-lab (GBX, France).

2.3. Solid state fermentation (SSF)

In a glass flask, 5 g of solid substrate was autoclaved at 121 °C and 3 bars for 20 min. The substrates were inoculated with spore suspensions at pH 5 to achieve an initial content of 10^6 spores/mL. Initial moisture was adjusted to 120% for wheat bran and 90% for corn cobs (both corresponding to an Aw close to 1). After thorough mixing, the flasks were covered with gas exchange film and incubated at 30 °C for 6 days. Unless otherwise specified, these fermentation conditions were maintained throughout the study. All the experiments were conducted in duplicate. After fermentation, the samples were recovered by mixing the fermented substrate with sterilized distilled water (7 mL/g of initial dry substrate). The preparations were centrifuged (8000 g for 15 min) to eliminate residual solids. Then, another round of centrifugation was performed on the supernatants to remove mycelia and spores (13600 g for 30 min). The resulting liquid was finally filtered through a 0.2-µm membrane. The samples were then analyzed by high-performance liquid chromatography (HPLC) or stored at -20 °C for additional analysis.

The fermentations for enzyme cocktail preparation were performed under equivalent conditions, with the exception that the inocula were prepared at pH 10 with Tris buffer. The incubation temperature was 25 °C. Samples were analyzed to determine corresponding enzyme

activities. For large-scale enzyme cocktail production by *A. tubingensis* and *A. oryzae*, fermentation was performed in plates with 200 g of wheat bran, and were recovered as 1 L of enzyme cocktail was recovered.

2.4. Submerged fermentation

Five grams of autoclaved corn cobs and wheat bran were inoculated with spore suspensions at 10^5 spores/mL in 125-mL Erlenmeyer flasks with 60 mL of H_2O at pH 5. The flasks were incubated on a rotatory shaker (Infors Multitron, Switzerland) for 7 days at 33 °C with shaking at 120 rpm. Samples of 500 µL were taken at regular intervals, filtered through a 0.2-µm membrane and stored at -20 °C for further analyses. At 96 hours, samples were collected to measure enzyme activities. All experiments were conducted in duplicate.

2.5.Simultaneous saccharification and fermentation

Two conditions, (i) a simultaneous submerged fermentation (SmF) and (ii) a simultaneous SSF, were employed, both with the enzyme cocktails from *A. tubingensis* and *A. oryzae*. The conditions were equivalent (pH 5 and 30°C) with the exception that the enzyme cocktails were used for spore inoculation and biomass humidification.

2.6.Analytical procedures

2.6.1.Enzyme assays

Enzyme cocktail activities were measured using chromogenic substrates, specifically azurine-crosslinked polysaccharides (AZCL-polysaccharides) such as AZCL-HE-cellulose, AZCL-xylan, AZCL-xyloglucan or AZCL-amylose (Megazyme, Ireland). The corresponding activities were determined by monitoring the solubilization of dyed compounds and measuring absorbance of the supernatant at 595 nm. The substrates were prepared at 0.4% (w/v) in 0.1 M sodium acetate buffer at pH 5. The reactions were performed with 950 µL of AZCL substrates and

50 µL of diluted enzyme cocktail samples for 30 min and incubated with overhead rotation at 25 °C. The supernatants were isolated by centrifugation at 13,600 g for 1 min. All spectrophotometric measurements were performed using a Genesys 10 Bio spectrophotometer (Thermo, USA). Given that the molar extinction coefficient of AZCL (azurine cross-linked) was unknown, the enzyme activities were expressed in arbitrary units corresponding to optical density variations per minute and per milliliter of the supernatant.

2.6.2.Sugar assays

To determine the total available sugar contents in wheat bran and corn cobs, 0.5 g of solid substrates were subjected to acid hydrolysis using incubation in 5 mL of 0.4 M HCl at 100 °C for 2 hours (Sørensen et al., 2007). Glucose was measured by a colorimetric method at 420 nm (Okuda et al., 1977). Reducing sugars were determined by the DNS (dinitrosalicylic acid) method with spectrophotometric measurements at 550 nm (Miller, 1959).

2.6.3.Organic acid assays

Chromatographic separation was achieved on a hypercarb porous graphitic carbon LC column (150 x 3.0 mm i.d., 3 µm, Thermo Scientific, USA) at 70 °C. HPLC analysis was performed using a 616 pump, a 2996-photodiode array detector operating in a range from 200 to 450 nm, and a 717 Plus autosampler controlled with Empower 2 software (all from Waters, USA). The mobile phase consisted of water:formic acid (99.9:0.1 v/v, phase A) and acetonitrile:formic acid (99.9:0.1 v/v, phase B) at a flow rate of 0.55 mL/min. Elution was performed with a gradient as follows: (i) 0% B (0-5 min), (ii) 0-27% B (5-18 min), and (iii) 27-27% B (18-26 min). Finally, phase B was decreased to its initial concentration (0%) in 1 min, and the column was re-equilibrated for 14 min. The injection volume was 20 µL. The calibration was performed using commercial IA and FA with

99.9% purity (Sigma-Aldrich, USA). Each sample was supplemented with 10 ppm IA or FA as an internal standard, and the organic acids were detected at 205 nm.

Table 3.1. Substrates characterization. Wheat bran and corn cobs were crushed to get particles of 0.5-1 mm in size. Physico-chemical analyses were subsequently performed.

	Wheat bran	Corn cobs
Dry substrate Aw	0.629	0.391
Hydrated substrate Aw[a]	0.982	0.984
Carbohydrate composition (% dry wt)	(Miron et al., 2001)	(Cao et al., 1997)
Cellulose	10.5 – 14.8	32.3 – 45.6
Hemicellulose	35.5 – 39.2	39.8
Lignin	8.3 – 12.5	6.7 – 13.9
Sugars	(mg/g of dry wt)	
Reducing sugars	387.1	389.2
Glucose	261.2	57.5
Nitrogen total (% dry wt)	2.95	0.44
Metals	(mg/g of dry wt)	
Calcium total	1078.9	280.4
Iron total	120.61	119.05
Magnesium total	4988.4	275.6
Manganese total	167	8
Potassium total	14414.6	7094.4
Sodium total	53.13	11
Zinc total	89	20
Anions	(mg/L)	
Orthophosphates	15300	669

a. 120% (w/v) water for wheat bran and 90% (w/v) water for corn cobs

3. Results and discussion

3.1.Solid State Fermentation (SSF)

Raw biomasses were fermented by four *Aspergillus* strains. Total available glucose, determined from the liberated glucose after acid hydrolysis, was found to be 261.5 mg of glucose per g of wheat bran and 57.5 mg per g of corn cobs (Table 3.1). These results were corroborated by the mycelial development of the four strains, in which mycelia were clearly larger on wheat bran compared to corn cobs. FA was produced by the four strains from both biomasses (Figure 3.1). When yields were calculated with respect to the total glucose, corn cobs gave better results for the production of FA for all strains (i.e., *A. oryzae* produced 0.31 and 0.03% FA from total glucose with corn cobs and wheat bran, respectively). FA is a primary metabolite, it is not surprising that this acid was concomitantly produced as the four strains grew. Conversely, IA is a secondary metabolite and was produced only by *A. oryzae* and only with corn cobs, with a yield of 0.05 mg of IA/g of biomass. IA production was previously demonstrated to be optimal under phosphate limitation (Willke and Vorlop, 2001). Phosphate content is 23 times lower in corn cobs compared to wheat bran (Table 3.1). This gap explains the highest results obtained with corn cobs. This is the first time that this strain has demonstrated the ability to produce IA in SSF as previously demonstrated for liquid state fermentation (Jiménez-Quero et al., 2016). *A. oryzae* also produced the highest quantity of FA at 0.18 mg/g of corn cobs. The strain *A. terreus* 826, even though it is industrially employed as an IA producer, did not yield IA under these conditions but produced 0.09 mg of FA/g of corn cobs. Thus, the combination of *A. oryzae* and corn cobs produced the most promising results, converting 0.31% FA and 0.09% IA from the total available glucose.

IA production is dependent on cis-aconitate decarboxylase (CAD), an enzyme found in *A. terreus* (Okabe et al., 2009). The production of IA by *A. oryzae* suggests the presence of CAD in

this species. Interrogation of the *A. oryzae* genomic database indicated a gene-encoding protein (AO090010000161) exhibiting 54% identity and 69% similarity to CAD of *A. terreus* (ATET_09971), supporting this hypothesis.

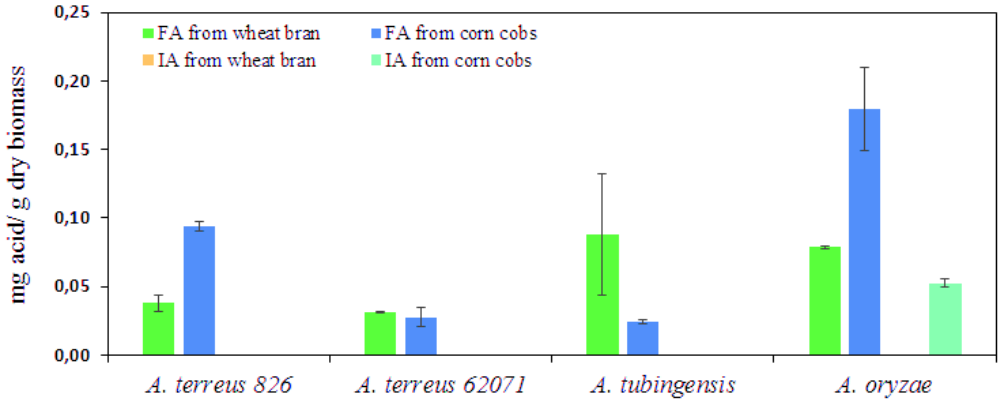

Figure 3.1. Production of itaconic and fumaric acids using SSF by the four Aspergillus strains on the biomasses.

3.2.Submerged fermentation

Submerged fermentation was performed with the 4 strains and produced varied results for acid production (Figure 3.2). *A. tubingensis* gave the best results for FA production with the two biomasses, yielding 0.19 and 0.11 mg/g of biomass for wheat bran and corn cobs, respectively. Both *A. terreus* strains produced FA only with corn cobs. *A. oryzae* produced FA with wheat bran (0.065 mg of FA/g) and demonstrated a lower yield with corn cobs (0.011 mg of FA/g).

IA was produced by *A. oryzae* from corn cobs with a yield of 0.076 mg of IA/g, which was 1.5 times higher than production in SSF (3.1.1). Furthermore, SmF facilitated IA production by the two strains of *A. terreus* in contrast with SSF. As with *A. oryzae,* these *A.* terreus strains produced IA only from corn cobs. This is likely due to phosphate availability (Willke and Vorlop, 2001). Furthermore, the higher cellulose content in corn cobs (roughly 3 times more than in wheat bran,

Table 3.1) eases the liberation of glucose from this biomass as already reported by (Dashtban et al., 2009).

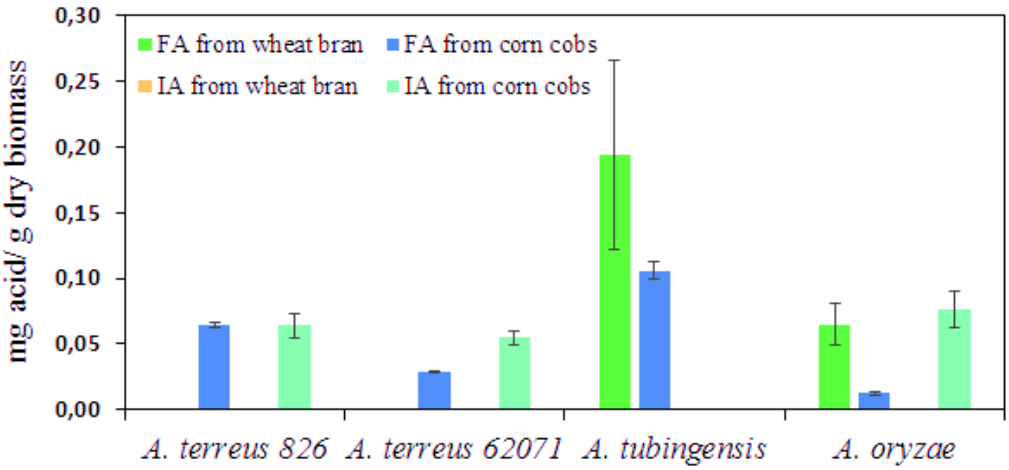

Figure 3.2. Production of itaconic and fumaric acids using SmF by the four Aspergillus strains on the biomasses.

3.3. Enzymatic treatment of biomasses

The biological treatment of lignocellulosic biomasses represents an interesting approach for the better solubilization of sugar from native biopolymers (Van Dyk and Pletschke, 2012) (Begum and Alimon, 2011). Such enzymatic treatments employ cocktails of enzymes containing different types of cellulases and hemicellulases that are able to convert biopolymers into assimilable sugars.

To generate an appropriate enzyme cocktail adapted to the complexity of the biomasses of interest, and also to test the feasibility of coupled biomass valorization (IA/FA and enzyme production), fermentation was performed with the four strains and two biomasses under investigation in solid and in submerged conditions. Enzyme activities obtained by SmF (Table S3.1) were lower for the four microorganisms than those from SSF (Table 3.2). These results are consistent with previous studies (Prévot et al., 2013). As a result, SSF was chosen to produce the

enzyme cocktails. Differential growth with wheat bran and corn cobs was evident (Figure 3.3). After two days of cultivation, fungal mycelia and spores were formed with wheat bran. In contrast, fermentation of corn cobs clearly resulted in delayed development for the four strains. In relatively good agreement, all enzymatic activities (Table 3.2) obtained with corn cob fermentation were null or very low, in contrast to the enzyme activities obtained with wheat bran. *A. tubingensis* and *A. oryzae* exhibited higher xylanolytic activities, which were more than 30 times greater compared to the activities of *A. terreus*. Endoxylanase activity, which is crucial for sugar release from the hemicellulose fraction, was higher for *A. tubingensis*. In contrast with the *A. tubingensis* cocktail, the *A. oryzae* cocktail exhibited higher amylase activity. However, starch constitutes only a small portion of these agricultural resources.

Figure 3.3. Pictures of SSF for enzymatic cocktail production with the two biomasses (two left glass flasks: wheat bran; two right flasks: corn cobs). a: A. terreus 826. b: A. terreus 62071. c: A. tubingensis. d: A. oryzae.

Table 3.2. Enzymatic activities of Aspergillus strain cocktails in SSF on wheat bran and corn cobs. All experiments were performed at pH 5 and at 25°C.

	A. terreus 826		*A. terreus* 62071		*A. tubingensis*		*A. oryzae*	
	Wb	Cc	Wb	Cc	Wb	Cc	Wb	Cc
α-Amylase activity	1.63 ± 0.24	0.18 ± 0.00	1.59 ± 0.36	0.22 ± 0.04	24.90 ± 2.31	0.17 ± 0.04	76.25 ± 3.56	0.22 ± 0.05
Cellulase activity (cellulose)	0.23 ± 0.06	0.03 ± 0.00	0.75 ± 0.08	0.00 ± 0.00	2.70 ± 0.63	0.01 ± 0.00	2.47 ± 0.56	0.00 ± 0.00
Endoxylanase activity	1.75 ± 0.21	0.00 ± 0.00	2.10 ± 0.12	0.00 ± 0.00	72.81 ± 4.23	0.33 ± 0.01	63.70 ± 2.39	0.07 ± 0.00
Cellulase activity (xyloglucan)	0.19 ± 0.03	0.01 ± 0.00	0.27 ± 0.07	0.02 ± 0.00	3.10 ± 0.25	0.05 ± 0.00	1.96 ± 0.41	0.02 ± 0.00

*Activity in OD/g*min.*

3.4.Simultaneous saccharification and fermentation

Previous studies have shown that simultaneous saccharification and fermentation appears to be an interesting process for the optimization of biomass valorization (Hendriks and Zeeman, 2008). In this process, the enzyme cocktail is directly added to wet the biomass, becoming the liquid phase during fermentation. Simultaneous saccharification and fermentation were first performed in a submerged manner (SmF) and then in a solid state. In both cases, the enzyme cocktails of the *Aspergillus* strains (*A. tubingensis* or *A. oryzae*) prepared as described in section 3.3 were used as the liquid phase.

3.4.1.Fermentations using A. tubingensis enzyme cocktail

The production of FA was generally similar for all conditions in simultaneous SSF (Figure 3.4a). FA yields were quite similar to those from SSF with raw biomasses (3.1). The yields calculated from total glucose indicated that *A. tubingensis* gives the highest overall yield for FA production, with 0.09% recovery from simultaneous SSF of corn cobs. When production was determined in relation to the biomass weight, higher FA levels were obtained using SmF of wheat bran (Figure 3.4b).

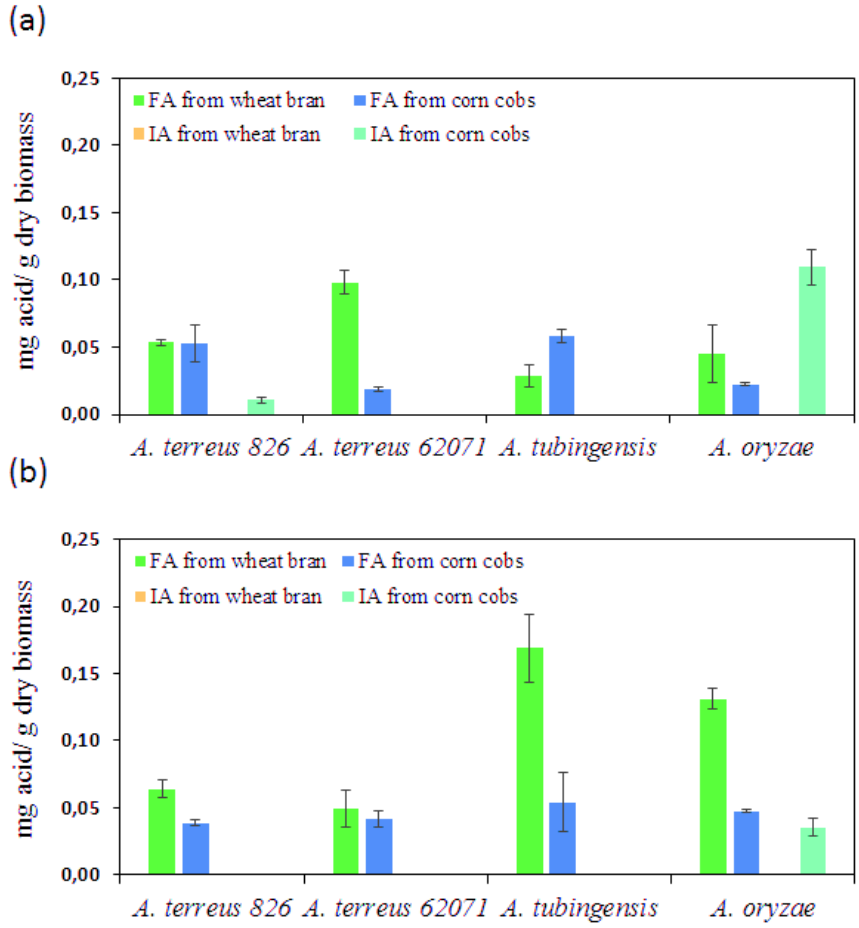

Figure 3.4. Production of itaconic and fumaric acids using simultaneous saccharification and fermentation (SmF and SSF) with the enzymatic cocktail of A. tubingensis by the four Aspergillus strains. a: SSF and b: SmF.

As already observed for SSF and SmF in this study, IA was produced by *A. oryzae*. However, the highest yield was observed for simultaneous SSF (Figure 3.4a) with 0.11 mg of IA/g corn cobs, which is more than twice that obtained with SSF (0.05 mg of IA/g, Figure 3.1). In simultaneous SSF, *A. oryzae* demonstrated a yield of 0.19% IA from total available glucose, more than twice the production observed in SSF with corn cobs (0.09% IA from total glucose). These results show the large effects of the enzyme cocktail for the liberation of sugars (Table S3.2) to

facilitate microbial fermentation and therefore enhance organic acid production as reported previously for *Aspergilli* (Viktor et al., 2013). For the same reason, the enzymatic treatment of biomasses in simultaneous SSF allowed IA production for *A. terreus* 826, whereas IA was not produced in SSF (Figure 3.1). However, glucose availability is not sufficient to explain all of the obtained results. Indeed, both strains of *A. terreus* produced IA from corn cobs in SmF (Figure 3.2) but not in simultaneous SmF (Figure 3.4b). It could be that inhibitory compounds are released by the action of enzymes and solubilized in the aqueous environment to disturb the metabolism (Jiménez-Quero et al., 2016). This is another illustration of the versatile nature of secondary metabolite production.

Additionally, simultaneous fermentation permits a significant reduction in process duration in comparison with current processes for liquid state fermentation of biomass hydrolysates, which involve two successive unitary operations of biomass hydrolysis and subsequent fermentation; for instance, 4 days instead of 6 days for IA production from corn starch (Okabe et al., 2009).

3.4.2.Fermentations using A. oryzae enzyme cocktail

To further investigate IA production, and considering the results obtained with the *A. tubingensis* cocktail, we decided to use the enzymatic cocktail produced by *A. oryzae* for the two strains (*A. terreus 826* and *A. oryzae)* that demonstrated the ability to produce IA.

Experiments with the *A. oryzae* enzymatic cocktail resulted in the highest FA yield observed in this study by SSF on wheat bran (Figure 3.5), with 0.54 mg/g of dry biomass. This was 12 times the yield obtained under the same conditions with the cocktail of *A. tubingensis* (0.045 mg of FA/g). This result can be explained by the use of a more specific and adapted cocktail for this biomass as it was produced by the same strain. Moreover, the use of this cocktail facilitated IA

production with wheat bran in SSF and in SmF for *A. oryzae*, which was not observed before (0.010 and 0.012 mg/g of biomass, respectively). Conversely, the yields from the corn cob biomass were lower than the yields obtained with the *A. tubingensis* cocktail: 5 and 2 times less than SSF and SmF, respectively. IA was also produced by *A. terreus* 826, both in simultaneous SSF and simultaneous SmF.

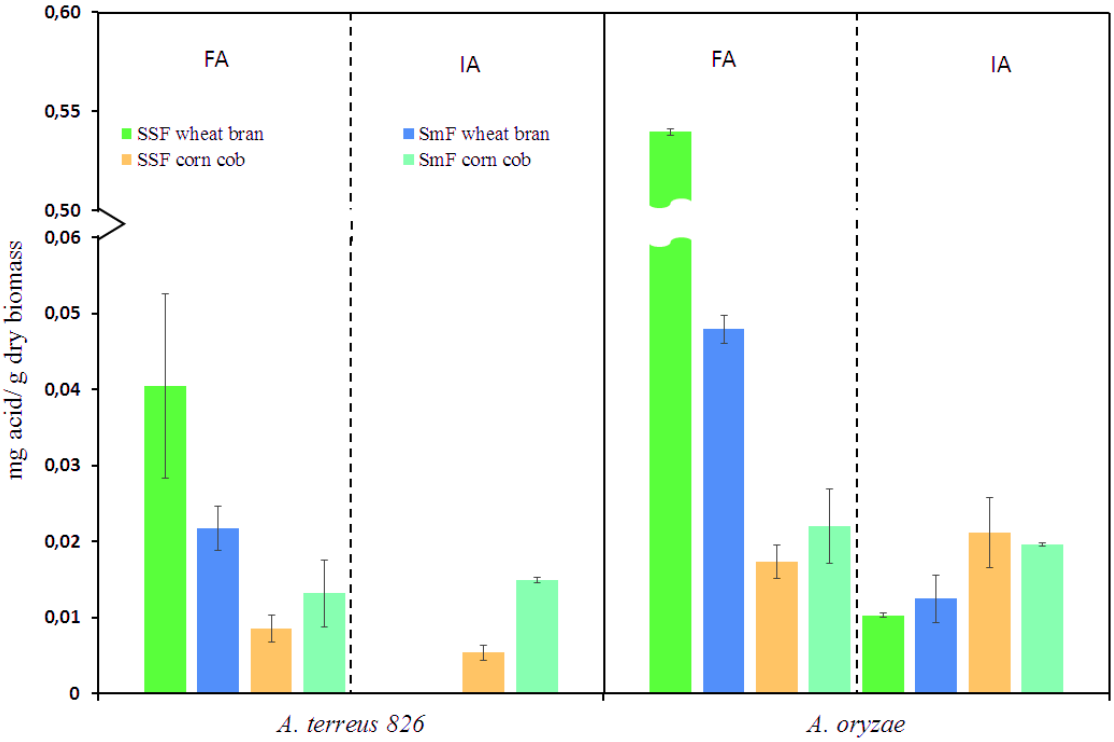

Figure 3.5. Production of itaconic and fumaric acids using simultaneous saccharification and fermentation (SmF and SSF) by A. terreus 826 and A. oryzae with the enzymatic cocktail of A. oryzae.

Corn grains have previously been used as a resource for FA production(West, 2008). In comparison, our experiments with SSF produced lower yields. However, cobs are sustainable by-product resources and do not compete with food. Concerning IA production by SSF, the literature is poor, and the only data available indicate the production of 0.036 mg/g of sugarcane pressmud (0.004% total glucose (Tsai et al., 2001)). In that paper, however, IA production was supported by

a supply of glucose (100 g/l), nitrate and other salts. Peeled sugarcane pressmud was added to support growth. Our system (*A. oryzae*/corn cobs) proved to be more efficient for IA production in simultaneous SSF with the *A. tubingensis* cocktail: 0.11 mg/g of corn cobs and 0.03% of total glucose. Conditions for the optimal growth of *A. oryzae* in solid-state and submerged fermentations have been described in different studies (te Biesebeke et al., 2002) (de Castro and Sato, 2014). However, enzyme and organic acid production by this fungus could be further improved.

4. Conclusion

This paper demonstrates that (1) the most promising strategy for the production of itaconic and fumaric acids with lignocellulosic biomass is simultaneous SSF; (2) the entire process can be achieved with a single fungal species, *A. oryzae*, which is described here as an IA producer in SSF; (3) *A. oryzae* can be cultivated to produce enzymes, organic acids, and cell biomass within the biorefinery concept; and (4) the results obtained from simultaneous SSF were promising and should be further optimized to improve enzyme production for corn cob-specific degradation.

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Supplementary data

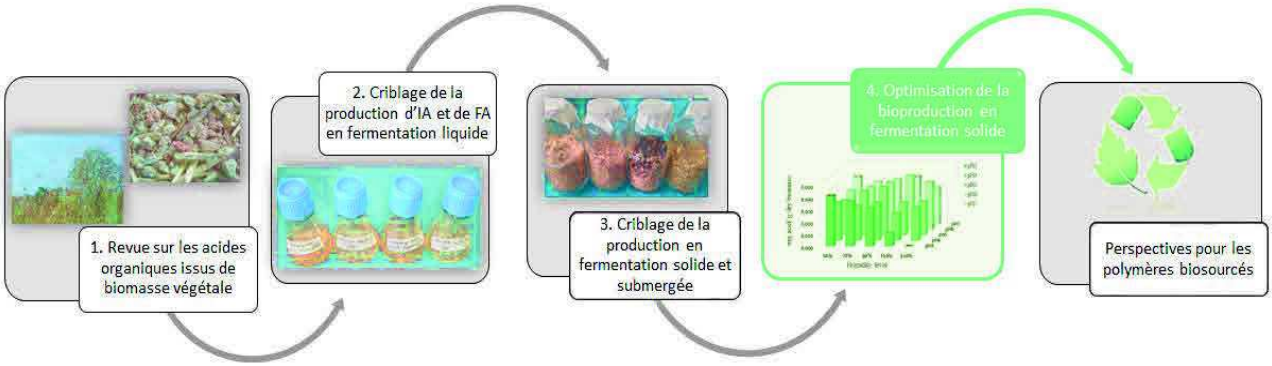
Table S3.1. Enzyme activities from SmF of Aspergillus strains with wheat bran and corn cobs biomasses.

	A. terreus 826		*A. terreus* 62071		*A. tubingensis*		*A. oryzae*	
	Wb	Cc	Wb	Cc	Wb	Cc	Wb	Cc
α-Amylase activity	0.00 ± 0.00	0.04 ± 0.00	0.00 ± 0.00	0.08 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.00
Cellulase activity (cellulose)	0.32 ± 0.02	0.02 ± 0.00	0.86 ± 0.05	0.11 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0.13 ± 0.02	0.02 ± 0.00
Endoxylanase activity	0.76 ± 0.11	0.08 ±0.01	1.08 ± 0.04	0.00 ± 0.00	2.16 ± 0.07	0.40 ± 0.07	2.12 ± 0.23	0.32 ± 0.04
Cellulose activity (xyloglucan)	0.06 ± 0.00	0.03 ±0.00	0.02 ± 0.00	0.08 ± 0.01	0.21 ± 0.03	0.13 ± 0.01	0.03± 0.00	0.02 ± 0.00

Table S3.2 Sugar liberation from biomasses after 216 h of enzyme treatment.

	A. tubingensis enzyme cocktail		*A. oryzae* enzyme cocktail	
(g/L)	Wb	Cc	Wb	Cc
Glucose	8.06	2.61	12.94	7.71
Reducing sugars	34.54	25.24	36.52	27.15

Chapitre 4. Optimisation de la production d'acide itaconique et fumarique en fermentation solide



Chapitre 4

Introduction

Le chapitre précédent montre la capacité d'*A. terreus* et d'*A. oryzae* à produire l'acide fumarique et l'acide itaconique que ce soit en fermentation solide ou submergée de rafles de maïs. Le rendement obtenu pour la production d'acide itaconique est le plus élevé répertorié dans la bibliographie. Il est notable que certaines conclusions sont communes en FMS/submergée (chapitre 3) et en FML (chapitre 2): meilleures performances *d'Aspergillus oryzae*, sur rafles de maïs et à l'issu d'un traitement biologique. Toutefois la conclusion majeure est que les procédés qui utilisent la biomasse solide sont les plus productifs à l'issue des criblages décrits dans les chapitres 2 et 3.

Le chapitre 4 utilise cette dernière conclusion comme base de travail et a pour objet d'optimiser la production des deux acides en fermentation en milieu solide. D'abord, la cinétique de production des deux acides est étudiée. L'influence du pH initial de fermentation et de l'humidification sont analysés. Finalement, et en fonction des résultats obtenus par l'optimisation, une étude comparative est développée à plus grande échelle (100 g de biomasse sèche, représentant une augmentation de la masse de biomasse d'un facteur 20) pour la FMS d'*A. oryzae* sur rafles de maïs à pH 6 et 110% d'humidité. Deux types de réacteurs sont comparés: un réacteur en couche mince classique et un réacteur prototype aéré.

Ce chapitre est présenté sous forme d'une publication « Optimization solid state fermentation of lignocellulosic biomass into itaconic and fumaric acid » par Amparo Jiménez-Quero, Eric Pollet, Luc Averous et Vincent Phalip qui est sera prochainement soumise pour publication.

Optimization of the solid state fermentation of lignocellulosic biomass into itaconic and fumaric acid

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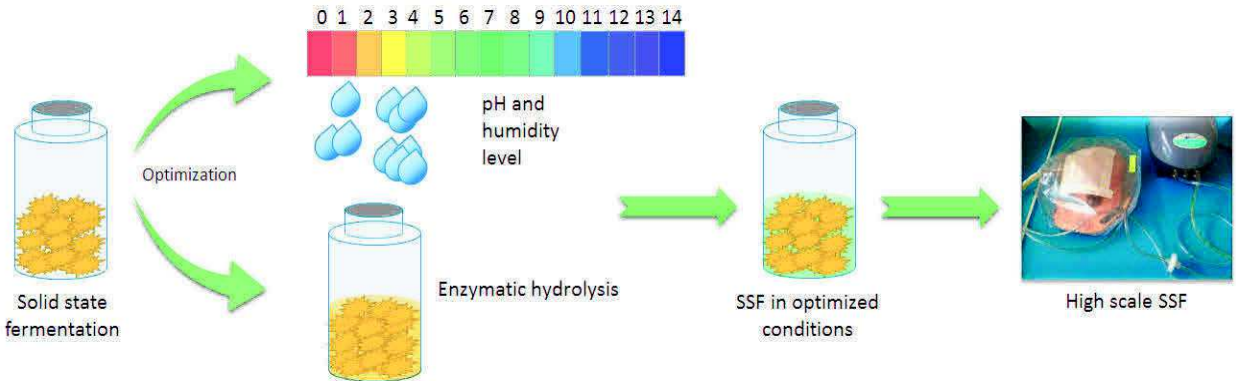

Abstract

The production of high-value chemicals such as itaconic and fumaric acid (IA and FA) from renewable resources via solid state fermentation (SSF) represents an alternative to the current bioprocesses of submerged fermentation using refined sugars. The use of lignocellulosic biomass instead of starch or grains promotes sustainable development in the IA and FA bioproduction field. Both acids are excellent platform chemicals with a wide range of applications with huge markets. Filamentous fungi, especially belonging to the *Aspergillus* genus, have shown a great capacity to produce these organic dicarboxylic acids. This study attempts to optimize the SSF conditions with lignocellulosic biomasses by using *A. terreus* and *A. oryzae* to produce itaconic and fumaric acids. First, a kinetic study of SSF was performed with wheat bran and corn cobs with two fungal species. Then, a panel of pH and moisture conditions was studied in corn cob fermentations. Next, a simultaneous SSF with an *A. oryzae* enzyme cocktail was investigated. Finally, a large scale fermentation process was developed for SSF using corn cobs with *A. oryzae*. The yields achieved were 0.05 mg of itaconic acid and 0.16 mg of fumaric acid per gram of biomass after 48 hours with *A. oryzae*. These values currently represent the highest reported production for solid-state fermentation from raw lignocellulosic biomass.

Keywords: lignocellulosic biomass; solid state fermentation; enzymatic hydrolysis; aerated fermenter; *Aspergillus oryzae*

1. Introduction

Solid state fermentation (SSF) has emerged in the last decades as an industrial process for several products, especially using agricultural byproducts as the substrate (Pandey, 2001) (Hölker and Lenz, 2005). SSF involves the growth of a microorganism on solid particles in the near absence of free water, and the majority of processes are performed by filamentous fungi under aerobic conditions (Hölker et al., 2004). The substrates used in SSF are often the source of nutrients for the microorganisms, and the inter-particle spaces allow the gas and nutrient exchanges between fungal hyphae and the medium. Fungi also behave as biocatalysts for the bioconversion of the substrates into specific target products such as bio-based fuels, commodity chemicals, enzymes, bioactive compounds or food products (Dashtban et al., 2009).

SSF offers several advantages over submerged fermentation (SmF) such as high volumetric productivity, product concentration, simpler and smaller bioreactors because of the minimal free water, a lower sterilization cost, less generation of effluents (reduced cost of effluent treatment) and easier aeration (facilitated by spaces between particles) (Krishna, 2005a) (Rodriguez-Leon et al., 2008). Finally, the conditions of SSF are similar to the natural environments of the filamentous fungi, which facilitates inoculation by fungal spores and disfavors the risk of contamination by bacteria. However, this type of bioprocess is slower, and all the fermentative factors cannot be controlled precisely. The main factors affecting fungal growth and metabolism in SSF are the selection of a suitable microorganism and substrate for the target product generation, the pre-treatment of the substrate, the moisture, the temperature and the removal of metabolic heat and transfers (Singh nee' Nigam and Pandey, 2009).

One of the most interesting biotechnological applications of SSF is the production of commodity chemicals, as evidenced by the large quantity of published studies (Christensen et al.,

2008) (Bozell, 2008). The biosynthesis of chemicals from biomass creates a sustainable alternative to the conventional chemical synthesis based on fossil resources (Willke and Vorlop, 2004) (Gallezot, 2012). In the last two decades, many molecules produced from biomass with a large range of applications have been described (Werpy et al., 2004) (Bozell and Petersen, 2010). Many of these building blocks are organic acids because of their capacity to generate high value products for widespread industries such as food, pharmaceuticals or polymers (Magnuson and Lasure, 2004) (Goldberg et al., 2006) (Tsao et al., 1999). The biosynthesis of organic acids by filamentous fungi has been studied extensively, and *Aspergilli* are often used for industrial production (Liaud et al., 2014) .

The two targeted products studied in this paper are fumaric and itaconic acid (FA and IA), dicarboxylic acids included on the DOE's (Unites State Department of Energy) list as part of the top twelve biomass-derived platform chemicals (Werpy et al., 2004). Both acids are intermediates in the tricarboxylic acid (TCA) cycle and are produced under aerobic conditions. Currently, the industrial production of FA is via catalytic isomerization of petroleum-derived maleic acid, but FA could also be produced biologically as an intermediate of the TCA cycle that is present in most aerobic organisms. Laboratory-scale fermentations with *Rhizopus oryzae* have shown interesting yields in SmF with sugars (Roa Engel et al., 2008) (Xu et al., 2012). IA is produced industrially by *Aspergillus terreus* in SmF with glucose as the principal source and a yield of 100 g/L (Yahiro et al., 1997) (Hevekerl et al., 2014) (Willke and Vorlop, 2001). The biosynthesis involves the action of the cis-aconitate decarboxylase (CAD) enzyme to transform the cis-aconitate into itaconate. The presence of CAD in *A. terreus* has been demonstrated in different studies, but this enzyme is also present in another *Aspergillus* species, *A. oryzae* (Kanamasa et al., 2008) (Okabe et al., 2009). This aerobic filamentous fungus is frequently used in SSF processes due to the capacity to hydrolyze

the lignocellulolytic substrates by enzyme degradation (Begum and Alimon, 2011). Nevertheless, the production of IA and FA by SSF with lignocellulosic biomass has not been studied extensively in the literature. A method of SSF using sugarcane pressmud for IA production, which yielded 0.0003 g kg^{-1} h^{-1}, was patented in 2001 (Tsai et al., 2001), and a maximum productivity of 0.021 g kg^{-1} h^{-1} of FA was reported via SSF of corn distiller grains by *R. oryzae* (West, 2008). In a previous study, we have shown the capacity of *A. terreus* and *A. oryzae* to produce both acids in the SSF process (Jiménez-Quero et al., 2016b). The yields obtained by *A. oryzae* from corn cobs were the most interesting, with 0.05 and 0.18 mg acid/g biomass of IA and FA, respectively. Both productivities are lower than those values reported from SmF processes utilizing soluble sugars in liquid media.

The goal of this work was to optimize the production in SSF of the two dicarboxylic acids (IA and FA) by two *Aspergillus* species (*A. terreus* and *A. oryzae*) using lignocellulosic biomass as the carbon source (wheat bran and corn cobs), without competition with food resources. Some factors were studied to enhance organic acid production yields (pH, moisture content, enzyme hydrolysis), and a larger scale fermentation was tested using the optimized factors.

2. Materials and methods

2.1. Feedstock and microorganisms

The two agricultural waste biomasses used as carbon sources were wheat bran and corn cobs obtained from Comptoir Agricole (France). The lignocellulosic material was crushed (SX 100, Retsch) to obtain particles that were 0.5-1 mm in size. The water activity (Aw) was measured on 1 g of dry substrate by an Aw meter Fast-lab (GBX, France).

A. terreus (DSM 826) was provided by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany). *A. oryzae* (UMIP 1042.72) was provided by the Fungal Culture Collection of the Pasteur Institute (France). The strains were revived on potato dextrose broth medium (PDB) for 5-6 days at 25 °C. The microorganisms were then grown and sporulated on potato dextrose agar (PDA). The spore suspensions were harvested from 5-6 day-old PDA plates with 0.2% (v/v) Tween-80. The spores were counted using a Malassez counting chamber and stored at -20 °C.

2.2. Solid state fermentation (SSF)

In glass flasks, 5 g of solid substrate were autoclaved at 121 °C and 3 bar, for 20 min. The substrates were inoculated with spore suspensions to have an initial concentration of 10^6 spores/mL. Initial moisture was adjusted to 120% for wheat bran and 90% for corn cobs (both corresponding to an Aw near 1). After thorough mixing, the flasks were covered with a porous adhesive film (VWR, USA) and incubated at 30 °C for 6 days. Unless specified otherwise, these fermentation conditions were maintained throughout the study. All the experiments were conducted in duplicate. After fermentation, the samples were recovered by mixing the fermented substrate with sterilized distilled water (7 mL/g of initial dry substrate). The preparations were centrifuged (8000 g for 15 min) to eliminate residual solids. Then, another centrifugation step was performed on the supernatants to remove mycelia and spores (13600 g for 30 min). The resulting solution was finally filtered through a 0.2 µm membrane. The samples were analyzed by high performance liquid chromatography (HPLC) and were stored at 20 °C for additional analysis.

2.2.1. pH and humidity level optimization

The initial pH and humidity levels were varied to screen the best conditions for organic acid production. Citrate-phosphate buffer solutions, pH 3 to 7, were prepared. The moisture content was

set at 50, 70, 90, 110 and 130 v / w. Five pH conditions were crossed with five humidity level conditions, generating 25 different conditions performed with biological duplicates in SSF as in section 2.2.

2.2.2. Enzyme production for biomass hydrolysis

The fermentation of wheat bran by *A. oryzae* for enzyme cocktail preparation was performed in a plate with 250 g biomass. The inocula were prepared with 300 mL of Tris buffer at pH 10 to achieve a final spore concentration of 10^6 per g of biomass, and the incubation temperature was 25 °C during the 4 days. The enzymatic cocktail was recovered with 1500 mL of sodium phosphate buffer at pH 6 and filtered with a Vivaflow 200 system (Sartorius, Germany). The cocktail was stored at 4 °C, and enzyme activities were determined.

2.3. Scale-up fermentation

Fermentation at a higher scale, with 100 g of biomass, was performed in two different types of reactor. A monolayer reactor consists of a glass plate covered with a gas exchange Miracloth film (Millipore, USA). The second reactor is a prototype of an aerated reactor (Figure 4.1). This fermenter was made from an autoclaved polypropylene laboratory bag with a central opening covered with a gas exchange film of Miracloth. Two PVC tubes were introduced and connected to stone ceramic air diffusers (3 mm in diameter). Another PVC tube was added for inoculation and water addition. The entire reactor was autoclaved with the biomass inside. During fermentation, water was added at the rate of 11 mL per day to keep the moisture constant (considering 10% evaporation/day). The aeration was provided by an air pump AC-9906 (Resun, China). A rocker mixer was used to shake the fermenter.

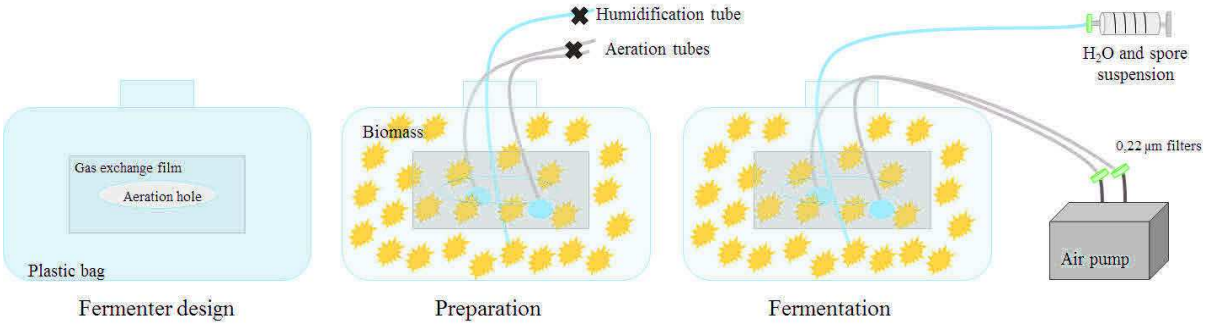

Figure 4.1. Model of aerated plastic bag fermenter (at left), made from autoclavable biohazard bags (polypropylene) with an aeration hole covered by a gas exchange Miracloth film (Millipore, USA). Prior autoclaving (middle), corn cobs were introduced as well as the air and humidification tubes (autoclavable tubes in PVC used for the liquid bioreactor). Right: operative fermenter with aeration and the inocula to be injected.

2.4. Analytical procedure

2.4.1. Mycelial growth (protein assays)

To determine the fungal proteins produced during the fermentation, the Bradford method was used (Bradford, 1976). All the samples were centrifuged and filtered (0.22 µm) before analysis to eliminate the spores. The protein assay was calibrated using BSA (bovine serum albumin) as the standard.

2.4.2. Organic acid assays

A chromatographic system based on a 616 pump, a 2996 photodiode array detector operating in a range of 200 to 450 nm, and a 717 Plus autosampler (Waters, France) controlled by Empower 2 software (Waters) was used to analyze the samples, as previously described (Jiménez-Quero et al., 2016a). The columns were calibrated using commercial IA and FA samples with a 99.9% purity (Sigma-Aldrich, USA) and a UV measurement at 205 nm. Each sample was supplemented with 10 ppm IA or FA as the internal standard to confirm the acid production.

2.4.3. Enzyme activity assay

Chromogenic substrates, especially azurine-crosslinked (AZCL) polysaccharides such as AZCL-HE-cellulose, AZCL-xylan, AZCL-xyloglucan or AZCL-amylose (Megazyme, Ireland), were used to measure the enzyme cocktail activity. The samples were collected and analyzed as previously described (Jiménez-Quero et al., 2016b).

3. Results and discussion

3.1. Solid state fermentation kinetics

Fermentations were performed for both fungal species using both biomasses. Organic acid production and fungal growth were examined. *A. terreus* and *A. oryzae* showed a different development on wheat bran and corn cobs (Figure 4.2). The growth of *A. oryzae* on wheat bran reached a plateau after 120 hours whereas *A. terreus* grew more slowly and regularly for more than 200 h on both substrates. These behaviors obtained from protein secretion were also confirmed by visual observations. For both fungi, the growth was vastly more important on wheat bran. However, both fungi produced more FA with corn cobs with 0.8 and 0.6 mg/g biomass for *A. terreus* and *A. oryzae*, respectively, at the end of the fermentation with a regular increase in the yields. On corn cobs, FA production displayed a completely different profile, with a maximum after 48 h (0.14 and 0.12 mg/g for *A. terreus* and *A. oryzae*, respectively) and then a regular decrease.

Although the growth was significantly higher on wheat bran, IA was produced only on corn cobs for both fungi (Figure 4.2). The different composition of both biomasses (Table 3.1) may explain this behavior. A maximum IA yield of 0.025 mg/g corn cobs was produced by *A. oryzae* at 168 h of fermentation. At the same fermentation time, *A. terreus* produced half of this amount (0.012 mg IA/g biomass). The fungal biomass of *A. oryzae* was 15 times lower on corn cobs than on wheat bran and almost 2.5 times lower than the one of *A. terreus* on corn cobs. In our

experiment, IA production was inversely linked to the growth. Both acid yields were lower than the yields from the current SmF (Mondala, 2015) (te Biesebeke et al., 2002) (Kautola et al., 1989), so the optimization of the fermentative conditions is necessary to enhance the acid production.

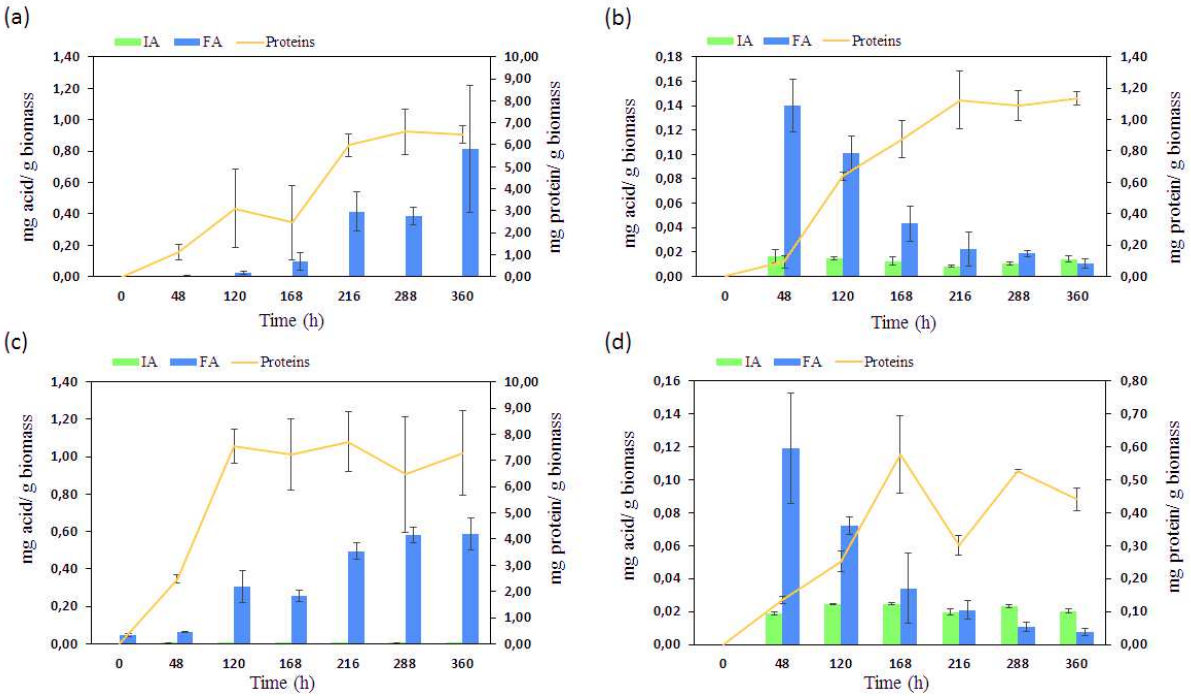

Figure 4.2. Kinetics of the fermentation of lignocellulosic biomasses: IA and FA yields and protein production (growth) from wheat bran (a and c) and corn cobs (b and d) by A. terreus (a and b, respectively) and by A. oryzae (c and d, respectively).

3.2. Optimization of the solid state fermentation steps

After testing the fermentations with both biomasses and both fungi, the optimization steps were performed in two different ways. First, a study with varying pH and humidity levels was used with corn cobs, to allow IA production. Second, an optimization of *A. oryzae* fermentation (the species displaying the higher yields in IA production) of wheat bran and corn cobs was performed by adding an enzyme cocktail to hydrolyze the lignocellulosic biomasses better.

3.2.1. pH and moisture level

Optimum pH and moisture level are crucial factors in the SSF processes to obtain maximum yield of the products of interest (Gervais and Molin, 2003) (Rodriguez-Leon et al., 2008). The initial and previously tested conditions for corn cob fermentation (section 3.1.) were pH 5 for the inoculation and 90% humidity. To optimize the pH and moisture conditions, 5 different pH values and 5 different moisture levels were evaluated for the inoculation step of the biomass culture (Figure 4.3).

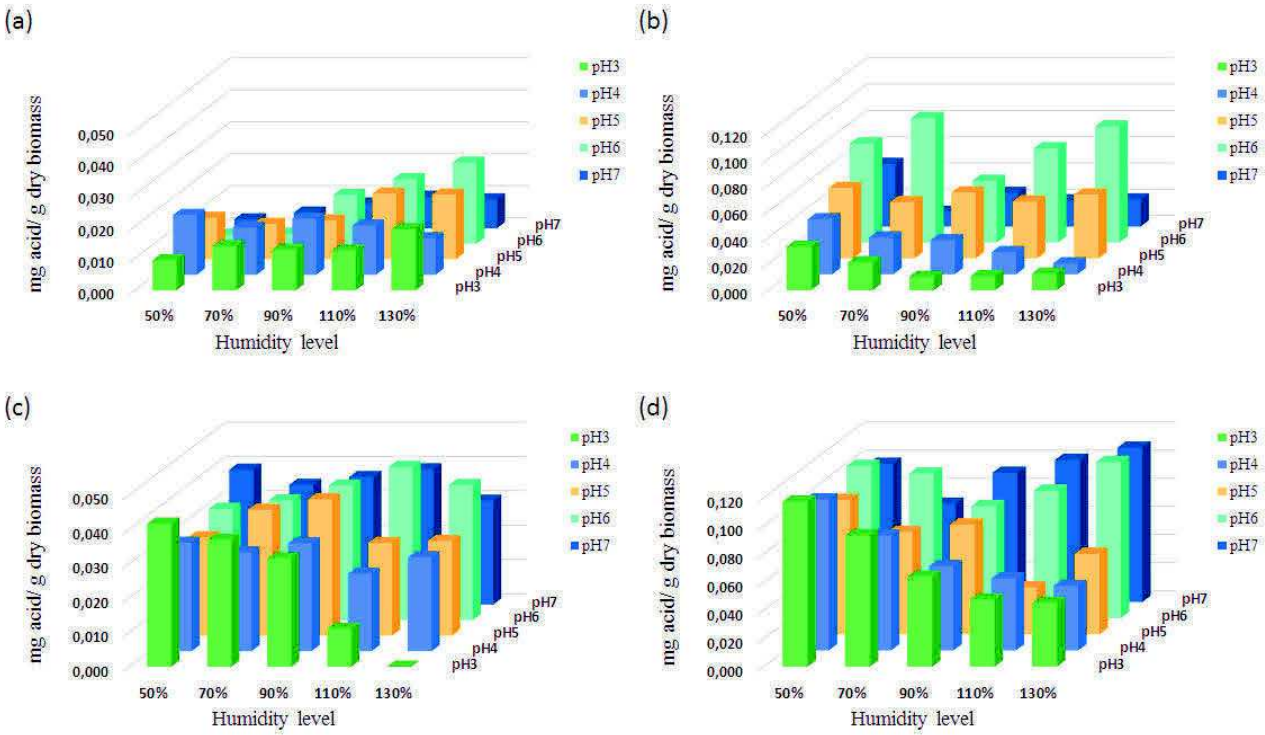

Figure 4.3. SSF on corn cobs at different pH and moisture levels by A. terreus (IA and FA yields: a and b, respectively) and A. oryzae (c and d, respectively).

For *A. terreus,* both acids were produced in high yield at pH 6 but the moisture content influenced the IA and FA production differently. The best IA production, 0.025 mg IA/g corn cobs, was observed at pH 6 and 130% humidity (Figure 4.3a), a doubling of the production compared

with the initial conditions (pH 5 and 90% humidity). FA was produced at a yield of 0.095 mg/g biomass (pH 6 and 70% humidity), also almost double the production under the initial conditions (Figure 4.3b). For both acids, a clear trend is that a neutral pH (pH = 7) seems too high (Figure 4.3a-b). This observation is in good agreement with previous results obtained for SmF (Mondala, 2015).

A. oryzae also showed a preference for pH 6 for the production of both acids (Figure. 4.3c-d). In the case of IA, the highest yield was 0.045 mg/g biomass at 110% humidity (Figure 4.3c), which is slightly higher than the yield under the initial conditions (0.039 mg IA/g biomass) and almost double the IA yield by *A. terreus.* Under the same conditions (pH 6, 110% humidity), 0.091 mg FA/g was produced (Figure 4.3d). However, 0.091 mg FA/g was not the highest production because at the same pH but at 130% humidity, the production was 0.111 mg/ g biomass. Low moisture content causes slower enzyme secretion from the fungus due to the lower solubility of the nutrients and the low level of growth (Koser et al., 2014) (Nuñez-Gaona et al., 2010) (supplementary data). However, acidic pH (3-5) and low moisture often allowed better production of the acids (Figure 4.3b-d). This behavior is not observed at higher pH levels. These observations are in agreement with the fact that in SSF, pH variation significantly impacts the production and the stability of enzymes by microorganisms (Viniegra-González et al., 2003), with many enzymes responsible for biomass hydrolysis during growth.

3.2.2. Enzymatic hydrolysis

As it is a complex and recalcitrant structure, lignocellulosic biomass is often predigested to be used as a sugar source for fermentation (Kumar et al., 2008). The bioconversion is carried out by enzymes produced under specific conditions by many microorganisms (de Vries and Visser, 2001). This saccharification process requires several hydrolytic enzymes such as cellulases,

hemicellulases, xylanases, etc. (Sandhya et al., 2005). In addition, the process could be coupled with the fermentation in a simultaneous saccharification–fermentation step.

Table 4.1. Enzymatic activity from solid state fermentation of wheat bran by A. oryzae.

	A. oryzae
	Wheat bran
α-Amylase activity	18.10
Cellulase activity (cellulose)	4.69
Endoxylanase activity	70.30
Cellulase activity (xyloglucan)	10.11

*Activity in OD/g*min.*

The enzyme cocktail produced by *A. oryzae* grown on wheat bran showed the best enzymatic activity for endoxylanases (Table 4.1) responsible for hemicellulose hydrolysis. Hemicellulose is the most abundant part of corn cobs (Miron et al., 2001) (Cao et al., 1997). However, cellulase and xyloglucanase activity was also found. When the enzymatic cocktail was used for simultaneous saccharification-fermentation of corn cobs, *A. oryzae* rapidly secreted proteins (i.e., it grew) in the first 20 hours, and a plateau was subsequently reached (Figure 4.4b) approximately at the same level as with the raw biomass (Figure 4.2d). Surprisingly, with the treated wheat bran (Figure 4.4a), the fungi secreted half of the proteins compared to the untreated biomass (Figure 4.2c). Moreover, the treatment had a dramatic negative effect on the production of FA from wheat bran (Figure 4.4a), almost 8 times less (0.08 mg/g biomass compared with 0.6 mg/g without pretreatment, Figure 4.2c). In contrast, for corn cobs, the FA yield was feebly increased to 0.15 mg/g biomass. The profile of FA production from corn cobs (Figure 4.4b) was

similar to the profile without the enzyme cocktail (Figure 4.2d), with a maximum (~ 50 h) followed by a decay.

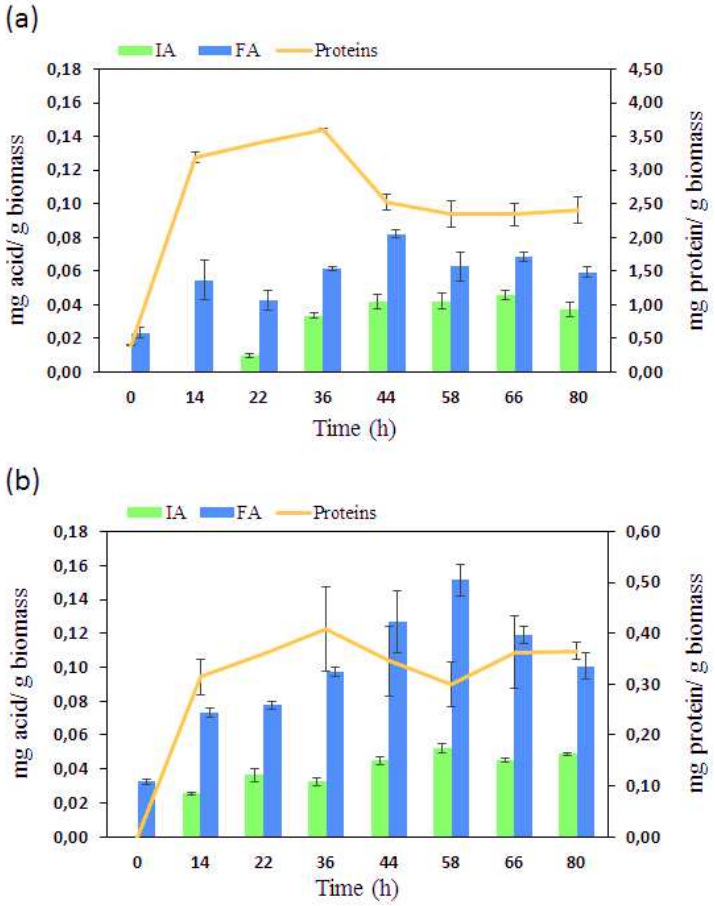

Figure 4.4. Kinetics of simultaneous saccharification and fermentation of wheat bran (a) and corn cobs (b) by A. oryzae.

The best contribution of the enzyme cocktail was linked to the production of IA. The use of the cocktail allowing IA production from wheat bran was reported for the first time in this study, according to our knowledge. IA production was detectable from 22 h, and a maximum yield of 0.046 mg/g biomass was produced after 66 hours (Figure 4.4a). With corn cobs, IA production is clearly detected earlier (14 h), and a yield of 0.052 mg/g biomass was achieved (Figure 4.4b) that

was twice the maximum yield produced with pH and moisture screening (Figure 4.3c). To summarize, even if the cocktail did not improve the growth of the fungus or FA production, the cocktail created better conditions to produce IA. FA is an intermediate metabolite of fungal fermentation, so its production is directly linked with the development of the microorganism unlike IA, a secondary metabolite (Mondala, 2015), and the time lag between growth and IA production is a behavior perfectly consistent with this classification.

In comparison with the best results obtained in pH and moisture optimization (at pH = 6 and 110% humidity) with corn cobs and *A. oryzae* (Figure 4.3c and 4.3d), the acid yields were increased, but the production of the enzyme cocktail required an additional 4 days for the entire process. Therefore, the most interesting strategy for the acid production was the kinetic fermentation of corn cob biomass by *A. oryzae* with the optimized conditions of pH and humidity.

3.3. Kinetic solid state fermentation with optimized conditions

From previous results, the optimum fermentative process was 80 hours at pH 6 and 110% humidity. Figure 4.5 presents the glass flasks and shows the development of *A. oryzae* through the fermentation (green color indicating a high spore concentration). The fungus grew progressively during the fermentation until 0.22 mg of protein/g of biomass was obtained, as in section 3.1 (at pH 5 and 90% moisture level) at the same time of fermentation (Figure 4.2d). In relatively good agreement, FA production was only slightly enhanced (+ 10%) with a maximum yield of 0.16 mg/g biomass within 48 hours (Figure 4.5). However, for IA, the enhancement was higher because the production was more than doubled (0.051 mg/g biomass) after 48 h and higher (0.061 mg/g) after 80 hours (factor 2.4). As generally described for fungi (Nuñez-Gaona et al., 2010), *A. oryzae*

metabolism is greatly influenced by pH and the humidity level at the start of the fermentation step (Koser et al., 2014) (Ayyachamy et al., 2013).

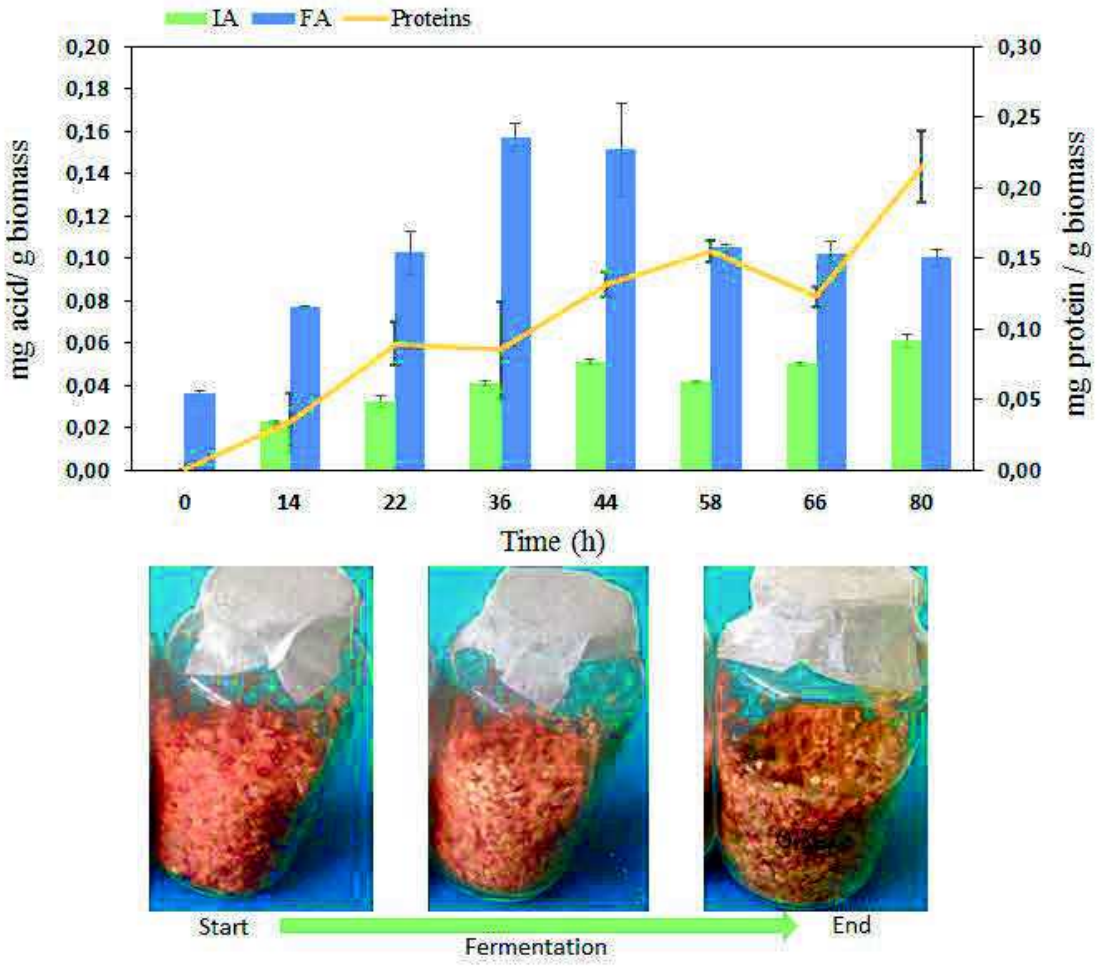

Figure 4.5. : Kinetics of SSF on corn cobs by A. oryzae under optimized conditions (pH 6 and 110% moisture).

The literature is deficient concerning solid state fermentation of FA and IA. To our best knowledge, the best result obtained for FA production was 5.09 mg/g of acid-pretreated corn grains after 10 days of fermentation by *R. oryzae* (West, 2008). Even if our result was lower (0.16 mg/g biomass), our result was obtained in only 36 hours with a non-food substrate and without biomass

pretreatment. For IA, a mutant of *A. terreus* displays a productivity of 0.0003 mg/g h with sugarcane pressmud supplemented with sugars and nutrients (Tsai et al., 2001). The result obtained in this study was more than two times higher (0.00076 mg/g h) by *A. oryzae* (novel IA producer) with a lignocellulosic substrate without any nutrient addition.

3.4. Larger scale fermentation

To develop and analyze scaling up the production, the fermentation was performed with 100 g of corn cobs, 20 times more compared to the previous glass flasks. The optimized conditions of pH and moisture (i.e., 110% moisture and pH = 6) were applied for the fermentation. Aeration plays an important role in SSF for the transfer of oxygen and the evacuation of the carbon dioxide produced. Aeration is also used to dissipate the joules generated by the process (Krishna, 2005b). However, too much aeration could decrease the moisture level during the fermentation by evaporation. The mixture of substrates (solid lignocellulose particles and fungal mycelium) also helps to equilibrate the gas exchange, temperature and moisture level (Hölker et al., 2004). However, mixing that is too strong could induce the disruption of the mycelial-substrate contact, which is particularly important for *A. oryzae*, for instance, to produce the degrading enzymes to hydrolyze the biomass (Yamane et al., 2002).

Two different processes were used to test the influence of both aeration and mixing. A monolayer reactor presenting the same conditions as the glass flasks except for size was compared with an aerated plastic bag fermenter. The plastic bag fermenter was gently mixed on a rocker shaker and distilled water was added in a timely manner to equilibrate the moisture level. The fungal growth was clearly different between the two fermenters (Figure 4.6). The aerated plastic bag yields nearly twice the amount of proteins (0.26 mg/g biomass) as the monolayer fermenter (0.15 mg/g biomass) at the end of the fermentation. Compared with the glass flask fermentation

(0.22 mg protein/g biomass; Figure 4.5), the monolayer fermenter produced less protein whereas the aerated one displayed an amount of protein similar to the small-scale fermentation. This difference in fungal growth was also obvious in observing *A. oryzae* sporulation, which was earlier for the monolayer fermenter (until the second fermentation day) than for the aerated fermenter. This premature sporulation indicates that mycelial development was interrupted by inadequate conditions. The aeration and the loss of humidity correction increased the fungal development and also delayed the sporulation.

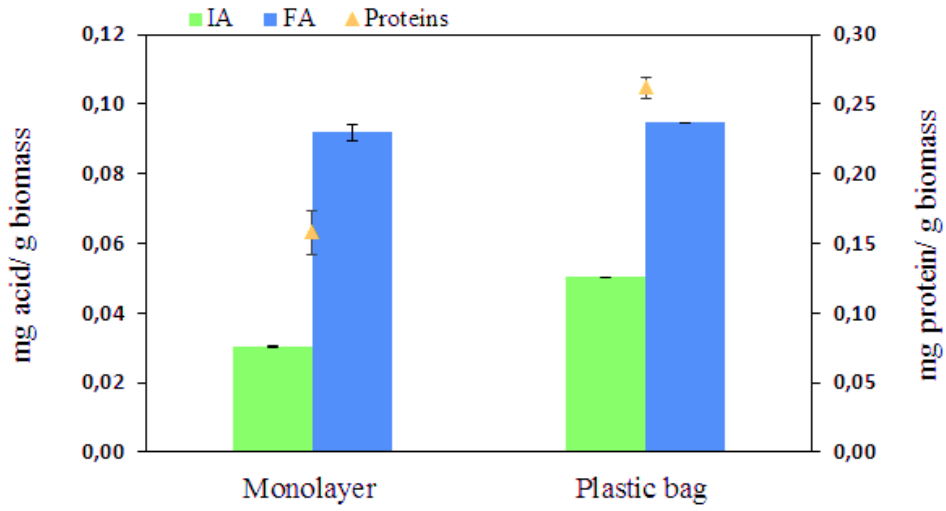

Figure 4.6. SSF in larger scale fermenters of A. oryzae from corn cobs: organic acid productions and protein secretion (growth).

The FA production was not affected by the different conditions (0.09 mg/g biomass in both reactors), probably because 96 hours of fermentation was not an optimized time to recover FA as shown in the glass flask (Figure 4.5), where maximum FA yield was produced at 36 hours. Conversely, IA production was 60% higher in the aerated fermenter (Figure 4.6) than in the monolayer reactor. This improvement could be explained by the supply of oxygen and the mixing

of the fermenter. Indeed, IA fermentation is strictly aerobic, and previous studies showed that a gain in dissolved oxygen and agitation induced high yields (Gyamerah, 1995) (Park et al., 1993). The moisture level could affect the IA production. Below 70% humidity, the nutrient transfers are limited, and the metabolism is affected (Chenyu Du, 2014). In our experiment, water addition along with aeration may allow the equilibrium of the air-solids-water to enhance IA production. Most of these factors were studied for *A. terreus*, long known as an IA producer. However, for *A. oryzae*, conditions still need further optimization. Even if the IA yield obtained in the aerated fermenter (0.05 mg/g biomass) was similar to the small glass flask (Figure 4.4), the final production was multiplied by 20.

4. Conclusions

The IA production process appears to be ideally amenable to SSF conditions, as demonstrated in this work. However, the fermentation conditions still need further optimization to provide yields similar to the yields obtained by submerged fermentation, considering the use of lignocellulosic substrates. Additionally, the use of a novel species, *A. oryzae* (which is used industrially for enzyme production) opens up the possibility of creating a biorefinery process for the production of both organic acids and enzymes. The use of agricultural wastes and cheap and non-food substrates in the bioprocess could lower IA production costs and could therefore promote the use of bio-based IA in the polymerization process to replace petroleum-derived polymers.

Acknowledgments

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Supplementary data

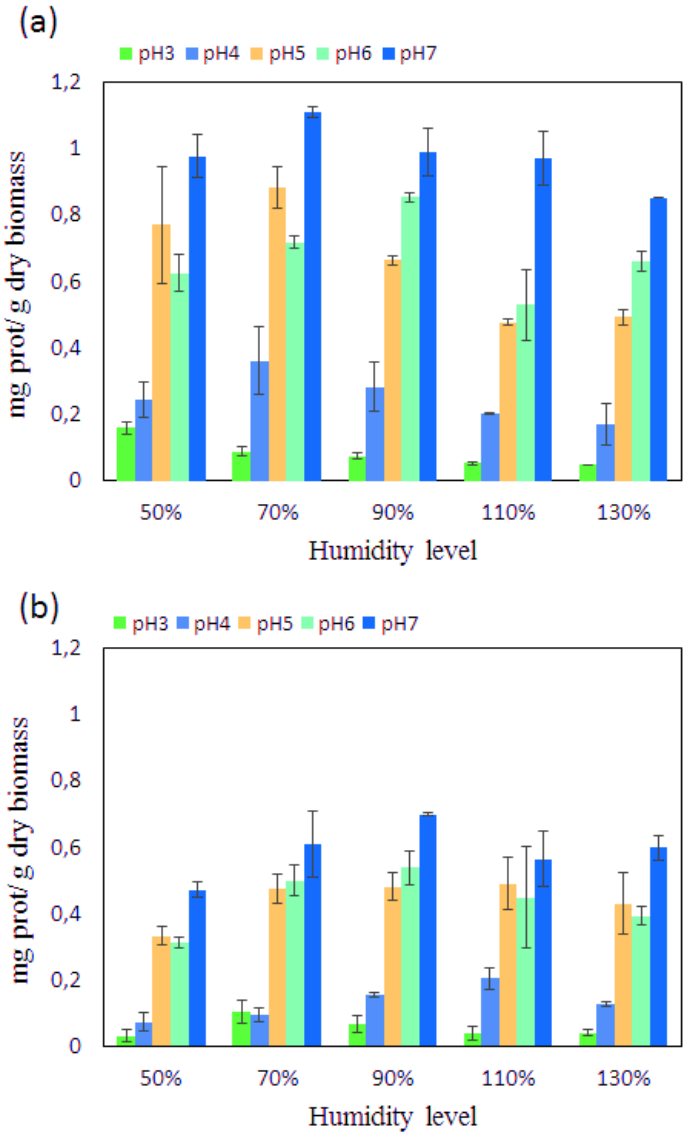

Figure S4.1. Fungal growth in SSF on corn cobs at different pH and moisture levels by A. terreus (a) and A. oryzae (b).

CONCLUSION GENERALE & PERSPECTIVES

Ce projet de thèse avait comme objectif la bioproduction de molécules d'intérêt, notamment pour la production de polymères, à partir de biomasse lignocellulosique. Différentes stratégies pour la valorisation des déchets végétaux ont été mises en place dans ce travail doctoral. Les molécules d'intérêt choisies sont deux acides organiques, l'acide itaconique et l'acide fumarique et la démarche scientifique de ce travail consiste en leur production par quatre souches de champignons filamenteux du genre *Aspergillus* par fermentation du son de blé et des rafles de maïs.

L'étude bibliographique décrit l'état de l'art sur la production des « building blocks » issus des ressources renouvelables. Elle permet d'appréhender les principales difficultés liées à l'utilisation de la biomasse végétale comme matière première dans la bioproduction des acides organiques. Ainsi, le caractère récalcitrant de la biomasse lignocellulosique et les prétraitements nécessaires pour la libération des sucres fermentescibles contenus dans les fibres lignocellulosiques peuvent présenter un frein à l'industrialisation de ces procédés. Le choix du prétraitement influence considérablement la fermentation fongique. La concentration des sucres libérés mais aussi celle des inhibiteurs dépendront non seulement du type de biomasse, mais aussi de ce prétraitement. C'est pourquoi, une connaissance préalable de la composition des biomasses (proportions de cellulose, hémicellulose et lignine) permet de s'orienter vers les traitements les plus adéquats. Ceux-ci sont le traitement à l'acide dilué et le traitement biologique (enzymatique) pour le son de blé et les rafles de maïs, du fait à leur forte concentration en hémicellulose. De plus, du fait de la nature des biomasses à valoriser, cette étude bibliographique a permis s'orienter vers les champignons filamenteux et en particulier du genre *Aspergillus* pour la production d'acides organiques. L'étude identifie également les procédés de fermentation potentiellement adéquats. Cette étude bibliographique a permis également de souligner qu'assez peu d'acide organiques complètement biosourcés, produits à partir de biomasse lignocellulosique, sont commercialisés. Seulement quelques exemples, tels que l'acide lactique ou l'acide succinique, sont bien développés. Concernant les acides itaconique et fumarique, et les polymères qui en sont dérivés, les travaux sont encore à un stade précoce de développement.

Avec la partie expérimentale, nous avons déterminé les meilleures conditions pour la bioproduction d'acide itaconique et fumarique par fermentation de ressources renouvelables, jusqu'au développement d'un prototype de fermenteur à plus grande échelle (Figure C1).

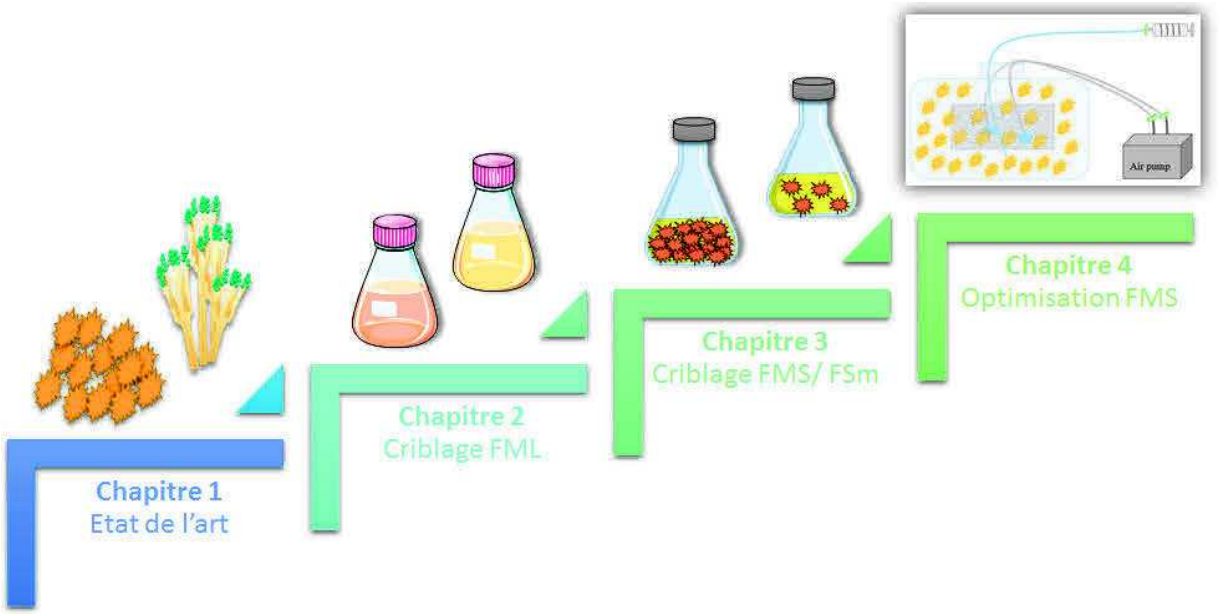

Figure C1. Récapitulatif de la démarche suivie vers la bioproduction d'acide itaconique et fumarique à une plus grande échelle.

Le criblage des conditions de fermentation liquide avec les quatre souches d'*Aspergillus* et les hydrolysats issus de treatment à l'acide sulfurique dilué et par hydrolyse enzymatique a montré une production différente des deux acides en fonction de la biomasse employée. Les meilleurs rendements de production ont été obtenus à partir des rafles de maïs. Les analyses chimiques des hydrolysats issus des deux biomasses ont dévoilé des concentrations différentes en nutriments et composés phénoliques. L'ensemble des résultats démontre que le prétraitement enzymatique des biomasses permet une meilleure bioproduction des acides organiques puisque la libération d'inhibiteurs est plus faible que lors d'un traitement chimique. L'un des résultats marquants est la capacité d'*A. oryzae* à produire l'acide itaconique. Cela n'avait jamais été répertorié dans la bibliographie préalablement. De plus, en fermentation liquide d'hydrolysats enzymatiques des rafles de maïs, ce champignon montre une production (0.14% d'acide itaconique à partir de glucose total disponible) supérieure à celle d'*A. terreus*, espèce productrice par excellence d'acide itaconique et utilisée industriellement. L'acide fumarique est en revanche produit en plus grande quantité par *A. terreus* (1.9% du glucose total), également à partir d'hydrolysats enzymatiques de rafles de maïs.

Le criblage des conditions de fermentation solide et la fermentation submergée a été réalisé à partir des biomasses brutes ou traitées avec des enzymes (traitement biologique). Les analyses chimiques des biomasses solides ont montré là encore des différences. Or, le manque ou l'excès de certains nutriments peut influencer le métabolisme des champignons et ainsi, avoir un effet direct sur la production des acides organiques. De meilleurs résultats ont été obtenus après le traitement enzymatique des biomasses, réalisé de façon simultanée à la fermentation. C'est le cas notamment pour la production d'acide itaconique. *A. terreus* et *A. oryzae* ont montré des rendements de 0.11 mg/g rafles de maïs, ce qui représente le plus haut rendement obtenu par fermentation solide à notre connaissance. En revanche, pour l'acide fumarique, c'est le son de blé qui a permis le plus haut rendement: 0.54 mg/g de biomasse par *A. oryzae*, également à partir d'une fermentation solide avec ajout d'enzymes.

Nous nous sommes ensuite concentrés sur l'optimisation de la fermentation solide, en prenant comme point de départ les conditions permettant les meilleurs rendements pour les deux acides organiques dans les chapitres précédents. En particulier, nous avons étudié les cinétiques de développement d'*A. terreus* et d'*A. oryzae* et de production des deux acides sur les deux biomasses. La production d'acide fumarique est clairement plus précoce que la production d'acide itaconique. Comme observé antérieurement, les rafles de maïs constituent la biomasse la plus adéquate pour la production des deux acides simultanément. De ce fait, l'optimisation du pH et du taux d'humidité a été réalisée avec cette biomasse. Pour *A. oryzae,* les meilleures conditions sont un pH de 6 et 110% d'humidité pour obtenir un rendement de 0.05 mg d'acide itaconique et 0.16 mg d'acide fumarique par gramme de biomasse après 48 heures. Il s'agit du plus haut rendement en fermentation solide pour ces acides à partir d'une biomasse lignocellulosique brute. Ces mêmes conditions ont été utilisées pour réaliser une fermentation à plus grande échelle (x 20). Un prototype de fermenteur en sac de polypropylène a été conçu et employé afin de permettre un apport d'oxygène (facteur déterminant pour la production d'acide itaconique par voie fongique). Le fermenteur avec aération a montré un rendement de production d'acide itaconique 60% plus élevé qu'un fermenteur sans aération. Le processus est plus court et donc plus intéressant en vue d'une fermentation industrielle. La figure C2 récapitule succinctement les principales conclusions.

Figure C2. Principaux résultats obtenus lors de ce travail de thèse.

Aux termes de ces volets expérimentaux, nous avons pu conclure que l'utilisation de la biomasse lignocellulosique constitue une alternative prometteuse pour la production d'acides organiques. Pour la valorisation de ces déchets végétaux, la stratégie la plus intéressante est la fermentation par voie solide qui peut permettre un développement du procédé au niveau industriel. De façon surprenante par rapport aux données bibliographiques, une nouvelle espèce productrice d'acide itaconique, *A. oryzae*, a montré les meilleurs rendements de conversion de la biomasse. Cependant, la production des acides reste faible et est très influencée par la composition chimique de la biomasse utilisée et par le traitement réalisé. De ce fait, la fermentation solide doit être encore améliorée afin d'augmenter les rendements pour égaler ceux des fermentations liquides actuelles, lesquelles sont non durables car réalisées à partir de sucres raffinés. Dans ce contexte, des méthodes actuelles de fermentation et d'analyses en microplaque (de type BioLector) pourraient permettre d'optimiser les conditions de fermentation d'une façon plus rapide et efficace. Les procédés devront aussi intégrer la séparation et la purification des biomolécules. Cette partie (le « down-stream process ») est actuellement la plus chère des procédés industriels de production des acides

organiques. Afin de rendre le procédé le plus rentable possible, l'intérêt doit se focaliser sur l'obtention d'une valorisation la plus complète possible de la richesse des ressources. Par exemple un même procédé de production des acides organique peut aboutir à la production d'autres molécules d'intérêt commercial. C'est le cas ici avec les enzymes produites en fermentation solide, qui devront donc être récupérés au même titre que les acides organiques. De plus, au-delà des exemples classiques d'utilisation en alimentation (humaine et animale), des études récentes ont montré que le mycélium formé par développement des champignons sur des substrats cellulosiques présente un intérêt en tant que potentiel matériau composite pour la construction. Le mélange, après séchage constitue une matière organique légère qui possède des caractéristiques similaires à des mousses ou des matériaux thermoplastiques.

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Bio-production d'acide itaconique à partir de biomasse végétale, pour une finalité matériaux

Résumé

Dans un contexte du développement durable, la bioproduction de synthons (molécules plateformes, ou building blocks) de façon biosourcée à partir de biomasse végétale, constitue une voie de remplacement des actuelles molécules prétrosourcées. Ce travail de thèse concerne spécifiquement l'utilisation de la biomasse lignocellulosique, renouvelable et abondante, pour la production de deux acides organiques d'intérêt : l'acide itaconique et l'acide fumarique. Ces molécules ont été choisies notamment car elles peuvent générer des polymères aux propriétés intéressantes. Les travaux expérimentaux ont consisté à utiliser le son de blé et les rafles de maïs, déchets agricoles, comme substrats pour la fermentation de quatre souches de champignons filamenteux du genre *Aspergillus*. Des criblages des meilleures conditions fermentaires montrent que les rafles de maïs permettent d'atteindre des rendements plus élevés, surtout en fermentation en milieu solide. Parmi d'autres résultats marquants, nous avons montré pour la première fois la capacité d'*Aspergillus oryzae* à produire l'acide itaconique. L'ensemble de nos résultats montrent que l'utilisation de la biomasse lignocellulosique est une alternative prometteuse pour la production de ces deux synthons d'intérêt industriel.

Mots-clés : biomasse lignocellulosique, molécule plateforme, *Aspergillus*, acide itaconique, acide fumarique

Abstract

In the context of sustainable development, the bioproduction of building blocks (chemical platforms) from biomass is way to substitute the current fossil-based chemical molecules. This thesis is focused on the use of lignocellulosic biomass, renewable and abundant, towards the production of two organic acids (potential building blocks): itaconic acid and fumaric acid. These molecules have been chosen especially because they can generated polymers with interesting properties. The experimental work consisted in using wheat bran and corn cobs, agricultural wastes, as substrates for fermentation by four strains of filamentous fungi from *Aspergillus* genus. Screenings of the best fermentation conditions show that enzymatically pretreated corn cobs, especially in solid state fermentation achieve higher yields, especially in solid state fermentation. Among other notable results, we have shown for the first time the ability of *Aspergillus oryzae* to produce itaconic acid. Overall, our results show that the use of lignocellulosic biomass is a promising alternative for the production of these two building blocks of industrial interest.

Keywords: lignocellulosic biomass, building blocks, *Aspergillus*, itaconic acid, fumaric acid.