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A todos aquellos que,
sin importar la
distancia física,
siempre están

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Table of acronyms

1G	First-generation biorefineries
2G	Second-generation biorefineries
3G	Third generation biorefineries
C5	Pentoses
C6	Hexoses
CCR	Carbon catabolite repression
COD	Chemical oxygen demand
DOE	US department of energy
GA	Glutamic acid
HCN	Hydrogen cyanide
HMF	Hydroxymethylfurfural
LAB	Lactic acid bacteria
MPF	Modified patent fermentation media
MPI	Modified patent inoculation media
MSG	Monosodium L-glutamate
PFM	Patent fermentation media
SFM	Synthetic fermentation media
SIM	Synthetic inoculation media
VRICR	Vertical Rotating Immobilized Cell Reactor
XOH	Xylitol
XR	Xylose reductase
YE	Yeast extract

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Introduction

Biorefinery concept first appeared in the late 1980s as a proposition to cope with climate change and the oil crisis in the world. It had a boost in the 2000s when bioethanol and biodiesel production began profitably. Once the researches found a competition in biofuel production with the alimentary, the concept of the circular economy appeared.

The main focus in circular economy is the reduction of the raw materials used, to achieve this, two main concepts are used: to repair and to reutilize. In the case of biorefineries, researches drive their focus on the raw material from sugars (1^{st} generation biorefineries) to the use of lignocellulosic biomass residues (2^{nd} generation biorefineries). From this moment, the scientific community is not only interested in the production of bioethanol and biodiesel, but also other different value-added molecules.

Because of their nature, lignocellulosic residues give a composition of hydrolysates containing a combination of sugars. These sugars are generally in C5 and C6, whose proportion and structure change depending on the type of plant, the environmental conditions it faced, and the pretreatment used for hydrolysis.

Even after nearly twenty years of research, biorefineries have not been completely adapted to be out in the market. Different reasons have been mentioned, such as irregularity of the raw material, the sugar selectivity of microorganisms, the inhibition due to the media composition after hydrolyzing, and recovery yield after fermentation.

Within the different issues facing biorefineries preparation to market, to limit this work, we focus on the step of the biological transformation of lignocellulosic hydrolysates in value-added molecules. The strategy consists on the fermentation of both types of sugars without preliminary separation. It would allow not only to minimize process times by eliminating the sugar separation stage, but it will also save energy. The biorefinery products selling price must at least be competitive with the market prices from the same molecules issues from oil. Then, by producing different molecules with added value and industrial diversification, the investment and operation costs of the biorefinery may become rentable with the sales.

Over the years, and since the start of second-generation biorefineries, one of the main concerns and problems is the selectivity of the microorganisms in sugar that they can metabolize. Due to the combination of sugars found, three strategies have mainly been used: 1) Genetical modification of the strain to metabolize several sugars, 2) Selection of a couple of microorganisms (genetically modified or not) to maximize one molecule production, 3) Use of different microorganisms to produce various products. This last method has been studied especially for xylitol and bioethanol production.

This work is a feasibility study, in which, with bibliographic research, we propose to select a couple of non genetically modified microorganisms with compatible fermentation conditions in which one of them will metabolize glucose to produce organic acids, and the second one will metabolize xylose to produce xylitol. In this work, we chose to work with a model medium which simulates agave bagasse composition.

In the first chapter, bibliographic research is presented to detail the issues confronted nowadays for optimizing biorefineries. For this first part, the selection of the couple of microorganisms is realized according to their reported culture conditions. The choice of compromised compatible fermentation conditions for mixed culture is defined at the end.

In the second chapter, the final materials and methods used in the process are presented.

For the third chapter, the first single culture assays and results in Erlenmeyer are presented. Here, the best medium composition and culture conditions are selected. These conditions were validated primarily for cell growth and metabolites production for both strains before being tested in bioreactors.

The last chapter shows the mixed culture tests realized with the chosen conditions in the previous chapter. The influence of aeration on metabolite production is investigated as well as the combined effect with pH regulation and organic acid presence. The second part of the results is shown in the form of an article.

I. Literature review

In this first part, we will talk about the biorefineries, the issues encountered actually for the biorefineries competitivity in the market, and the strategy that has been proposed by different authors: the production of multiple molecules in the process. This strategy is suggested to minimize production costs, and augment the raw material transformation yield.

I-A. Biorefinery concept

Over the last years, the interest in exploiting biomass has grown. The objective of biorefineries to reduce petroleum-derived chemicals is seen as a way to re-direct the industrial chemistry towards a more sustainable economy. The principal difference between those two types of industry is the raw material; in a standard refinery, petroleum is transformed, and in the case of biorefineries, biomass feedstocks are used.

The most important factor when thinking about a biorefinery is that it has to be competitive with the current oil refineries. When comparing each other, some characteristics are highlighted as renewability, sustainability, and carbon-neutral (zero emissions) (Choi *et al.*, 2015). In biorefineries, each product or energy type can be developed in a single process. More integrated production of chemicals, materials, energy, and food would have a more efficient approach for the valorization of biomass resources (Moncada, Aristizabal and Cardona, 2016).

There are different ways to classify biorefineries. The most common is by the raw material used for the conversion. First-generation biorefineries (1G) used as a raw material edible crops, mostly sugars, and vegetable oils. The principal advantage of this technology is that the biomass is ready to use, edible crops composition are simple molecules, and there are no complex treatments before the transformation. However, the main problem is the competition with the food sector. The generation of special crops to produce biofuels could lead to an increase in food prices and generate pressure in the use of the land. With those disadvantages, 1G biorefineries will no longer meet with sustainability principles.

Second-generation biorefineries (2G) aim principally to valorize lignocellulosic biomass, such as agricultural or industrial wastes. Those are non-edible, biodegradable, and can grow in all types of lands. Because of their raw-materials choice, 2G biorefineries avoid competition with the food sector and meet with sustainable principles.

Third generation biorefineries (3G) focuses on the use of micro-algae, and their advantages lie in the low- cost of culture, renewability, and low energy maintenance.

In this work, the interest is centered in a transformation by biochemical conversion in a 2G biorefinery. There are mainly two ways that can be used for further transformation: thermo-chemical conversion and biochemical conversion. Figure I-1 presents a schema made by Menon and collaborators (Menon and Rao, 2012a) describing the different ways to generate different sources of energy and chemicals from lignocellulosic biomass. It’s observed that the type of transformation depends on the final product targeted.

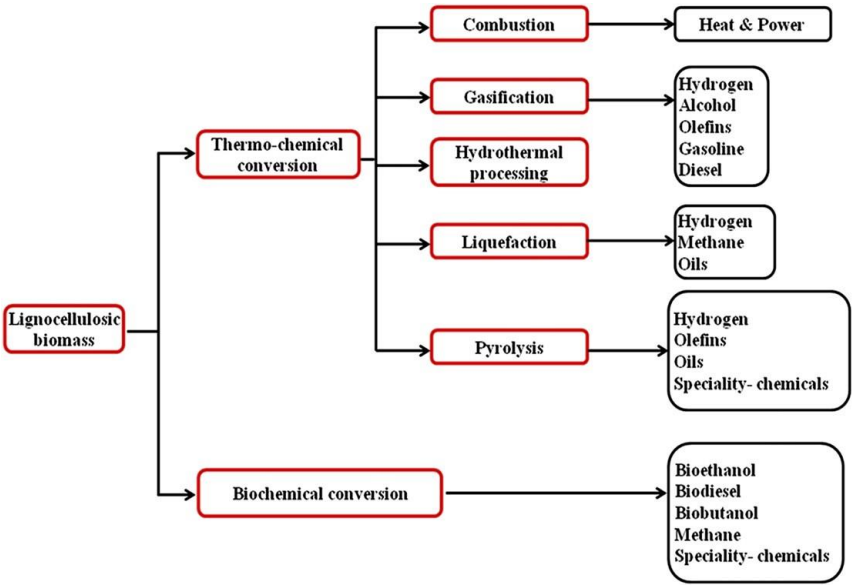


Figure I-1 Thermochemical and biochemical processing of lignocellulosic biomass
(Menon and Rao, 2012a).

Figure I-2 presents the four main steps necessary for the biochemical conversion. When using lignocellulosic biomass, obtained at the end of a production chain or by forest residues, a pre-treatment stage is required to obtain a fermentable fluid. This step contains primarily: hydrolysis, clarification, and pH regularization. The hydrolysis stage is the critical stage in the pre-treatment; its principal purpose is to obtain free monomers available for fermentation. Different types of hydrolysis exist; the most current are acidic hydrolysis, thermolysis, and enzymatic hydrolysis. Each one has its advantages and withdraws. The yield and final composition of the hydrolysate depend on the nature of the biomass and the pre-treatment conditions (Limayem and Ricke, 2012). The final choice of an optimal pre-treatment depends on the nature of the feedstock and the economic and environmental impact (Menon and Rao, 2012a).

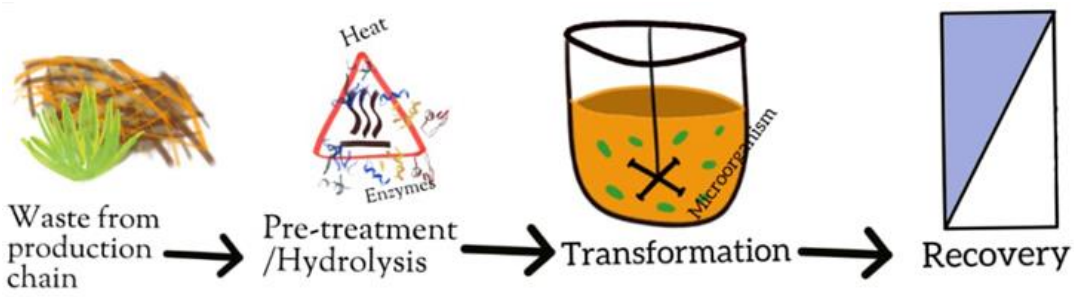


Figure I-2. Biotransformation biorefinery simplified schema

The biological conversion is followed by further recovery and/or purification. To increase productivity and efficiency, the use of unit operations that lower the overall energy consumption is needed. The global strategy will be to try to maximize the use of all feedstock components, byproducts, and waste streams, and to reduce costs by increasing productivity, common processing operations, materials, and equipment to drive down all production costs (Werpy *et al.*, 2004).

There are different ways of optimizing biorefineries. In this work, the focus is only on the biotransformation stage of the chain to maximize sugar utilization by the microorganisms. Nevertheless, it is essential to understand that everything is interconnected and that any change in any stage, could modify the entire chain. For example, if the raw material or the hydrolysis process changes, this has a direct impact on product yield and productivity. If the microorganism used changes, the metabolites would change because it has to be adequate for the hydrolysate used. Also, the change in metabolites modifies the unit operation used in the recovery stage. Because of these constraints, a specific type of lignocellulosic material has to be chosen.

I-A.1 Lignocellulosic residues in fermentation

Lignocellulosic biomass represents 85 % of the total biomass available in the world. Its composition is mostly cellulose, hemicellulose, and lignin. As indicated in Table I-1, the composition differs between the sources of the feedstock. In general, it is composed of three polymers (in dry weight): 30 to 50 % of cellulose, 25 to 30 % of hemicellulose, and around 10 to 20 % of lignin. The structure and the quantity of the components vary according to species, tissues, and maturity of the cell wall (Isikgor and Becer, 2015).

Table I-1 Composition of representative lignocellulosic feedstocks. Modified from (Menon and Rao, 2012b)**.**

	Carbohydrate composition (% dry wt)		
	Cellulose	**Hemicellulose**	**Lignin**
Corn cob	32.3-45.6	39.8	6.7-13.9
Corn stover	35.1-39.5	20.7-24.6	11.0-19.1
Cotton	85-95	5.0 - 15	0
Cotton stalk	31	11	30
Coffee pulp	33.7-36.9	44.2-47.5	15.6-19.1
Eucalyptus	45-51	11.0-18	29
Hardwood stems	40-55	24-40	18-25
Rice straw	29.2-34.7	23-25.9	17-19
Rice husk	28.7-35.6	11.96-29.3	15.4-20
Grasses	25-40	25-50	10-30
Sugarcane bagasse	25-45	28-32	15-25
Sugarcane tops	35	32	14
Pine	42-49	13-25	23-29
Olive tree biomass	25.2	15.8	19.1
Jute fibres	45-53	18-21	21-26
Switchgrass	35-40	25-30	15-20
Grasses	25-40	25-50	10-30
Winter rye	29-30	22-26	16.1
Oilseed rape	27.3	20.5	14.2
Softwood stem	45-50	24-40	18-25
Oat straw	31-35	20-26	10-15
Nut shells	25-30	22-28	30-40

The structures of the three polymers are presented in Figure I-3.

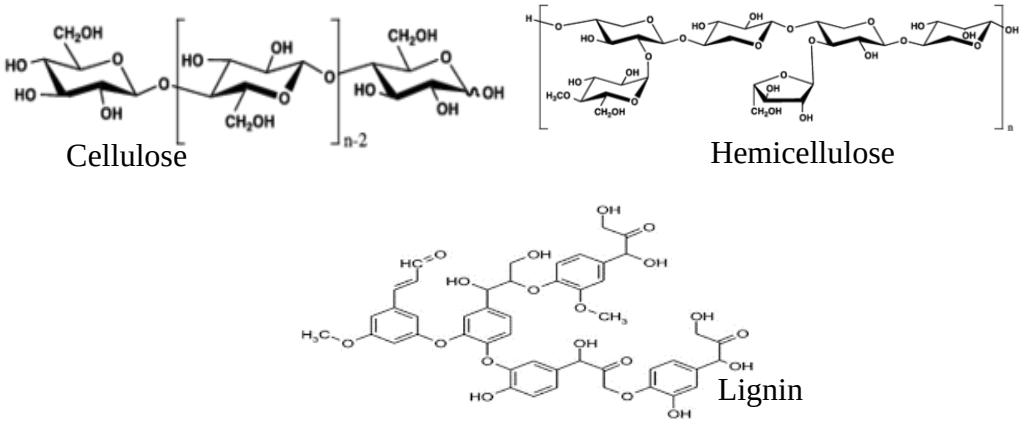


Figure I-3 Structure of cellulose, hemicellulose, and lignin

Cellulose is a glucose linear polymer constituted of repeating glucose monomers linked by β1→4 bonds. Cellulose forms microfibers, and those are arranged in a crystalline or amorphous form. The concentration of this amorphous form affects ultrastructure.

Hemicellulose is the second most abundant polymer. In contrast with cellulose, it is not chemically homogeneous and has a random and amorphous structure. Its composition is mostly different types of sugars: pentoses (C5) (xylose and arabinose), hexoses (C6) (glucose, mannose, and galactose), and uronic acids. Depending on the biomass, the hemicellulosic composition will differ. Softwood hemicellulose contains mostly glucomannans, and on the contrary, hardwood hemicellulose contains mostly xylans.

Lignin is a complex, large molecular 3D structure containing cross-linked polymers of phenolic monomers. It is present in plant cell walls and confers a rigid, impermeable resistance to microbial attack and oxidative stress (Isikgor and Becer, 2015; Bajpai, 2016).

One of the main challenges with lignocellulosic residues fermentation is the presence of mixed sugars, C5 and C6, and inhibitors released during the pre-treatment. In lignocellulosic biomass, inhibitors are formed from carbohydrate degradation like aliphatic carboxylic acids, acetic, formic, and levulinic acids; and the furan aldehydes, furfural, and hydroxymethylfurfural (HMF). The nature and concentration of these inhibitors differ according to the hydrolysis treatment, and the source of the biomass. Also, the inhibitory capacities depend on the microorganism used for the fermentation and on its resistance to the specific molecules (Limayem and Ricke, 2012; Jönsson and Martín, 2016).

Each microorganism is different regarding the type of assimilable sugars but no native microorganism can convert both glucose and xylose into one product (Chen, 2011). Because of this, energy-consuming pre-treatments have been proposed to separate the sugars before the bio-transformation. This means that the recycled-raw material will not be used entirely and that supplementary energy is used, making the process less environmentally friendly, expensive, and time-consuming.

I-A.2 Agave bagasse residues

In the case of this research, the lignocellulosic biomass that we are interested in is agave bagasse residues coming from the production of Mexican distilled alcoholic beverages. Mesoamerican civilizations had a variety of fermented beverages coming from native plant species, this was reported on codices written before European contact. Historical data shows high cultural importance on the agave and its different derivates (food, textile, and fermented beverages) (Villarreal *et al.*, 2009). Nowadays, Mexico counts with four origin denominations for fermented beverages coming from agave, or maguey as is commonly known in Mexico. They are Mezcal, Tequila, Sotol, and Bacanora (Figure I-4).

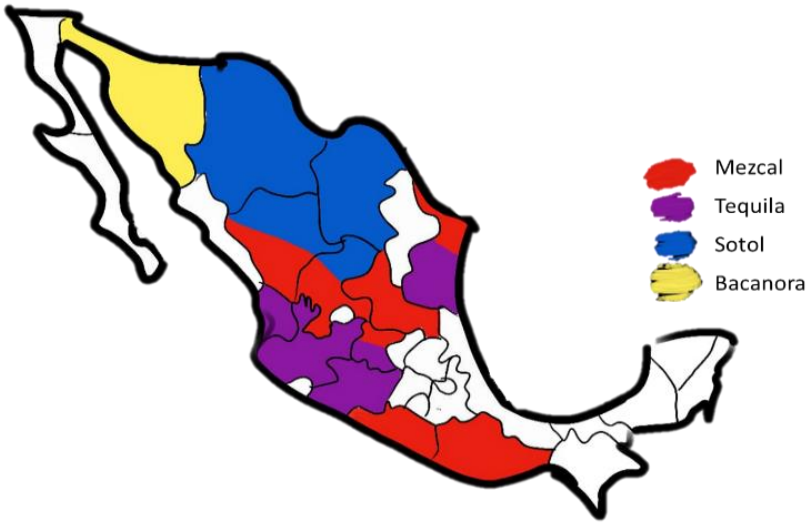


Figure I-4 Mexican states with their "denomination of origin" from agave beverage

Tequila

As described by the Mexican official norm (Mexican Secretariat of Economy, 2006) describes it; Tequila is the alcoholic beverage coming from the "*agave tequilana weber*", blue variety. The reduced sugars from blue agave juice have to be at least at 51% of the total fermented sugars and all process has to be done at the factories in the states with the designation of origin.

Mezcal

Its name came from the Nahuatl vocables "metl" which means Maguey and the word "ixcalli" which means cooked. Mezcal production is mainly artisanal in comparison with tequila production (Carrillo Trueba, 2007). Organoleptic properties will depend on the agave species, as well as the transformation process. Agave quality will depend on the soil, topography, weather, climate, techniques and the microorganisms used. The most common species used are *Agave angustifolia, Agave cupreata, Agave duranguensis, Agave salmiana, Agave tequilana,* and *Agave Americana* (Mezcal-Regulatory-Council, 2018).

Sotol

Sotol is the alcoholic beverage obtained from the agave plant known as Sotol or sereque (*Dasylirion spp.*). These are obtained from wild and cultivated plants in the states of Chihuahua,

Coahuila, and Durango. The *Dasylirion* is not an *agavacea*, even if it looks like the agave. Sotol has been produced for over 800 years for the people in the village of Paquime (Instituto-Mexicano-de-la-Propiedad-Industrial, 2002).

Bacanora

Is the regional alcoholic beverage of Sonora state in the north of Mexico. It's obtained with the must prepare directly from the juices of the mature plants of *Agave angustifolia*. In 1915 general Plutarco Elías Calles prohibit the production, commercialization, and consumption. This "dry law" was kept for 70 years (Salazar Solano, 2016).

Bacanora production is informal, because of the 70 years banned, the productors didn't had the chance to optimize and automatize this process (Gutiérrez-Coronado, Acedo-Félix and Valenzuela-Quintanar, 2007). Nevertheless, 300 000L of Bacanora are produced per year and the Sonora Regulatory Council of Bacanora said that 30 thousand liters are exported per year (Valenzuela, 2019).

Each year, the production of these four spirituous beverages has increased. Indeed, in 2018, México produced 309.1 million liters of tequila. By 2019, these numbers were already reached 351.7 million liters of tequila (Tequila-Regulatory-Council, 2020). Because of this production, 1 mil million tons of agave were needed, and 418 000 tons of bagasse were produced as a byproduct. This production continues to increase over the years. In the case of Mezcal, in 2018, the production increased by 18 % for national trade and 22 % for exportation (Mezcal-Regulatory-Council, 2018).

The production of agave fermented beverages follows the same process divided in seven stages, as observed in Figure I-5.

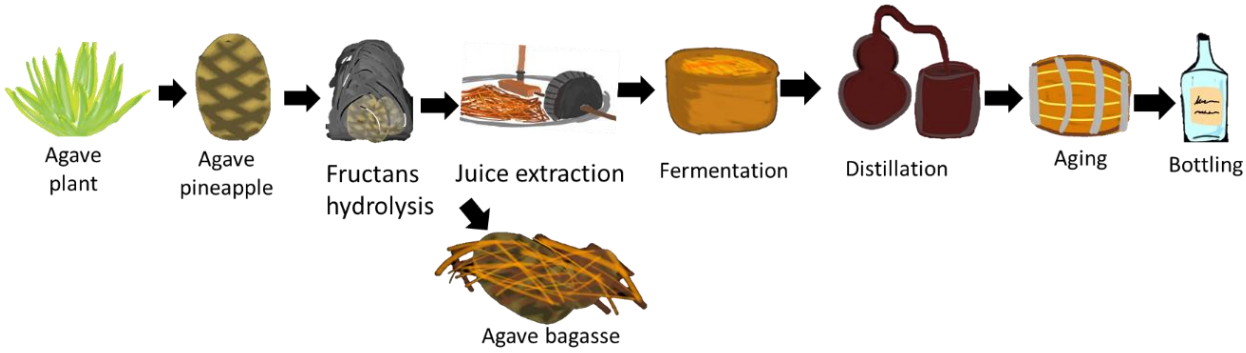


Figure I-5 Schema of the production chain of agave fermented beverages

Sugar extraction is the most crucial stage in the process because it will be at the origin of the overall juice composition, including the production of some fermentation inhibitors resulting from the degradation of carbohydrates and lignin (HMF, phenols, acetic and formic acid (Saldaña Robles *et al.*, 2012; Rodríguez-Garay *et al.*, 2016; Palomo-Briones *et al.*, 2018).

Sugar extraction can be realized by two methods. The first one is cooking, in which the pineapple is divided into several parts and placed in masonry furnaces. It's cooked for 24 h to degrade carbohydrates into simpler sugars, follow by milling for the extraction of the juice along with simple sugars. The second method is diffusion. The pineapple is not cooked before the sugar extraction. Instead, it is crushed and shredded into fine fiber strips that are washed to

extract the sugars from the plant. After, the juice is hydrolyzed through thermal and acidic treatment (Saldaña Robles *et al.*, 2012).

The choice of the technology used at this stage has an impact on the sugar composition to be fermented. Nowadays, diffusion is the method most used in the Tequila houses. And, cooking is mostly used for mezcal production.

At the end of the process, large amounts of residues are generated, among which bagasse is one of the most important. Bagasse is the residual fiber that comes after the cooking, milling, and extraction of the "mosto". It represents about 40 % of the total weight of the agave (Gonzalez-García, Gonzalez-Reynoso and Nungaray-Arellano, 2005).

In Mexico, several studies have been done to propose another use of this residue. In the state of Jalisco, there's a center of research (CIATEJ), which has as their main purpose of giving technological and human solutions to enhance the competitiveness in the agricultural, food, health, and environment sectors.

Various researches have been realized on these residues. For example, they can be used as a base for animal feed as recommended by different authors (Iñiguez-Covarrubias, Lange and Rowell, 2001; Ramírez-Cortina, Alonso-Gutierrez and Rigal, 2010). Moran and collaborators studied different degradation conditions for the agave bagasse and its possible use as a fertilizer (Moran-Salazar *et al.*, 2015). Also, several authors have been studied different hydrolyzation techniques (Arreola-Vargas *et al.*, 2015; Olivares Cantú *et al.*, 2016). In the work presented by Arreola-Vargas and collaborators, they evaluated acid hydrolysis for sugar extraction on *Agave tequilana* bagasse. Those hydrolysates were then tested for methane production, having interesting results in which the addition of supplementary nutriments presented a suppression in methane production. Without nutriments addition, they obtained a methane production of $0.26 L/g_{COD}$ (chemical oxygen demand) in anaerobic sequencing batch reactors (AnSBR). The bioethanol produced from the bagasses or its calorific force can be used as fuel (Alonso Gutierrez, 2005; Chávez-Guerrero and Hinojosa, 2010; Valenzuela, 2011; Flores-Gómez *et al.*, 2018). Another use was the production of biochemicals and biopolymers tested by Gonzalez-García and collaborators and Palomo-Briones and his team (Gonzalez-García, Gonzalez-Reynoso and Nungaray-Arellano, 2005; Palomo-Briones *et al.*, 2018) and, as a second research line, the production of phenolic compounds from the lignin in the fibers.

Because of the high quantity of residues due to a yearly increase in agave-based beverages, the interest, and concerns about different ways to valorize this residue have increased. The economic interest in the use of lignocellulosic waste material for 2nd generation biorefinery has led us to choose the agave bagasse as the raw material of interest for the transformation of its sugars into value-added molecules. Arrizon *et al*., (2012); Hernández-Salas *et al*., (2009), and Saucedo-Luna *et al*., (2011) have reported sugar composition in different agave bagasse hydrolysates. They all confirmed that the main sugars were glucose and xylose.

Now, as we are interested in producing different molecules with two different microorganisms, the next stage is to define both of them.

I-B. Selection of chemical building blocks and types of microorganisms

For this research, different products other than ethanol will be targeted. One of the main reasons is that bioethanol production has been largely studied in the last years. Also, its low cost of commercialization does not allow the investment of the biorefinery to be profitable.

Several multicriteria analyses have been realized to determine the best-case scenario for a good economical-environmental compromise (Vlysidis *et al.*, 2011; Cheali *et al.*, 2015; Nieder-Heitmann, Haigh and Görgens, 2019). The authors agree that the best case is presented when value-added molecules are produced. That is why we chose the production of molecules with high added value in the market. Vlysidis *et al*., (2011) reported that that the main capital expense is the cost of fermenters with 34 % of the total investment cost, in the process cost, the raw material is the most expensive expense with 80 % of the total value. They concluded that in the case of a bioethanol biorefinery, biorefinery will be profitable with the sale of side-products.

In the Biobased Chemical Building Blocks summary report, "Overcoming hurdles for innovation in industrial biotechnology" (BIO-TIC, 2014), the co-production of high-value products is proposed. Indeed, the best use of biomass feedstock would be reached once developing multi-step and multi-product biorefineries (Menon and Rao, 2012a).

In 2004 the US department of energy (DOE) (T. Werpy and Petersen, 2004; Koops *et al.*, 2010) reported and listed 12 molecules (succinic, fumaric, malic, aspartic, glucaric, glutamic, itaconic, levulinic acid, xylitol, sorbitol, glycerol, 3-hidroxypropionic acid) metabolized from simple sugars which have a large interest in the industry. Indeed, as chemical building blocks, these molecules can be further converted into other valuable or commodity products.

Our work aims to propose the co-production of at least 2 value-added molecules. For this purpose, it's necessary to find a couple of microorganisms that will be able to produce, in similar culture conditions, different molecules from each sugar present in the hydrolysate (glucose and xylose).

Villela-Alonso, (2016) realized a bibliographic research work, in which she compiled for pure micro-organisms, different fermentation conditions, substrates, yields, and productivities reported by different authors. Based on the DOE's list, she compiled the fermentation conditions of various microorganisms that are reported as producing the chemical building blocks. One of the microorganisms uses glucose while the other consume xylose.

One of the main issues of this bibliographic research was to select microorganisms not genetically modified to keep strain stability over the cycles. In the end, she made a first proposition of five couples of microorganisms for which fermentation conditions could be matched, and that could potentially be used to co-produce interesting metabolites.

In her work, Villela-Alonso proposed couples along with some compromise conditions to test according to the conditions already tested by the authors in pure cultures. The couples she suggested are summarized in Table I-2. The product concentration, yield, and productivity presented in the tables are the ones obtained by the referenced authors in the second column during the pure cultures.

Table I-2 Summary table of possible pairs of strains proposed by (Villela-Alonso, 2016)**.**

	Strains	Ref.	Products (g/L)	Yield (mol/ mol)	Productivity, (g/L.h)	Substrate	Conditions to be tested proposed by Villela-Alonso, (2016)
1	***Candida tropicalis* ATTC 13803**	(Choi *et al.*, 2000)	18.7 Xylitol	0.74	3.90	Xylose 25 g/L Glucose 20 g/L	Fed-batch aerobic 40 mMO2/L·h
	Corynebacterium glutamicum	(Amin, 1994)	11.9 Glutamic acid	0.73	4.00		
2	***Candida tropicalis* ATTC 13803**	(Choi *et al.*, 2000)	18.7 Xylitol	0.74	3.90	Xylose 25 g/L Glucose 20 g/L	Fed-batch aerobic Saturated air 60 %, pH 7.6, 39 °C
	***Corynebacterium glutamicum* 2262**	(Delaunay *et al.*, 1999a)	7.3 Glutamic acid	0.45	3.54		
3	***Candida tropicalis* KFCC 10960**	(Kwon, Park and Oh, 2006)	21.8 Xylitol	0.86	2.91	Xylose 25 g/L Glucose 20 g/L	Batch, Aerobic, 170 rpm, pH 7, 30 °C
	Corynebacterium glutamicum	(Otten, Brocker and Bott, 2015)	5.7 Itaconic acid	0.4	0.27		
4	***Candida tropicalis* KCTC 7221**	(Kwon, Park and Oh, 2006)	19.5 Xylitol	0.77	2.58	Xylose 25 g/L Glucose 20 g/L	Batch aerobic pH 6.5, 30 °C
	Brevibacterium	(US Patent-3,128,237, 1961)	9.9 Glutamic acid	0.61	1.00		

From the table, we can observe that the pair of molecules with higher productivity and similar conditions of production are: xylitol (XOH) obtained from xylose and glutamic acid (GA) obtained from glucose. Xylitol is proposed to be produced by a *Candida* yeast and glutamic acid by a *Corynebacterium* family bacteria.

Coryneform bacteria are known to produce several bulk products (amino acids and organic acids). They became a notable species at an industrial level due to the different products that can be obtained with some changes in the fermentation conditions (Hermann, 2003). The multiple metabolites obtained by this microorganism is interesting information that must be kept in mind later in the work.

I-C. Selection of strains

I-C.1 Strains for glutamic acid production

From the literature study (Table I-2), we decided to work with *Coryneform* bacteria to produce glutamic acid from glucose. In Table I-3 different essential parameters for several - *Coryneform* bacteria strains are presented. Parameters like temperature, oxygen supply, and pH were studied for their impact on the specific growth rate, maximal production and productivity.

Table I-3 Summary table of different *Corynebacterium* and *Brebivacterium* strains and their glutamic acid production conditions.

Strain	Max specific growth rate (h^{-1})	Substrate (g/L)	Production (g/L)	Productivity (g/L.h)	Yield (g/g)	T (°C)	pH	Aeration (vvm)	Support	Mode	Exp time (h)	Ref
***Brevibacterium sp.* DSM. 20411**	0.021	Corn steep liquor and 1:100 ml starch hydrolysate 7.5 - 45	21	0.15	0.196	30	7.5	0.36	-	Batch + Fed-batch	50	(Nampoothiri and Pandey, 1999)
***Brevibacterium sp.* Tc452**	0.47	Glucose 120	41.4	1.18	0.53	30-38	7.8	-	-	Batch	25	(Choi, Nihira and Yoshida, 2004)
***Corynebacterium glutamicum* ATCC 13058**	n/a	Glucose 100	50.7	4	0.64	30	slightly alkaline	0.09 MO2/L.h	Polyurethane foam disc - VRICR[1]	Continuous	-	(Amin, 1994)
***Corynebacterium glutamicum* ATCC 13022**	-	Sugarcane molasses 175	93	3.83	0.55	30	7.8	-	Carrageenan gel beads	Batch ICR	144	(Amin and Al-Talhi, 2007)
***Corynebacterium glutamicum* ATCC 13022**	0.16	Sugarcane molasses 100	72.8	29.12	0.6	30	7.8	-	Carrageenan gel beads	Continuous ICR	336	(Amin and Al-Talhi, 2007)
***Brevibacterium lactofermentum* ATCC No.13869**	0.02	Glucose 110	50.1	1.252	0.45	31	7	-	Water medium	Batch	40	(Shinichi *et al.*, 1963)
***Brevibacterium saccharolyticum* ATCC No. 14066**	0.05	Glucose 85	49.6	1.241	0.45	30	7	-	Water medium	Batch	40	(Shinichi *et al.*, 1963)
***Brevibacterium flavum* ATCC No.14067**	0.05	Glucose 110	55.6	1.391	0.5	31	7	-	Water medium	Batch	40	(Shinichi *et al.*, 1963)
***Brevibacterium roseum* ATCC No.13825**	0.05	Glucose 110	52.9	1.324	0.47	31	7	-	Water medium	Batch	40	(Shinichi *et al.*, 1963)

[1] Vertical Rotating Immobilized Cell Reactor

Finally, *Brevibacterium sp.* Tc452 and *Corynebacterium glutamicum* ATCC No. 13022 were selected to be compared more precisely because their production yield was the highest. By comparing the values in the table above, we find that *Corynebacterium glutamicum* ATCC No. 13022[2] is more suitable to be used for the production of glutamic acid. This strain has better performance and productivity than *Brevibacterium sp.* The *C. glutamicum* soil strain (ATCC 13032, original strain from which all the different *C. glutamicum* strains derived) was first discovered in 1957 by Kinishota and collaborators (Kinoshita, Udaka and Shimono, 1957), who studied different strains to determine which one produced glutamic acid most efficiently. At that time, the strain named *Micrococcus glutamicus* was the one with the higher yield. This gram-positive actinobacterium was after called *Corynebacterium glutamicus.* The fermentation time for that study showed the highest accumulation of glutamic acid in 3 days.

I-C.2 Strains for Xylitol production

Yeast from the *Candida* family are known for having high yield in xylitol production. In 1988 Barbosa and their team (Barbosa, M. de Medeiros, *et al.*, 1988) discovered that *Candida tropicalis* and *Candida guilliermondii* were capable of producing around 70 g/L of xylitol from 104 g/L of xylose. *Candida* is a yeast from the group ascomycetes. Like in the case of the *Corynebacterium* strain, a summary table was made to compare specific growth rate, xylitol yield, and productivity for different operation conditions (Table I-4).

[2] It is very likely that the strain "ATCCC 13022" described in the article is a Typing-mistake and that they worked on ATCC 13032. The list of strains in the ATCC and the 13022 corresponds to a BR1N-129.5 [PTA-4304]. Yet in the article they had said *Corynebacterium glutamicum* to produce Glutamic acid.

Table I-4 Summary table of different *Candida* strains and their production conditions

Strain	Specific growth rate (h^{-1})	Substrate (g/L)	Production (g/L)	Productivity (g/L.h)	Yield (g/g)	T (°C)	pH	Aeration (vvm)	Support	Mode	Experimental time (h)	Ref
***C. guilliermondii* FTI 20037**	0.042	xylose ; glucose 30 - 75	55	0.57	0.87	30-35	6	1.25-1.5	Biocomposite-based supports	Batch	144	(Soleimani and Tabil, 2014)
***Candida magnoliae* TISTR 5663**	0.072	xylose ; glucose 21 - 60	44	1.1	0,719-0,727	30	7	1	no support	Repeated fed-batch	94 -272	(Sirisansaneeyakul, Wannawilai and Chisti, 2013)
***C. mogii* ATCC 18364**	0.013	glucose/xylose ratio of 10 % 50 - 60	16.0	0.8	0.7	30	5	-	-	Batch		(Sirisansaneeyakul, Wannawilai and Chisti, 2013)
***C. guilliermondii* FTI 20037**	0.046	xylose ; glucose	20.6	0.44	0.47	30		-	Ca-alginate beads	Batch	48	(Carvalho *et al.*, 2002)
***C. guilliermondii* NCR 5578**	-	xylose ; glucose 14.1 – 83	3.10	0.02	0.46	30	6-7.5	-	Ca-alginate beads	Batch	164	(Converti, Perego and Domínguez, 1999)
***C. tropicalis* DSM 7524**	0.412	D-xylose 100	90-95	1.35	0.77-0.80	30-37	2.5	0.012	Porous glass	Batch + continuous	-	(da Silva and Afschar, 1994)
***C. guilliermondii* FTI 20037**	-	Xylose 0.8 - 70	52	0.52	0.8	30	5	0.011	Porous glass particles	Semi-continuous	96	(Silva *et al.*, 2003)
***C. guilliermondii* FTI 20037**	0.38	Xylose 50 - 170	21	0.44	0.54	30	-	-	Ca-alginate beads	Batch	48	(Carvalho *et al.*, 2003)
***C. guilliermondii* FTI 20037**	0.109	Xylose 45	23.5	0.39	0.58	30	-	1.063	Ca-alginate beads	Batch	60	(Santos *et al.*, 2005)
***C. guilliermondii* FTI 20037**	0.045	Xylose 45	15	0.21	0.46	30	-	0.062	Ca-alginate beads	Batch	72	(Santos *et al.*, 2008)
***C. guilliermondii* FTI 20037**	0.082	Xylose 30	39.7	0.53	0.77	30	5.5	1.04	Polyvinyl alcohol (PVA) hydrogel	Repeated Batch	120 (1 cycle)	(Cunha *et al.*, 2007).

Among these, most of the authors have focused their research on studying the yeasts in different supports. Two microorganisms appear to be the most interesting for the production of xylitol. *Candida guilliermondii* FTI 20037 and *Candida magnoliae* TISTR 5663. Those strains had the best values achieved among all strains and satisfactory productivity. Guo and collaborators (Guo *et al.*, 2006) made comparative research between strains producing xylitol from xylose. They compared different *Candida* strains. At the end of their study, *C. guilliermondii* had the second maximal production, although it was the one with the least byproducts. Because of the least byproducts, *Candida guilliermondii* is the strain selected for this work.

I-D. Knowledge of selected strains

When strains were chosen, the metabolism and nutritional needs need to be understood to be used at their maximum potential in the production stage. *Corynebacterium glutamicum* is known for being a bacteria with important industrial applications by producing different metabolites when it is cultivated in specific conditions.

I-D.1 Corynebacterium glutamicum ATCC13032

Also known as *Micrococcus glutamicum*, *Corynebacterium glutamicum* was first reported by Shukuo Kinoshita and his team in 1957 (Kinoshita, Udaka and Shimono, 1957). The main objective of their research was to compare different microorganisms to determine the one with higher glutamic acid production capacity. The group isolated several bacteria and compared them with strains in the fungi kingdom, obtaining the highest yield (0.2 g/g) with the strain now known as *Corynebacterium glutamicum wt*. In 1961, a patent (Patent- 3002889, 1961) was filed for glutamic acid production by several strains derivated from *Mircococus glutamicum wt*, which be later renamed as *Corynebacterium glutamicum ATCC13032*.

The C*orynebacterium* genera belong to the family of actinomycetes. The bacteria is non-sporulating, auxotrophic for biotin, gram-positive, aerobic, or anaerobic facultative. The culture medium and the cultivation time can directly influence its physical qualities. It is used mainly in amino acid and organic acid production. Some examples of products obtained by its culture are lysine, succinic acid, glutamic acid, or lactic acid (D'Este, Alvarado-Morales and Angelidaki, 2018).

The soil provenance of the strain gives it the ability to grow in a medium with simple mineral salts. As reported by Kalinowski et al., (2003), *Corynebacterium glutamicum* is capable of synthesizing all cell constituents except for D-biotin from only simple precursors. Glucose is the preferred sugar source, but it can metabolize others like sucrose, fructose, xylose, ribose, mannose, and maltose (D'Este, Alvarado-Morales and Angelidaki, 2018).

This soil extracted strain has been studied by several authors principally for glutamic acid production, as can be observed in Table I-5.

Table I-5 Recapitulative of studies with the strain *Corynebacterium glutamicum* ATCC 13032 used to produce glutamic acid. All cultures were realized at 30 °C.

Reference	Fermentation conditions		Glucose (g/L)	Nitrogen (g/L)	pH	Glutamic acid (g/L)
	Culture media	Aeration				
(Schneider, Niermann and Wendisch, 2011)	Brain heart infusion medium (BHI). To trigger l-glutamate production: 500µg/ml ethambutol was added to the minimal medium after 27 h of fermentation.	500 ml Erlenmeyer flasks with 60 ml medium at 120 rpm.	90	Urea 5	-	6
(Follmann *et al.*, 2009)	Minimal medium by Nakayama	pO2>30 % 1 vvm		$NH_4H_2PO_4$ 1	Optimized buffers 6 to 9	
(US-Patent 3002889, 1961)	Glucose (100g/L) (NH4)2SO4 (2 g/L) K2HPO4 (1 g/L) MgSO4*7H2O (0.25 g/L) FeSO4*7H2O (0.01 g/L) MnSO4*4H2O (0.01 g/L)	220 rpm. 60 mL of media in 500 mL Flask	100	$(NH_4)_2SO_4$ 2	Manual with 10 % Urea	38.2
(Kinoshita, Udaka and Shimono, 1957)	Meat extract (2 g/L), peptone (2 g/L) KH2PO4 (0.5 g/L) MgSO4.7H2O (0.2 g/L)	-	50	Urea 8	Manually with diluted ammonia to 7.2	21
(Georgi, Rittmann and Wendisch, 2005)	300 mg/ml ethambutol was added to the minimal medium to trigger GA production	60ml in 500mL flask. 120 rpm agitation	40	Urea 5	-	4.85
(Vijayalakshmi and Sarvamangala, 2011)	Biotin1.0 µ g/L and penicillin 1.0 U/ml, 10g of M calabura fruit	120 rpm. 100ml of media in a 250 ml Flask	17[3]	Urea 2	Manual 1N HCl or 1N NaOH to pH 7	15.1

a) Metabolism

Corynebacterium glutamicum has specific needs to excrete glutamic acid like: biotin limitation to thin the cell wall, temperature changes, the addition of fatty acid surfactants or antibiotics (Yamashita *et al.*, 2013). Biotin has been proven as a key compound in the controlled fermentation of glutamic acid. Maximal production is achieved with a limited supply of biotin in the medium. A lack of biotin decreases the activity of the fatty acid synthases. This decrease subsequently alters the physiological properties of the membrane, which becomes more permeable to glutamate. That's why when the biotin concentration is not optimal for maximum growth, maximum accumulation of GA is obtained (Tryfona and Bustard, 2004; Kumar and Sharma, 2008). Niaz and collaborators (Niaz et al., 2009) studied different parameters to determine the influence of nitrogen and carbon sources. They observed a maximum excretion of glutamic acid at 10 mg/L biotin. In their work, glutamic acid production started after 24 hours

[3] Total 17g of sugars for 100g of fruit

of incubation and reached a maximal concentration at 72 hours (4.2 g/L). Yet, at 96 hours of fermentation, the glutamic acid concentration decreased at half of maximum due probably to glutamic acid reabsorption. Wen and collaborators (Wen *et al.*, 2018) found in their research that Corns stover, rice straw, and sugarcane bagasse collected in China had a high content of biotin and other vitamin B compounds. So, they implemented glutamic acid excretion by penicillin induction to be able to use these lignocellulosic materials to produce glutamic acid. Another glutamic acid excretion technique reported is the use of microorganisms auxotrophic for glycerol or oleate, that allowed the bacteria to produce large amounts of glutamate without biotin limitation.

Moreover, as can be observed in Figure I-6, *Corynebacterium glutamicum* has three main pathways: Embden-Meyerhof-Parnas (glycolysis), pentose phosphate pathway, and the Krebs cycle (tricarboxylic acid cycle) (D'Este, Alvarado-Morales and Angelidaki, 2018). Because of these multi pathways, different products apart from glutamic acid can be obtained from *Corynebacterium glutamicum*. Some amino acids (the L forms of lysine, valine, isoleucine, threonine, aspartic acid, and alanine) and organic acids (lactic and succinic acid) are obtained as byproducts (Hermann, 2003; Amin and Al-Talhi, 2007).

Apart from those three main metabolic pathways, this bacteria has the enzyme "pyruvate carboxylase", which produces oxaloacetate in the presence of CO_2 vía what is called the "Anaplerotic pathway" (Papers *et al.*, 2002). Because bicarbonate is a co-substrate for anaplerotic enzymes, its addition increases the glucose consumption rate, and the organic acid production rates in *C. glutamicum* (Okino, Inui and Yukawa, 2005). In general, the anaplerotic pathways exist as a shortcut to regenerate the used molecules in the Krebs cycle.

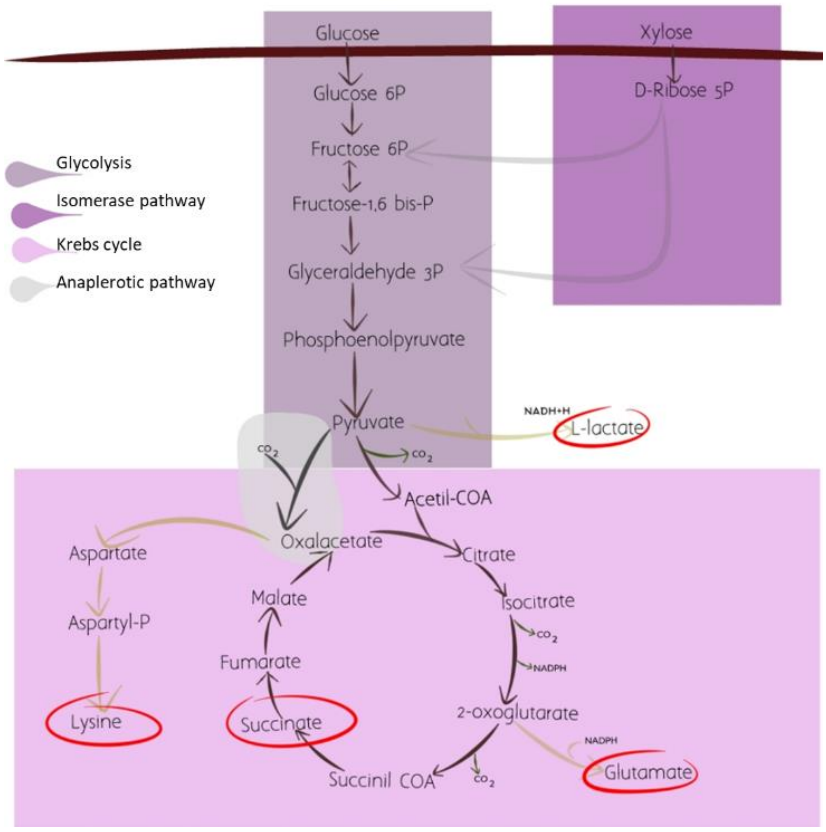


Figure I-6 Biological pathways for *Corynebacterium glutamicum*.

Metabolites obtained with *Corynebacterium glutamicum* are circled in red in Figure I-6 to show the parts of the chain where they are founded. The light-gray arrows going from the isomerase pathway through the glycolysis represents the Pentose phosphate pathway. Several strains of *Corynebacterium glutamicum* can produce different metabolites. The soil extracted strain (ATCC 13032) is known to produce glutamic acid in micro-aeration conditions. These micro aeration conditions haven't been clearly defined by the authors. The only reference is the one in which Follmann and collaborators (See Table I-7) specified oxygenation, dissolved oxygen being always superior at 30 % of saturation. However, several authors agree (Okino *et al.*, 2008; Wieschalka *et al.*, 2013) that if aeration is lower than needed for glutamic acid production, then lactic acid and small amounts of succinic acid are obtained; excess oxygen caused growth inhibition and production of a-ketoglutarate (Amin, 1994).

Strains derivated or genetically modified from 13032

In 1989, Hirao and collaborators (Hirao *et al.*, 1989) obtained a mutant of *Corynebacterium glutamicum.* They obtained a strain capable of producing lysine at a high lysine concentration with a continuous culture. The maximal productivity obtained was 105 g/L.h.

Micro-aerobic conditions in *Corynebacterium glutamicum* have a direct and strong influence on organic acid production. Kaboré and collaborators (Kaboré *et al.*, 2017) tested different micro-aeration conditions for succinic and acetic acid production on a genetically modified strain of *C. glutamicum*. In their modified strains, they defined the conditions in respect of the percentage of dissolved oxygen available. The percentage had to be under 10 % but not equal to zero. They found out that the transition from aerobic to micro-aerobic conditions augmented seven times succinic acid production compared to their control conditions (in the lysine producing and the modified strains). These micro aeration conditions were valid for all different strains derivated from *Corynebacterium glutamicum* ATCC13032, where the nature of the metabolite and the concentration depend on the oxygen transfer during production.

As stated, the soil extracted *Corynebacterium* has been studied over the years since its discovery in the early 1960s. Strains mutations of the original have been created to obtain producers of specific molecules throughout the years. Because of its metabolic complexity coupled with its low nutritional requirements for production, different genetic modifications have been carried out to optimize the production of a single metabolite or to deviate the balance in the Krebs cycle to pass through different metabolic pathways and obtain organic amino acids or organic acids. All these genetic modifications have been summarized in different reviews throughout the last years (Hermann, 2003; Becker and Wittmann, 2012; Wieschalka *et al.*, 2013; Chen and Nielsen, 2016; Wendisch *et al.*, 2016).

The strain *C. glutamicum* 2262, a temperature-sensitive strain, has been largely studied by the research team at the Laboratory of agro alimentary bioprocess in Nancy, led by S. Deaulnay. Stansen *et al.*, (2005), worked with the strain ATCC 13032 and the 2262 in their work, the main characteristics between both strains is that wild-type *Corynebacterium* is biotin auxotroph and 2262 produces glutamic acid with a process started with temperature changes. Different aeration values had been tested for this strain. For example, Briki *et al.*, (2020) realized a study in which they compared different kLa values in Erlenmeyer flasks, using a kLa correlation

obtained by Seletzky *et al.*, (2007). The results showed that oxygen plays a different role at different values. From 0.6 to 118 h^{-1} cell concentration augments; between 0.6 and 44 h^{-1} there's an influence in the maximal specific growth rate, at values under 20 h^{-1} glucose is not entirely consumed, and at values superiors from 31 h^{-1} the lactate produced is reabsorbed due to glucose depletion.

Because aeration rate has a different role at different values for the strain *C. glutamicum* 2262, it is probable that, according to the aeration conditions and the amount of biotin in the medium, cell growth, glutamic or lactic acid could be prioritized in *Corynebacterium glutamicum* ATCC13032.

b) Some products obtained from Corynebacterium glutamicum and their industrial interest

Corynebacterium glutamicum ATCC13032 can produce different value-added molecules. In this section, we will talk about the importance of two of their products in the industry and the chemical transformation's options for the obtention of bioproducts.

Glutamic acid

The biosynthesis of GA is an aerobic process requiring oxygen throughout the fermentation.

Glutamic acid (glutamate as an ion) is an α-aminoacid with molecular formula $C_5H_9NO_4$. It is one of the 20 proteinogenic amino acids. It is a crucial molecule in cellular metabolism. Glutamate is the most abundant fast excitatory neurotransmitter in the mammalian nervous system. Also, it is the most common stimulating neurotransmitter in the central nervous system as well as may impose protective effects on the heart muscles in heart patients. (Niaz *et al.*, 2009).

Physically is a white powder, with a molecular weight of 147.13 g/mol and a sublimation point of 175 °C. It is moderately soluble in water and almost insoluble in ethanol or ether. It has three dissociation constants, pKa's at 1.88, 4.3, and 9.47. Its isoelectric point is 3.2, when in a saturated solution, the pH value is 3 to 3.5. Its 2D structure is represented in Figure I-7.

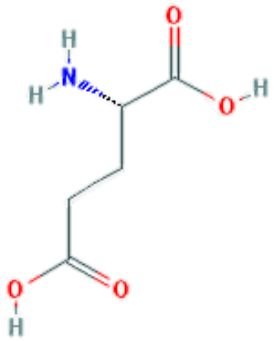


Figure I-7 Glutamic acid's structure

In general, amino acids are used as food additives, food supplements, cosmetic, pharmaceutical, and agricultural chemicals. The main glutamic acid industrial application is in

the food industry as a flavor enhancer [in the form of monosodium L-glutamate (MSG)]. Its annual production (in 2013) was 2.1 million tons, with a 3-5 % growth rate per year (Yamashita *et al.*, 2013; Kumar, Vikramachakravarthi and Pal, 2014).

Gutamate production started first in 1908 (Boulahya-Brhimouche, 2010). Glutamine was extracted from wheat gluten, hydrolyzed with HCl, and finally purified by crystallization. To avoid HCl released gases, in the 1950s two new processes emerged using chemical synthesis and fermentation.

By chemical synthesis, a racemic product is obtained. They could be separated by specific conditions in which the crystallization rate of each monomer is higher than the crystallization rate from the mix of monomers. The production by fermentation is specific for a selected amino acid. The *Corynebacterium glutamicum* ATCC13032 strain releases the L-form of the amino acid into the culture medium (Sano, 2009).

Amino acids structure has two functional groups: the carboxylic acid and the amine function. They're capable of being transformed in either of their functional groups. Because of its structure, they have a large variety of reactions to be modified and obtain molecules with interesting properties. They also have some reactions specific to the structure of the alpha-amino acids. One example is presented in Figure I-8.

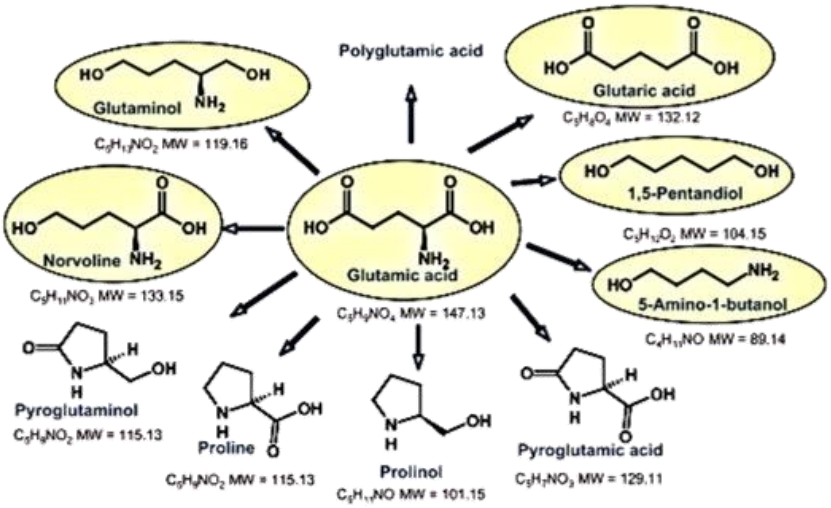


Figure I-8 Glutamic acid and their derivatives (Werpy and Petersen, 2004)

Commercial fermentation process to obtain glutamic acid

Glutamic acid production by fermentation was first developed by Kyowa Hakko Koygo Co Ltd in 1956 (Sano, 2009). Today, most acids are produced by fermentation (over 0.2 M tons of acid per year) in neutral or even slightly basic medium based on the process scheme shown in Figure I-9

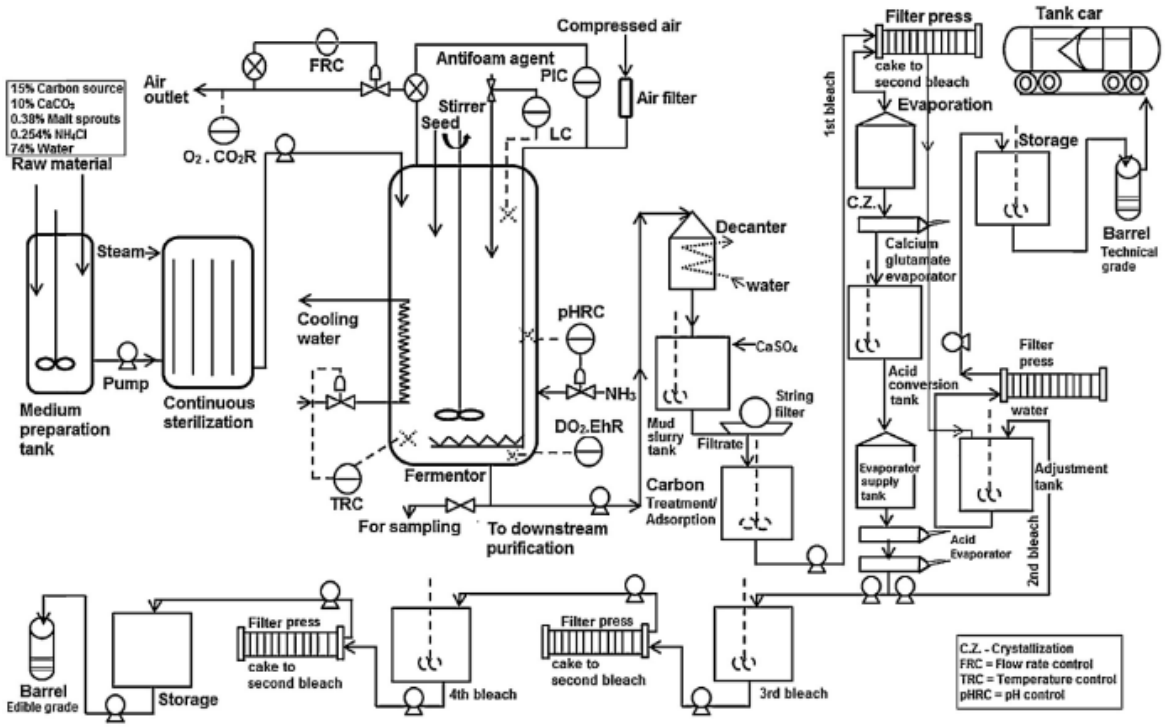


Figure I-9. Conventional scheme of the process for the production of glutamic acid (Kumar et al., 2014)

One of the methods of the industrial output of glutamic acid is the fermentation of sugars with nitrogen nutrients and biotin limitation. After the fermenter output stream is filtered, activated carbon is used to decolorize the calcium glutamate. This salt is then used to produce food-grade acid. Then, the calcium glutamate is concentrated by evaporation at about 70 °C until the concentration of 37 % is reached. The next step is the acidification with concentrated sulfuric acid. The calcium sulfate precipitate is removed by continuous filtration and recycled for reuse. The filtered glutamic acid is then treated with activated charcoal for decolorization and concentrated by evaporation in a stainless-steel tank. The heavy metals present in the acid are removed by ion exchange resins, where other co-produced amino acids are likely to be eliminated at the time.

Crystallization is carried out in a single unit. A double stainless-steel evaporator is used. Liquid-liquid extraction is another method for purifying the glutamic acid obtained using an immiscible solvent. The latter must have a very low water solubility, a high partition coefficient for glutamic acid, and a low partition coefficient for impurities such as residual sugars (Kumar et al., 2014).

Then, the conventional purification process is constituted by a series of unit operations such as precipitation, standard filtration, acidification, adsorption with activated carbon, evaporation, etc. Therefore, it is a rather complicated and energetic process. The process requires a lot of manpower. Another disadvantage of this process is the high demand for harmful chemicals such as sulfuric acid and ammonia. But, liquid-liquid extraction also creates a large amount of wastewater that would lead to environmental pollution due to the difficulty of treating them with end-of-line treatment.

Lactic acid

Lactic acid is a carboxylic acid with a molar mass of 90 g/mol. It's a transparent viscous liquid with a density of 1.2 g/mL. The two reactive functions in its structure (hydroxyl and carboxyl) gives lactic acid a high industrial interest. The chemical reaction and products obtained from lactic acid are synthesized in Table I-6.

Table I-6 Chemicals produced from Lactic acid (Krishna *et al.*, 2018)

Reaction	Chemical produced
Hydrogenation	**Propylene oxide**
Decarboxylation	**Acetaldehyde**
Dehydration	**Acrylic acid**
Reduction	**Propanoic acid**
Condensation	**2,3-pentanedione**
Self-esterification	**Dilactide**

It has multiple applications. In the food industry, it is used to acidify and give flavor, also as a preservative in some preparations. Its fungistatic capacities are put in advance to prevent microorganisms from developing in the products and to add an acidic flavor. The majority of applications with 38 % are in the food industry. In pharmaceutical and cosmetic lactic acid is looked for its safety water retaining properties as moisturizing. These properties made the molecule interesting for the formulations. Self-care industry represents 13 % of applications for this organic acid. The polymers and bioproducts industry correspond to 39 % of the lactic acid market. To be more specific, the L form of lactic acid can be polymerized to polylactic acid. This polymer has a high interest because of its biodegradable qualities and biocompatibility (Komesu *et al.*, 2017; Krishna *et al.*, 2018). The market was valued at USD 2.64 billion in 2018 and is expected to grow at an estimated compound annual growth rate of 18.7 % from 2019 to 2025 (*Lactic Acid Market Size & Share | Global Industry Report, 2019-2025*, no date). Due to its high utility in various industries, the high production of lactic acid at low prices and at the needed purity is of importance to be competitive.

Because of one asymmetrical carbon in its structure (Figure I-10), it can be found in two isomeric forms L and D-Lactic acid. Two different processes are used to obtain Lactic acid: organic synthesis and microbiological transformation of sugars. The nature of the process determines which isomer will be obtained. The racemic mixture is obtained when the chemical production of lactic acid is used.

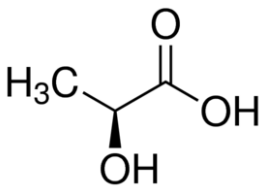


Figure I-10 L-lactic acid's structure

A racemic solution of lactic acid is produced via organic synthesis from petrol derivatives. In the industry, lactic acid is obtained by two reactions. First, the acetaldehyde reacts with

Hydrogen cyanide (HCN) in the presence of chlorhydric acid. The lactonitrile obtained is then hydrolyzed to obtain the racemic mixture of lactic acid (Garrett, 1930).

Synthesis by fermentation can be realized by bacteria, fungi, and yeasts. The bacteria group is divided into four categories: Lactic acid bacteria (LAB), *E. coli*, *Corynebacterium glutamicum*, and *Bacillus* strains (Krishna *et al.*, 2018). In the review, Krishna reveals that fungal fermentation's main advantage is the low nutrient requirements and the possibility of creating high concentrations of biomass used in the food industry. Indeed, the nutriment need for acid lactic bacteria increases by 35 % of the production costs (Singh, Ahmed and Pandey, 2006).

Some main optimization points in lactic acid production by fermentation discussed in the review by Cubas-Cano and collaborators (Cubas-Cano, González-Fernández and Tomás-Pejó, 2017) are the production of byproducts by pentose metabolizing organisms capable of using lignocellulosic raw materials; and the reduced tolerance to lactic acid of microorganisms. Research has been realized to magnified yield and productivity by changing the process, for example, a theoretical study to simulate a feed-batch with a membrane cycle bioreactor (Marques *et al.*, 2017) and immobilization due to cell recycling obtaining a yield of 92 % for glucose conversion (Zhao *et al.*, 2016). Others were concentrated on using the same systems but with genetic modification of the microorganisms (Zhang *et al.*, 2016; Kong *et al.*, 2019). Kong and collaborators (Kong *et al.*, 2019) engineered a *K. marxianus* strain capable of co-metabolize glucose and xylose. Obtaining a concentration of 103 g/L of L lactic acid from 180 g/L of corncob residue. Zhang and collaborators (Zhang *et al.*, 2016) did several metabolic modifications to a *Kluyveromyces marxianus* strain to optimize lactic acid production. At the end of their study, they obtained 103 g/L of L-lactic acid.

I-D.2 Candida guilliermondii FTI20037

Candida FTI 20037 is a strain that was first described by Barbosa and collaborators in 1988 (Barbosa, M. de Medeiros, *et al.*, 1988). In this study, various yeasts capable of transforming xylose into xylitol were compared. This strain initially came from the "Fundacao de Tecnologia Industrial" in Sao Paolo, Brazil.

Since its first apparition, the yeast has been widely studied by different authors to optimize xylitol production. Barbosa and collaborators (Barbosa, M. B. de Medeiros, *et al.*, 1988), found that ammonium is a better nitrogen source for xylitol production than urea for this yeast. Silva and Roberto (Silva and Roberto, 2001) have observed that initial xylose concentration and inoculum concentration have significant effects on xylitol production yield and productivity. They found that with a concentration of initial xylose at 82 g/L and 3 g/L of initial concentration in cells, maximal concentration of xylitol was produced (52 g/L with a yield of 0.65g/g).

a) Metabolism

Candida guilliermondii's metabolism to convert D-xylose into xylitol is linked to the concentration of NADPH in the cell. The increased concentration of the latter causes an increase in the production of xylitol.

The xylitol from xylose bioprocess is based on the ability of pentose-assimilating yeasts to reduce xylose to xylitol as the first step of xylose metabolism. It requires two key enzymes: xylose reductase, which depends on NADH, and xylitol dehydrogenase, which depends on $NADP^+$. The accumulation of xylitol is caused by an $NADH/NAD^+$ imbalance. This imbalance is a result of a limited O_2 supply. This prevents the oxidation of xylitol from xylose. This pathway is related to energy generation for cell growth and redox balance (Kim, Ryu and Seo, 1999)(Hernandez-Perez, de Arruda and Felipe, 2016)

A schema in Figure I-11 presents the part of *Candida guilliermondii* FTI 20037's metabolism to produce xylitol with xylose and ethanol by glucose fermentation. *Candida'*s ability to ferment hexoses was studied by Wen (Wen *et al.*, 2016). They report that this yeast consumes high glucose quantities to obtain high ethanol concentrations by metabolizing from 15 to 30 % of hexoses in an ethanol range from 6 to 9 %. The yeast needs glucose to develop, as stated before, but hexoses presence in the media could lead to ethanol production.

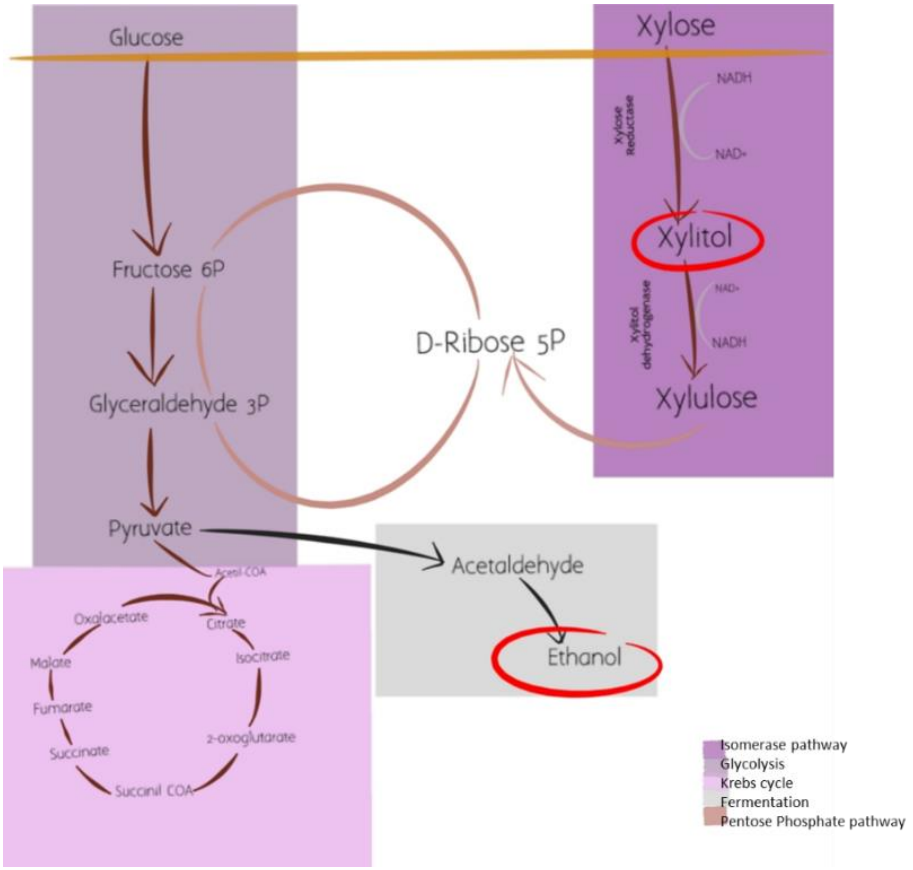


Figure I-11 Biological pathway to produce xylitol in *Candida guilliermondii* FTI20037.

One of the major studies about optimization of xylitol production by *Candida guilliermondii* FTI 20037 was realized by Silva and collaborators in 1997 (Silva, Quesada-Chanto and Vitolo, 1997). They studied the effect of inoculum age, the ratio between glucose and xylose, yeast extract concentration, and the concentration of several molecules obtained after hydrolysis that could have been presented in the raw material. They found that the inoculum age didn't affect productivity after 26 h of culture. For inoculum around 15 h of culture, cells weren't metabolically fully active. Yeast extract (YE) adequate concentration was 1 g/L. Superior YE concentrations switched the metabolism into cell development and no into

xylitol production. Glucose presence had two different effects on xylitol production, depending on the glucose:xylose ratio. At ratios superiors to 2:5 glucose inhibited the production. It's essential to precise that a small concentration of glucose helped to cell development before xylitol production from xylose.

In a sugar mix media, Gurgel (Gurgel *et al.*, 1998) confirmed that glucose was a more effective carbon source for growth than xylose for *C. guilliermondii*. In its study, the xylose consumption started after 12 hours of fermentation.

Da Silva and collaborators (Da Silva *et al.*, 2007) improved xylose metabolism on *C. guilliermondii* cultivated in hemicellulosic sugarcane bagasse hydrolysate. They obtained a maximum xylitol production when glucose:xylose ratio was at 1:2.5, the same ratio obtained 20 years before by Silva and collaborators (Silva, Quesada-Chanto and Vitolo, 1997). Indeed, cell growth was over 18 % higher than the control medium. In their study, it was observed that xylitol concentration decreased after 72 hours of fermentation. It indicated that *C. guilliermondii* can assimilate the polyol. Results reported also by Felipe and collaborators (Felipe *et al.*, 1997) and Walther, Hensirisak and Agblevor, (2001).

Some authors (Barbosa, M. de Medeiros, *et al.*, 1988; Lee *et al.*, 1988; Nolleau, Preziosi-Belloy and Navarro, 1995; Mussatto, Silva and Roberto, 2006) have studied aeration effects on xylitol production by *Candida guilliermondii* FTI20037. Barbosa and collaborators worked in flasks changing the volume of the media. They obtained a maximal yield production of 0.6 mol/mol with 300 mL of culture media in a 500 mL Erlenmeyer flask. Nolleau and collaborators studied the oxygen needs for *Candida guilliermondii* and C*andida parapsilosis.* They concluded that the maximum oxygen transfer rate needed for both *Candida* yeast was different: 2.2 mmoles O_2/L.h for *C. guilliermondii* versus 0.4 mmoles O_2/L.h for *C. parapsilosis*. These values show that the order of magnitude is not the same for the different types of *Candida* yeast. The authors agree with the fact that the aeration in the fermentation affects the pathways involved in the metabolism of xylose; high aerations lead to the development of more cells, while low aeration leads to xylitol production.

b) Products obtained from Candida guilliermondii FTI 20037

As observed in Figure I-11, the two main products obtained by *Candida guilliermondii* are ethanol and xylitol. In a media with glucose, in anaerobic or oxygen-limited conditions, glucose is transformed into growth, energy, or ethanol. When xylose is the main sugar, and in microaerobic conditions, xylitol will be produced. As stated before in this text, glucose:xylose ratio had a direct impact on xylitol production.

For this work, we will center only in xylitol production by the yeast, because the objective is to produce value-added molecules different from ethanol. After all, its production has been largely studied.

Xylitol

Xylitol is one of the 12 recognized chemical building blocks. It is a polyalcohol having five carbons found in the fibers of fruits and vegetables (Figure I-12). It has a great interest in

food (chewing gums, tablets, diet food) and cosmetic (toothpaste) industries, due to its sweetening and anti-cariogenic properties. Besides, it does not need insulin to be metabolized in the body, which makes it very interesting for diabetics (Silva, Quesada-Chanto and Vitolo, 1997; Rodrigues *et al.*, 2003). The xylitol market is increasing, it is estimated to be $340 million per year, and priced at $4–5/kg (Prakasham, Rao and Hobbs, 2009).

Its condensed formula is $C_5H_{12}O_5$. It has a molar mass of 152.15 g/mol and a boiling point of 345 °C. It is highly soluble in water (642 g/L) and almost not soluble in ethanol (only 12 g/L).

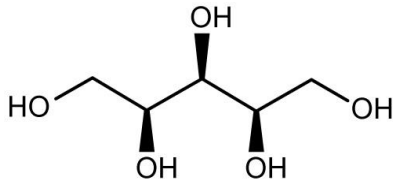


Figure I-12 Xylitol's structure

Xylitol was first synthesized in 1891 by Herman Emil Fischer from beech chips, using acid treatment. Xylitol can be produced by chemical synthesis with the hydrogenation of xylose by nickel plaques, or by fermentation. The chemical process has a yield of only 60 %. Still, the process becomes expensive for the transformation stage and energy-consuming for the recovery and purification stages (Prakasham, Rao and Hobbs, 2009).

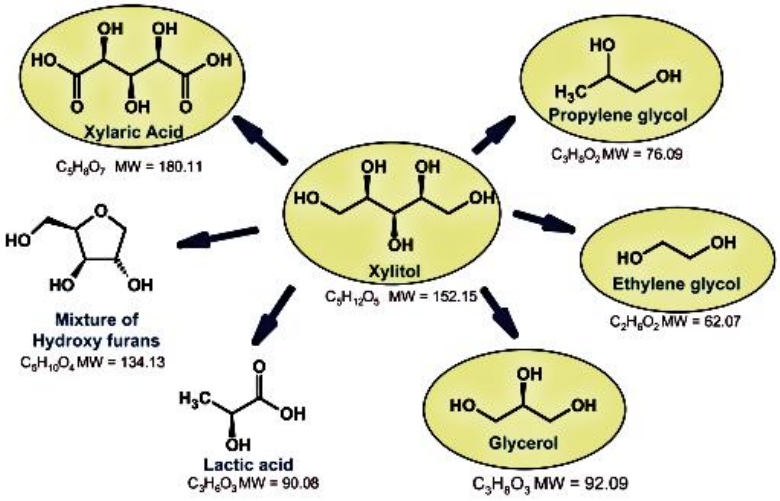


Figure I-13 Derivatives of xylitol (Werpy and Petersen, 2004)

Nowadays, xylitol is mainly produced on an industrial scale by the reduction of pure xylose in the presence of a nickel catalyst at elevated temperature and pressure. The actual chemical process is represented in figure I-12. The yield can be up to 60 %, but this requires a series of purification, which is energy-consuming. This is why there is an interest in the two biotechnological approaches: the fermentation process and the enzymatic pathway, which can potentially replace chemical production (Nigam and Singh, 1995; Parajó, Domínguez and Domínguez, 1998; Elseviers and Roper, 2002).

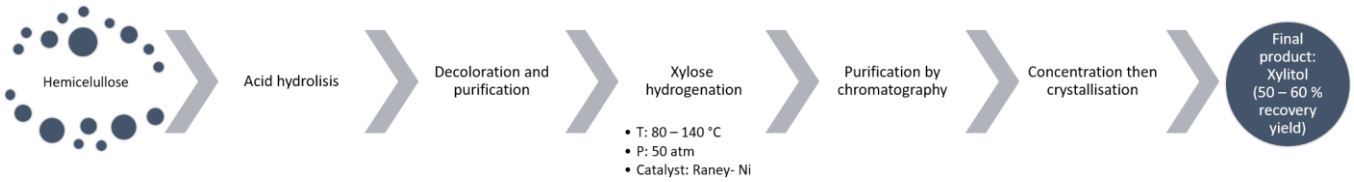


Figure I-14 Schema of xylitol's chemical production by a catalyst

Xylitol can also be produced via fermentation of pure xylose or hemicellulose, which hydrolysates via bacteria, fungi, or yeast. Among these three kinds of microorganisms, yeast is considered the best one for xylitol production. It has been extensively studied in recent years. Nevertheless, the production of xylitol by fermentation is often limited by various factors, such as precise control of cultural conditions, the need for expensive nutrients, high water consumption, and type of process (batch, fed-batch or continuous). The implementation of a fermentation process at the industrial scale requires many adaptations. It must be associated with preparatory steps such as sterilization and the development of inoculum (Gong, Chen and Tsao, 1981; Nigam and Singh, 1995; Sampaio *et al.*, 2008).

By comparing with the chemical production of xylitol, fermentation brings economic interest because it does not require as many hydrolysate purification steps. Thus, it could be a substitute for chemical processes provided that the operating parameters and the kinetics of microorganisms are optimized.

I-E. Strategies for sugar mix consumption

At the end of the biomass pre-treatment (hydrolysis step), a complex mixture is obtained containing sugars and potential inhibitors for microorganisms. Leaving aside the inhibitors found, one of the greatest challenges to face is the inability of the microorganism to convert the substrate completely because the microorganism is not able to metabolize both sugars, or because carbon catabolite repression which limits sugar consumption when the microorganism is in a sugar mix. This incapacity affects the economic use of lignocellulose as raw material. Which represents more than 80 % of the production cost in biorefineries (Vlysidis *et al.*, 2011).

Currently, the genetic modification of the selected microorganism is used to consume C5 and C6 sugars. Apart from this technique, in industry and nature, a collaboration between microorganisms called a consortium may be a strategy to consume both sugars. In industry, several types of consortium were found, which could also be called co-cultures. The main ones are i) a mixture of microorganisms to maximize the performance and productivity of one molecule (Bader *et al.*, 2010; Jiang *et al.*, 2017) and II) a mixture of microorganisms to co-produce two different molecules

This leads to 3 possible strategies that could be implemented to transform lignocellulosic hydrolysates by fermentation:

1. Some researchers chose to modify genetically a strain to metabolize different sugars. An example is given by Gonçalves and collaborators (Gonçalves *et al.*, 2014). In their work, they genetically modified a *Saccharomyces cerevisiae* strain overexpressing the production of the xylose reductase and dehydrogenase enzymes. They compared the effect of the initial sugar (glucose or xylose or a mix) and concluded that when they modify the strain, it is necessary to

consider the composition of the biomass material to have maximal ethanol productivity. Indeed, whereas the HXT1 transporter seemed more suitable for hydrolysates containing xylose/glucose mix, the HXT7 permease would be a better choice for a media rich in xylose, but with different sugars in the mixture.

Wen-Chao Li and collaborators (Li *et al.*, 2019) used an adaptive evolution to maximize ethanol production and resistance to inhibitors obtained on hemicellulose hydrolysates for an already modified *S. cerevisiae*. In the end, the strain showed an excellent performance in real lignocellulosic hydrolysates. They attained an augmentation by 20 % in bioethanol yield with this strategy.

Involving an energy-producing bacteria, Shuang Li, and collaborators (Li *et al.*, 2010) modified the strain *Thermoanaerobacterium aostearoense* to optimize H_2 production. They modified the L-lactate dehydrogenase gene. In the end, the engineered bacteria was able to double H_2 production yield on a medium with glucose as the only substrate. When they tested the strain's ability to produce H_2 from a glucose/xylose mix, they obtained a yield of 2.28 mol H_2/mol sugar.

In the US patent-9598689 B2 (Argyros *et al.*, 2017) the authors claim to have genetically modified a host strain and change it to metabolize arabinose into ethanol or obtain, by genetic recombination, a strain capable of metabolizing xylose into xylitol. The main objective of the patent is to modify the microorganism to ferment a different sugar than in nature, giving the desired product and besides, to obtain microorganisms capable of metabolizing arabinose and xylose.

2. The second strategy is to find a couple of microorganisms (genetically modified or not) to maximize one molecule production. Several authors (Bader *et al.*, 2010; Chen, 2011; Jiang *et al.*, 2017) have realized reviews where they list different couples of microorganisms that have been proposed to metabolize glucose and xylose.

From the couples listed on the reviews, the main interest is the production of bioethanol and energy. Indeed, more than 35 couples have been proposed to maximize ethanol production. The highest ethanol yield reported was obtained by Fu and collaborators (Fu *et al.*, 2009) by a mix of *Zymomonas mobilis* and *Pichia stipitis*; a yield of 0.5 g/g (98 % of the theoretical yield) and a productivity of 1.27 g/L.h were obtained (Chen, 2011).

Delgenes and its team (Delgenes *et al.*, 1998) studied two different mixes of microorganisms to maximize the production of bioethanol and later the same couple but for xylitol production. First, they studied the mix *Saccharomyces cerevisiae* and *Pichia stipitis* to produce bioethanol. They used a bioreactor adapted with a microfiltration membrane to separate the cells. In the end, they obtained a yield of 0.46 g/g and a substrate conversion yield of 94 %. They concluded in this first part that the selection of the mix is important because it's needed to have a high ethanol tolerant strain to continue with the conversion. For xylitol production, they studied the mix of *Candida guilliermondii* and *Lactobacillus reuteri.* They had a mix of glucose, xylose, and arabinose. After 100 h of fermentation, they obtained a yield of 0.54 $g_{xylitol}/g_{xylose}$. The acid was produced at high purity (94 %), which would help the subsequent recovery by crystal purification.

3. A third strategy is to use different microorganisms to produce different products. This method has been studied especially for xylitol and bioethanol production. To the best of our knowledge, the first paper that reports this strategy is the research made by (Arrizon *et al.*, 2012). They used two acetic acid –furfural tolerant strains to realize a sequential fermentation process. Their main purpose was to simultaneously produce bioethanol and xylitol from three industrial wastes: coffee husks, sugarcane bagasse, and *Agave tequilana* bagasse. They obtained in sequential co-fermentation yields of 0.22 $mol_{bioethanol}/mol_{glucose}$ and 0.4 $mol_{xylitol}/mol_{xylose}$.

Saccharomyces cerevisiae and *Candida tropicalis* were used by (Latif and Rajoka, 2001) for simultaneously consuming glucose and xylose from hydrolysates to produce two different molecules. They tested single and co-culture to study the production of ethanol and xylitol. The co-culture was not successful for ethanol production because co-culture was between 15-20 % less effective than when single strains were used. However, xylitol production wasn't affected by the yeast co-culture.

Zahed and collaborators (Zahed *et al.*, 2016) used a mix of *Saccharomyces cerevisiae* and *Candida tropicalis* to obtain yields of 0.44 g/g for ethanol and 0.55 g/g in xylitol. Volumetric productivity was enhanced by 15 % and 10 % in a continuous co-culture.

As Chen, (2011) said: co-culture fermentation provides the opportunity to achieve the simultaneous conversion of a sugar mixture, maximize substrate consumption rate, and increase yield and production rate, leading to higher production and a reduction in process costs. The co-culture as strategy has various advantages. The most important is the maximal profit of the feedstock, an increment of the production yield, with a non-negligible market advantage by diversifying the products obtained.

In our study, we will concentrate mainly in the third strategy in which two value-added molecules different from bioethanol and without non genetically modified strains. To achieve this, three co-culture process propositions will be defined for the continuation of our work:

1) Mixed fermentation: In this strategy, both microorganisms will be cultivated at the same time from the beginning of the fermentation
2) Sequential fermentation: This strategy consists of two parts. During the first one, one microorganism will consume one of the sugars to produce one of the molecules, and the second one will start with the inoculation of the second in the culture media. Fermentation conditions can be the same as in the first part or be changed.
3) Sequential fermentation with a filtration stage: This strategy also consists of two parts. The main difference is that between the first and second part, there is an intermediate separation stage, in which the first microorganism will be removed from the fermentation media.

I-F. Coupling the microorganisms

When choosing strains coupling, the two microorganisms should be compatible both technically, in their culture conditions, and biologically. A compromise between the optimal culture conditions values for each strain not only optimizes the productivity of both products but also facilitates bioreactor design minimizing the cost of operations.

I-F.1 pH

Microorganisms' growth is sensitive to change in pH. They can develop if and only if the pH of the medium is in its optimal range. As seen in Table I-7, the optimal pH for glutamic acid production is 7; nevertheless, as reported by Tavakkoli and Esfahani (Tavakkoli and Hamidi-Esfahani, 2012) pH changes along with GA production. At the end of their process, pH decreased from 7 to 5.8. If we want to maintain pH, a neutralizing agent such as calcium carbonate must also be added to the culture medium at the start of fermentation (Shah, Hameed and Khan, 2002).

In the case of *Candida guilliermondii*, the optimal production pH value is 5.5. Other pH values were tested by Rodrigues and collaborators (Rodrigues *et al.*, 2003). They compared 3.5 and 7.5 and concluded that at a high pH value, xylitol production was disadvantaged over cell production.

Accordingly, the production of the two products should be carried out in the same reactor to ensure optimal productivity. Taking a value between 5 and 7 to maintain homeostasis in *C. glutamicum* is recommended (Follmann *et al.*, 2009). From the information found in the bibliography, we decided to choose pH 7. This value was chosen because it is the pH of maximal production for glutamic acid by *Corynebacterium glutamicum,* and with the information on the work of Rodrigues and collaborators, it's known that *Candida guilliermondii* still produces xylitol at this value.

I-F.2 Culture media composition

For *C. glutamicum,* biotin is a key compound in the controlled fermentation of glutamic acid. Lack of biotin decreases the activity of the fatty acid synthases and makes the cell wall more permeable to glutamate. Niaz et al. (2009) obtained their maximal yield at 72 h and at 96 h the amino acid was reabsorbed by the cell. So, the production yield could decrease if the culture time is longer than necessary.

Candida guilliermondii can produce xylitol in a media with a mix of glucose and xylose. Glucose is used for growth and xylose to product xylitol but only in a xylose favorable ratio in the mix. Da Silva and collaborators (Da Silva *et al.*, 2007) improved xylose metabolism of *C. guilliermondii* cultivated in hemicellulosic sugarcane bagasse hydrolysate. They obtained a maximum xylitol production when glucose:xylose ratio was at 1:2.5. Xylitol can be reabsorbed by the yeast after 72h of culture (Felipe *et al.*, 1997; Walther, Hensirisak and Agblevor, 2001).

The molecules re-assimilation by the strains has to be taken into account to determine the fermentation times, as reported by Niaz in the case of *C. glutamicum* and Silva, Walthers, and Felipe for *Candida.* The assimilation test by both microorganisms has to be realized to verify fermentation times and compatibility between the strains.

In the case of the bacteria, among the literature, urea and ammonium sulfate are the main nitrogen sources used for glutamic acid production by *C. glutamicum* ATCC 13032 (Kinoshita, Tanaka and Akita, 1961; Delaunay *et al.*, 1999a; Sekine *et al.*, 2001; Amin and Al-Talhi, 2007; Kahina, 2012; Kumar *et al.*, 2013; Shyamkumar *et al.*, 2014; Kaboré *et al.*, 2017; Briki *et al.*,

2020). None of the existing papers compare the effect of this nutriment on the strain we will use. The only reference in nitrogen source comparison in a *Corynebacterium* strain are Niaz and collaborators (Niaz *et al.*, 2009). They worked on *C. glutamicum* NIAB BNS-14 ammonium sulfate was in the comparison, urea, however, wasn't chosen as one of the sources in the work.

Silva and collaborators (Silva *et al.*, 1994) compared the effect of different nitrogen sources in xylitol production by *C. guilliermondii*. They compared NH_4Cl, $(NH_4)_2SO_4$, and urea. As a result, they obtained yields around 0.7 mol/mol. Comparing productivities (0.54 g/L.h, 0.49 g/L.h, and 0.46 g/L.h respectively), they concluded that inorganic nitrogen is more suitable for xylitol production than organic nitrogen, no significant difference was observed between the anions used in the inorganic nitrogen. As a remark, they also found that yeast extract presence in the media had a significant impact on the production (no xylitol was obtained without yeast extract in the media). They suggest that the addition of vitamins with this product would be at the origin of these results.

The research wasn't conclusive in the bibliographical analysis, that's why a further selection will be realized in the experimental part.

I-F.3 Temperature

Given that the selected microorganisms are both mesophilic, production is maximized at a temperature between 20 and 40 °C. For both strains, the optimal production temperature reported is 30 °C; this parameter was kept as no compromise value has to be found.

I-F.4 Oxygen consumption

Oxygen supply to the culture medium is an essential element for microbial activities. In *Corynebacterium glutamicum*'s metabolism, oxygen limitation (for example a relative limitation like 30 % as studied by Follmann and collaborators (Follmann *et al.*, 2009)) is needed to produce glutamic acid. However, as stated before in the text, the authors didn't agree on the specific aeration needs for glutamic acid production in the strain ATCC13032. What they all agree on is that if the oxygen transfer in the media is lower than needed, it's possible to have a significant accumulation of lactate, pyruvate, and succinate.

Amin reported (Amin, 1994) that in a vertical rotating immobilized cell reactor for *Corynebacterium glutamicum* when aeration is equal to 90.2 mM O_2/L.h, 50 % of the consumed glucose was converted into byproducts instead of 40 % at 40 mMO_2/L.h aeration.

This suggests that the control of the oxygen transfer rate (OTR) is essential to reach the desired metabolite and have higher production. Products obtained from this strain are: Lysine, glutamic, succinic, acetic, and lactic acid (Okino, Inui and Yukawa, 2005). The main metabolite and the eventual byproducts will vary depending on the oxygen available in the media during fermentation.

In the case of *Candida guilliermondii*, the conversion depends on the amount of the enzymes (NADPH dependent xylose reductase (XR) and $NADP^+$ dependent xylitol

dehydrogenase). Nolleau (Nolleau, Preziosi-Belloy and Navarro, 1995) reported maximal xylitol production at an aeration rate of 2.2 mM O_2/L.h. for *C. guilliermondii* FTI20037. Lee and collaborators (Lee *et al.*, 1988) studied different aerations in Erlenmeyers (50 to 300 in 500 mL Erlenmeyers flasks) and they found that when aeration is lower, xylitol production is higher.

Both strains have respectable production of their specific molecule in micro-aeration conditions. As stated, micro aeration conditions have not been specifically defined by the authors. With the values presented in Table I-7 its adequate to suggest a start aeration value at 0.5 vvm. Oxygen transfer in the media will depend on the viscosity and the fermentation conditions, but 0.5 vvm seems a good value to start because is the media of the values tested for the different authors, for each strain. As stated before, for *Corynebacterium glutamicum* ATCC13032, the product obtained will depend on the oxygen available during fermentation, and for *Candida guilliermondii*, xylitol will be the only product with only xylose as substrate.

Table I-7 Selected strains to produce the chemical building blocks

Production	**Glutamic acid *Corynebacterium glutamicum* (ATCC13032)**	**Ref.**	**Xylitol *Candida Guilliermondii* (FTI 20037)**	**Ref.**
pH	6 - 7	(Follmann *et al.*, 2009)	5.5	(Rodrigues *et al.*, 2003)
Temperature (°C)	30	[4]	30	5
Aeration (vvm)	0.6	(Tavakkoli and Hamidi-Esfahani, 2012)	0.4	(Rodrigues, Silva and Felipe, 1999)

I-F.5 Microbial interactions

When trying to perform a co-culture, it is not only necessary to set fermentation conditions with which it is possible to increase the production of the metabolites of interest. It is equally important to understand the interaction that takes place between the two microorganisms.

Different interactions between organisms can be identified in nature. Interactions between species can be described as positive (mutualism, commensalism, and protocooperation), neutral and negative, like ammensalism (Figure I-15).

Amensalism is the interaction between two species in which the impact of one species on the other is negative, but where there is no noticeable impact of the second species on the first (Kitching and Harmsen, 2008). There are two types of ammensalism, competition, and antibiosis (The Editors of Encyclopaedia Britannica, 2010). In competition, one organism limits or deprives another of its shelter or food. In the case of antibiosis, one organism secret a chemical that kills the second, while the first one is unharmed.

[4] All the authors have worked at this temperature

Mutualism is the interaction where both species get benefited from their interaction. Not so different from the first one, commensalism is the interaction where one of the organisms gets a benefit, but this benefit doesn't affect the second party positively or negatively.

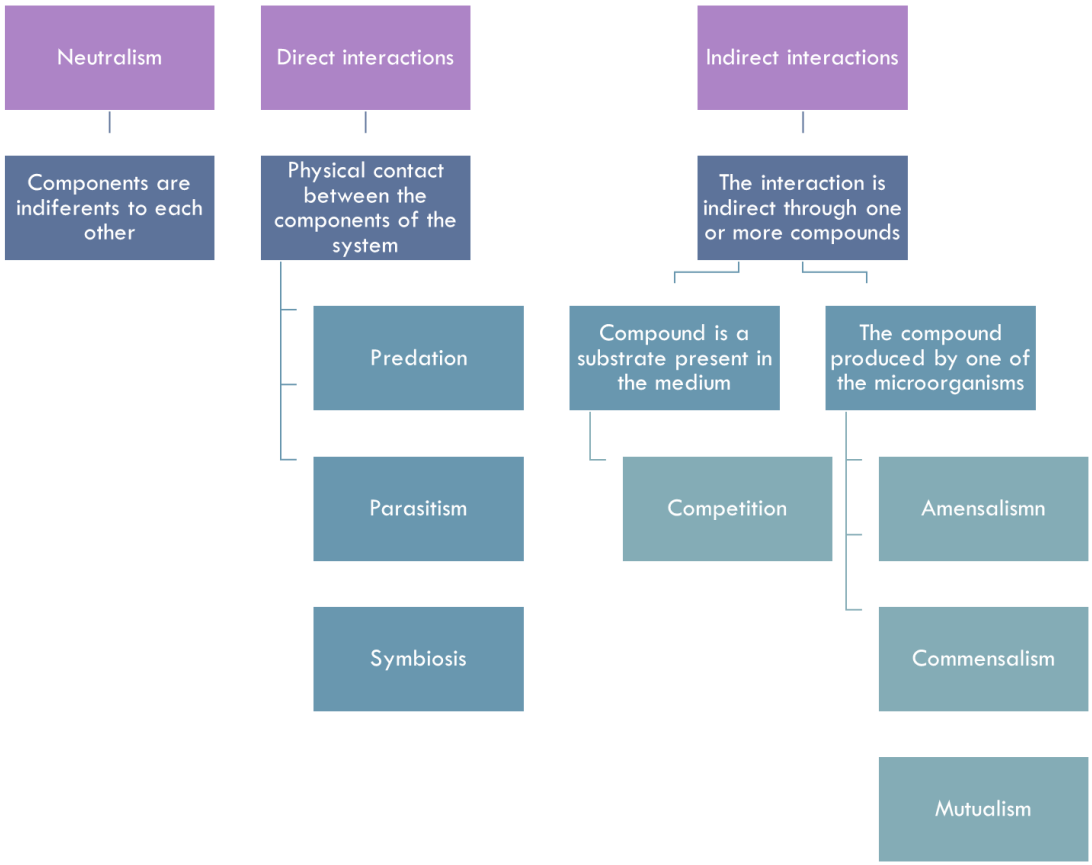


Figure I-15 Schema of the classification of the different microbial interactions (translated from Renouf, (2006))

On the other side, there's parasitism. In this interaction, the parasites benefit from the host, while this one is harmed. Competition is a particular interaction; in this one, the organisms compete for the same resource. Usually, there's a winner and a loser. But, sometimes, the interaction finishes by being harmful to both parties (Taniguchi and Tanaka, 2004; Braga, Dourado and Araújo, 2016; Sperandio and Ferreira Filho, 2019).

It has been reported (Holm Hansen *et al.*, 2001) that under different aeration conditions in the same culture media, one of the microorganisms could overgrowth the second one, this was observed in non-*Saccharomyces* yeasts during mixed culture with *Saccharomyces cerevisiae*.

One mechanism in microbial interactions is cell communication. *Quorum sensing* is a mechanism in which low molecular weight molecules produced during the growth phase will "activate" or inhibit some growth-related genes. Those molecules aren't toxic when they're in the required concentrations. Those molecules will act on the original strain and on the cells of different strains in mixed cultures (Brou, 2018).

Most research realized on microbial interactions is mainly focused on the food industry, in particular for yeast-yeast interactions for wine-making (Renouf, 2006; Brou *et al.*, 2018), Lactic acid bacteria interactions for dairy products (Viljoen, 2001; Narvhus and Gadaga, 2003), and yeast-lactic acid bacteria for bread production (Winters *et al.*, 2019). In winemaking, population dynamics are influenced by yeast interactions, three types are defined: indirect interactions, cell to cell contact, and competition for space (Brou *et al.*, 2018). In bread making, yeast and lactic acid bacteria coexist and the nature of their interactions depends on the metabolic pathway of each microorganism (Winters *et al.*, 2019).

The nature of the uses of the selected strains, to our knowledge, hasn't generated information related to the interactions between *Candida* and *Corynebacterium* species.

Through this bibliographic synthesis, the importance of suggesting new strategies for the development of biorefineries was discussed. High costs in the preparation processes, coming from the mixed sugar separation, and the low transformation yields are preventing the competitiveness of biorefineries in the market. The most recommended strategy was the co-production of various value-added molecules. Along with the production of multiple chemical building blocks, doing this by a co-culture will, at the same time, increase transformation yields and minimize time and costs.

I-G. Strategy and methodology

The objectives of this thesis are divided into two parts: bibliographical and experimental.

From the bibliographic review, two microorganisms with capacities to potentially metabolize lignocellulosic hydrolysates, and with similar fermentation conditions were identified: *Corynebacterium glutamicum* ATCC13032 to produce organic acids from glucose and *Candida guilliermondii* FTI 20037 to produce xylitol from xylose.

The experimental challenges will be:

- to find operating conditions compatible for both microorganisms: medium composition, temperature, aeration, pH.
- to propose a fermentation process minimizing the number of steps or reactors: simultaneous cultures or sequential cultures.

In the following chapters, first, a strategy will be proposed in Erlenmeyer to determine a culture medium for both strains. Then, bioreactor experiments will be carried out to find the optimal conditions. Several co-culture conditions (simultaneous, sequential) will be tested in bioreactors to propose a fermentation strategy for the obtention of two chemical building blocks.

II. Material and methods

II-A.Strains

II-A.1 Corynebacterium glutamicum ATCC13032 (DSM-20300)

Corynebacterium glutamicum DSM 20300 was furnished by Leibniz Institute. The technical fiche of the strain is presented in Table II-1.

Table II-1 *Corynebacterium glutamicum* safety sheet (Leibinz Institute DSMZ- German Collection of Microorganisms and cell cultures)[5]

Name:	***Corynebacterium glutamicum* (Kinoshita et al. 1958) Abe et al. 1967 emend. Nouioui et al. 2018**
Synonym(s):	*Brevibacterium divaricatum* Su and Yamada 1960
	Corynebacterium lilium Lee and Good 1963
DSM No.:	20300, Type strain
Strain designation:	534
Other collection no. or WDCM no.	ATCC 13032, NCIB 10025
Isolated from:	sewage
Country:	country of origin unknown
Date of sampling:	before 22.08.1990
Nagoya Protocol Restrictions:	There are NO known Nagoya Protocol restrictions for this strain.
History:	<- H.G. Schlegel <- K. Komagata <- ATCC <- Kyowa Ferm. Ind. Co., Ltd., 534 (*Micrococcus glutamicus*)
Cultivation conditions:	Medium 535a , 28°C To médium 535 add 5% blood after cooling
	Medium 535 (Trypticase Soy Broth Agar) Trypticase Soy Broth (BBL 11768, Oxoid CM129 or Merck 5459 30g Agar 15g Distilled water 1000ml pH 7.3
Summary and additional information:	<- H.G. Schlegel <- K. Komagata <- ATCC <- Kyowa Ferm. Ind. Co., Ltd., 534 (*Micrococcus glutamicus*). Sewage. Type strain.Taxonomy/description (1300, 1305, 3979). Murein: A31. Produces L-glutamic acid (U.S. Pats. 3,002,889; 3,003,925). (Medium 535a, 28°C).
Risk group	1 (Classification according to German TRBA)

[5] https://www.dsmz.de/catalogues/details/culture/DSM-20300

a) Other denominations for Corynebacterium glutamicum DSM-20300

ATCC 13032, 534T, AJ 1502, AS 1.1886, CCRC 11384, CCT 0542, CCT 2736, CCTM 2336, CCTM La 2336, CCUG 27702, CDBB 70, CECT 4157 , CGMCC 1.1886, CIP 82.08 . CIP 82.8, CIP 82.8T, DSMZ 20300, DSM 20300, HAMBI 2052, HNCMB 132501, strain 534, IAM 12435, IAW 26, IFO 12168, IMET 10482, IMET 10842, IMSNU 10063, IMSNU 21196, JCM 1318, KCTC 1445, KCTC 9097, KY 534, KY 9002, Kyowa Ferm Ind534, Kyowa Ferm. Ind. Co. 534, La 2336, LMD 72.28 , LMG 19741, LMG 3730, NBRC 12168, NCCB 70082, NCCB 72028, NCIB 10025, NCIM 2705, NCIMB 10025, NERE 10026, NRRL B-2784, ptcc1162

II-A.2 Candida guilliermondii FTI 20037 (DSMZ 7524)

Candida guilliermondii FTI20037, was furnished by Leibniz Institute. Technical fiche of the strain is presented on Table II-2.

Table II-2 *Candida guilliermondii* safety sheet
(Leibinz Institute DSMZ- German Collection of Micoorganisms and cell cultures)[6]

Name:	***Candida tropicalis* (Castellani) Berkhout**
DSM No.:	7524
Strain designation:	FTI 20037
Country:	Brazil
Date of sampling:	before 19.03.1993
Nagoya Protocol Restrictions:	There are NO known Nagoya Protocol restrictions for this strain.
History:	<- A. S. Afschar <- S. S. Da Silva (*C. guilliermondii* FTI 20037)
Cultivation conditions:	Medium 186 , 25°C (Universal medium for yeasts) Yeast extract 3g Malt extract 3g Peptone from soybeans 5g Glucose 10g Distilled water 1000ml
Summary and additional information:	<- A. S. Afschar <- S. S. Da Silva (*C. guilliermondii* FTI 20037). Xylitol production from D-xylose (5226). (Medium 186, 25°C).
Risk group:	2 (Classification according to German TRBA)

[6] https://www.dsmz.de/catalogues/details/culture/DSM7524.html?tx_dsmzresources_pi5%5BreturnPid%5D=304

II-B. Culture

II-B.1 Strain reactivation and conservation

Reactivation followed the schema in Figure II-1. 50ml of culture media were prepared in Erlenmeyer flasks as presented in Table II-3 and Table II-4. Petri dishes were prepared to confirm the purity of the strains.

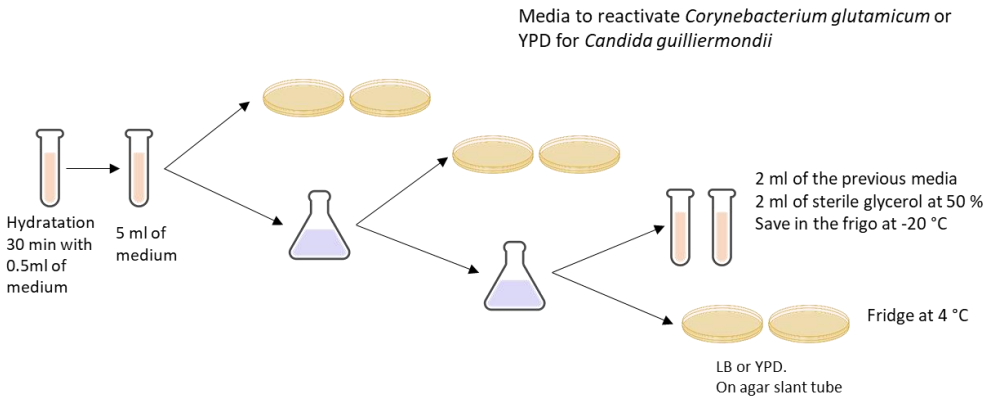


Figure II-1. Methodology for the microorganism reactivation[7]

Table II-3 Culture media used to reactivate *Corynebacterium glutamicum* (Amin and Al-Talhi, 2007)

	g/L
Glucose	40
K_2HP_4	1
$CaCO_3$	1.6
$MgSO_4*7H_2O$	0.5
Yeast extract	1
Urea	8

Table II-4 Culture media used to reactivate *Candida guilliermondii* FTI 20037

YPD	**g/L**
Yeast extract	10
Peptone soy	10
Glucose	20

Strains are conserved on two different conditions:

1. In 2 ml of sterilized glycerol (at 50 %), 2 ml of the reactivation culture-specific for each strain were mixed and further saved in the freezer at -20 °C. *C. glutamicum* is conserved on the freezer in the media presented mixed in Table II-3 with glycerol. *C. guilliermondii* is conserved in the freezer in the media presented in table Table II-4 with glycerol.
2. Agar slant tubes were inoculated with the reactivation culture and after incubation kept in the refrigerator at 4 °C. These tubes are the ones used directly for the experiments. For *C. glutamicum* LB medium agar slant tubes
3.

[7]Erlenmeyer flask and test tube from:https://www.vecteezy.com/free-vector/chemical"Chemical Vectors by Vecteezy

4. Table II-5) were used. For *C. guillermondii* the YPD Agar was used for slant tubes.

Table II-5 LB media composition

LB (Lysogeny broth)	g/L
Tryptone	10
Yeast extract	5
NaCl	10

II-B.2 Culture media used in the work

Two different culture media were used in this work. There were named “Patent” and “Synthetic." “Patent inoculation and fermentation media” were reported in the 1961 Patent to produce glutamic acid, work realized by Kinoshita and collaborators (Kinoshita, Tanaka and Akita, 1961). Whereas “synthetic media” origin is explained in the next chapter. Patent media was used only for *C. glutamicum* culture. Synthetic medium was used for both strains.

a) Patent inoculation and patent fermentation media preparation

Patent inoculation and patent fermentation media (PFM) were prepared with the compositions in Table II-6, and Table II-7. 60 ml of “Patent inoculation media” was prepared in a 500 mL Erlenmeyer flask. 100 mL of the “Patent fermentation media” were prepared in a 500 mL Erlenmeyer flask.

C. glutamicum was inoculated from an LB slant in patent inoculation media. The preculture was realized at 28 °C, agitation 200 rpm for 24 h. Then, 10 % of this preculture was added to the Patent fermentation media. Fermentation conditions for the culture were the same in agitation and temperature as the pre-culture.

Fermentation time was 48 hours. pH was adjusted manually with a solution of 10 % (by weight) of urea. pH was maintained in a range between 6 to 9 and verified with pH paper.

Table II-6 Patent Inoculation media

	g/L
Glucose	20
Peptone	10
Meat extract	5
Sodium Chloride	2.50

Table II-7 Patent Fermentation medium

	g/L
Glucose	100
$(NH_4)_2SO_4$	2
K_2HPO_4	1
KH_2PO_4	1
$MgSO_4*7H_2O$	0.25
$FeSO_4*7H_2O$	0.01
$MnSO_4*4H_2O$	0.01
Biotin	2.5E-06

Modified patent fermentation media

Patent fermentation media sugar composition was modified to realize some experiments. Glucose concentration passed from 100 g/L to 40 g/L, and xylose was added at a concentration of 35 g/L. The other components and fermentation conditions were kept the same.

b) Synthetic inoculation and fermentation media preparation

The media used for the culture were the "Synthetic inoculation media" and "synthetic fermentation media." The choice and evolution of this culture media are described in the Chapter "Choice of culture media in Erlenmeyer". The final composition is presented in Table II-8.

Table II-8 Synthetic inoculation and fermentation media

Synthetic inoculation media	
	g/L
Glucose	32.5
Xylose	17.5
K_2HPO_4	1.5
KH_2PO_4	1.7
$CaCl_2$ *$2H_2O$	0.1
$FeSO_4$ * 7 H_2O	0.01
Yeast extract	1
$CaCO_3$	1.6
$(NH_4)_2SO_4$	3
$MgSO_4$ * $7H_2O$	2.5
$MnSO_4$*H_2O	0.1

Synthetic fermentation media	
	g/L
Glucose	65
Xylose	35
K_2HPO_4	1.5
KH_2PO_4	1.7
$CaCl_2$ *$2H_2O$	0.1
$FeSO_4$ * 7 H_2O	0.01
Yeast extract	1
$CaCO_3$	1.6
Biotin	5. E-06
$(NH_4)_2SO_4$	3
$MgSO_4$ * $7H_2O$	2.5
$MnSO_4$*H_2O	0.1
Urea	5

c) Sterilization

For both media, nitrogen sources were added after autoclave to prevent Maillard reactions. Salts were also added after autoclave. Several solutions were prepared to be added after autoclave. The solutions concentrations and total added volume are presented in Table II-9 and

Table II-10.

The calcium carbonate solution had to be prepared in two phases. First, 24 g de $CaCO_3$ were dissolved on 75 ml of concentrated HCl, and then the volume was adjusted at 100 mL in a gauge flask.

Two mixes were prepared. A phosphate mix (K_2HPO_4 and KH_2PO_4) and a nitrogen mix (with urea and ammonium sulfate).

For the nitrogen mix, 50 mL of water, 50 g of urea, and 30 g of $(NH_4)_2SO_4$ were used. The solution is endothermic, so it had to be well agitated and heated to avoid crystal production.

Once everything was well dissolved, the real concentrations were calculated with the total volume of the prepared solution. This volume was measured with a test tube.

For the Phosphate mix, 100 mL were prepared with 35 g of K_2HPO_4 and 10g of KH_2PO_4, the salts were diluted and then adjusted at 100 mL in a gauge flask. After salts were added, inoculation and fermentation media were adjusted to 7 with NaOH 2 M.

Table II-9 Salt solution to add in Synthetic inoculation media for each 500 mL Erlenmeyer with 100 mL of culture media.

	Concentration (g/L)	Added volume (µL)
$FeSO_4 * 7H_2O$	200	5
$MnSO_4*H_2O$	200	50
$MgSO_4*7H_2O$	980	255
Mix K_2HPO_4/ KH_2PO_4	349/ 200	745
$CaCO_3$	240	666
$CaCl_2$	100	100
$(NH_4)_2SO_4$	300	1000

Table II-10 Salt solutions to add only on the Synthetic fermentation media. For each reactor with 1.2 L of culture media

	Concentration (g/L)	Added volume (ml)
$FeSO_4 * 7H_2O$	200	0.06
$MnSO_4*H_2O$	200	0.6
$MgSO_4*7H_2O$	980	3.1
Mix K_2HPO_4/ KH_2PO_4	349/ 200	8.9
$CaCO_3$	240	8
$CaCl_2$	100	1.2
Mix $(NH_4)_2SO_4$/Urea	296 /490	12
Biotin	0.1	0.06

Two tables are presented because the added volume depends on the devices used for the fermentation.

II-B.3 Fermentation devices

a) Culture in Erlenmeyer

Culture of *C. glutamicum* (Figure II-2)

From the agar slant tubes, *C. glutamicum* is reactivated on LB media. For this, 100 mL of the media were prepared on a 250mL Erlenmeyer flask. The flask was incubated at 30 °C and 200 rpm for 7 h. Then 10 mL of the LB culture were added to 100 mL of the synthetic inoculation media in a 500mL Erlenmeyer flask. After 24 h incubation (at 30 °C and 200 rpm), the volume needed for this preculture to obtain a cell concentration around 5.10^6 cell/ml was inoculated in 100 mL of the synthetic fermentation media in a 500 mL Erlenmeyer flask. Fermentation times on the synthetic fermentation media varied from 48 to 100 h, depending on the assay.

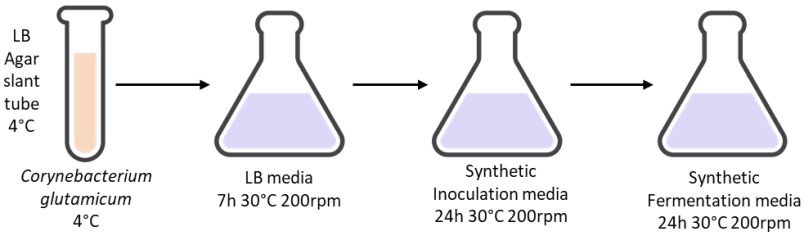


Figure II-2 Schema of *C. glutamicum* culture in Erlenmeyer

Culture of C. *guilliermondii* FTI20037 (Figure II-3)

To produce xylitol with *C. guilliermondii* FTI20037, a first reactivation phase in YPD media was required. For this, 100 mL of the media were prepared on a 250 mL Erlenmeyer flask. The strain was then incubated at 30 °C and 200 rpm for 48 hours. 100 mL of Synthetic inoculation and fermentation media were each prepared on a 500 mL Erlenmeyer flask. 5 mL of the YPD media culture were added to the synthetic inoculation media. After 24 h of incubation in the synthetic inoculation media, the volume needed to obtain a cell concentration of around 5.10^6 cell/ml was inoculated in the synthetic fermentation media.

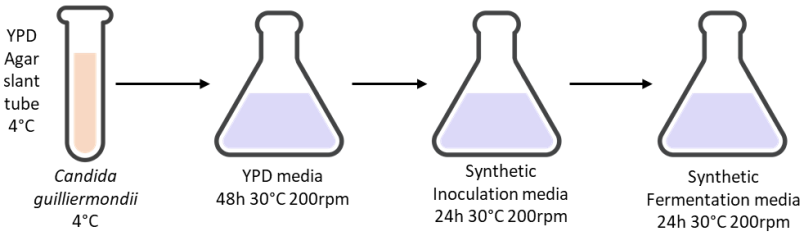


Figure II-3 Schema of *Candida guilliermondii* culture in Erlenmeyer

Fermentation conditions for both strains

Temperature and agitation were adjusted at 30 °C and 200 rpm, respectively, for both strains. Samples were taken every 3 hours. pH was measured and adjusted to pH 7±0.3 with NaOH and HCl 2 M.

Urea addition for both strains

Fermentation media had an initial concentration of 5 g/L of urea. Every 6 hours, 1 mL of urea 10 % v/w had to be added at the fermentation media until the concentration of total urea attainted 12 g/L.

b) Batch culture in reactors

Reactor batch fermentations were realized in Eppendorf Bioflow 110 and 120 bioreactors equipped with dissolved oxygen and pH probes meddler Toledo. NaOH and HCL 5 M solutions were used to maintain pH to 7. The reactivation and preculture steps in the synthetic inoculation media followed the steps presented in Figure II-2 and Figure II-3. General conditions of fermentation are specified in Figure II-4.

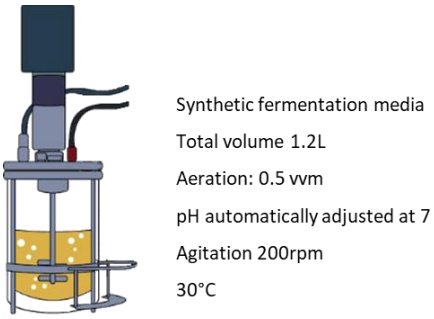


Figure II-4 Culture conditions in the bioflow bioreactors

Variation on the experimentation conditions for reactor cultures

Several conditions were tested in reactor cultures. The reasons will be explained in the next chapters. The main variations were:

1. Urea 10 % w/v was added continuously until the urea addition of 12 g/L, imitating the conditions in the culture realized on Erlenmeyer. The addition rate was 0.175 g/L.h
2. Calcium carbonate was eliminated from the medium composition.
3. A media with less glucose was tested (15 g/L instead of 65 g/L)
4. Lactic acid was added at a concentration of 25 g/L since it was produced in some *Corynebacterium glutamicum* fermentations.
5. A modification of the aeration from 0.5 vvm to 0.1 vvm was realized.

II-B.4 Sequential fermentation

Two sequential fermentations were tested: one keeping *C. glutamicum* after lactic acid production from glucose (Figure II-5) and the second with eliminating bacteria before the second stage with *C. guilliermondii* (Figure II-6).

After 48 hours of bacteria culture in the reactor at 0.5vvm and pH controlled at 7, residual glucose was around 15 g/L. The yeast was inoculated at this time in the same reactor, pH was not controlled anymore, and the aeration rate changed to 0.1 vvm.

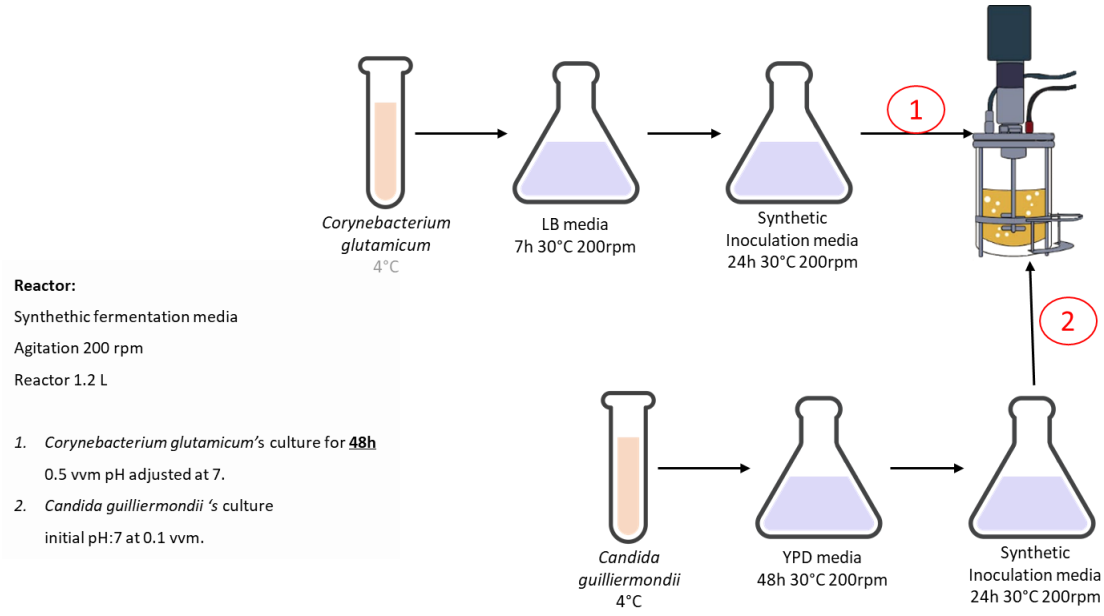


Figure II-5. Schema of sequential culture of *C. glutamicum* followed by *C. guilliermondii's* inoculation after 48h of culture.

For the sequential culture with eliminating *C. glutamicum*, the same fermentation conditions were used. The main difference was the removal of the *Corynebacterium* cells. For this, after the first stage of 48 h, pH was lowered to 5.5 with HCl 5 M to dissolve calcium carbonate in the medium. Culture media was centrifugated for 10 min at 4000 rpm in a Thermoscientific Megafuge 40R centrifuge, followed by a sterile filtration in a Thermofisher Nalgene rapid flow 90 mm filtration unit (0.2 µm). After filtration, the synthetic fermentation media was put in a new sterile reactor, pH was adjusted at 7, aeration changed at 0.1 vvm, and *C. guilliermondii* was inoculated.

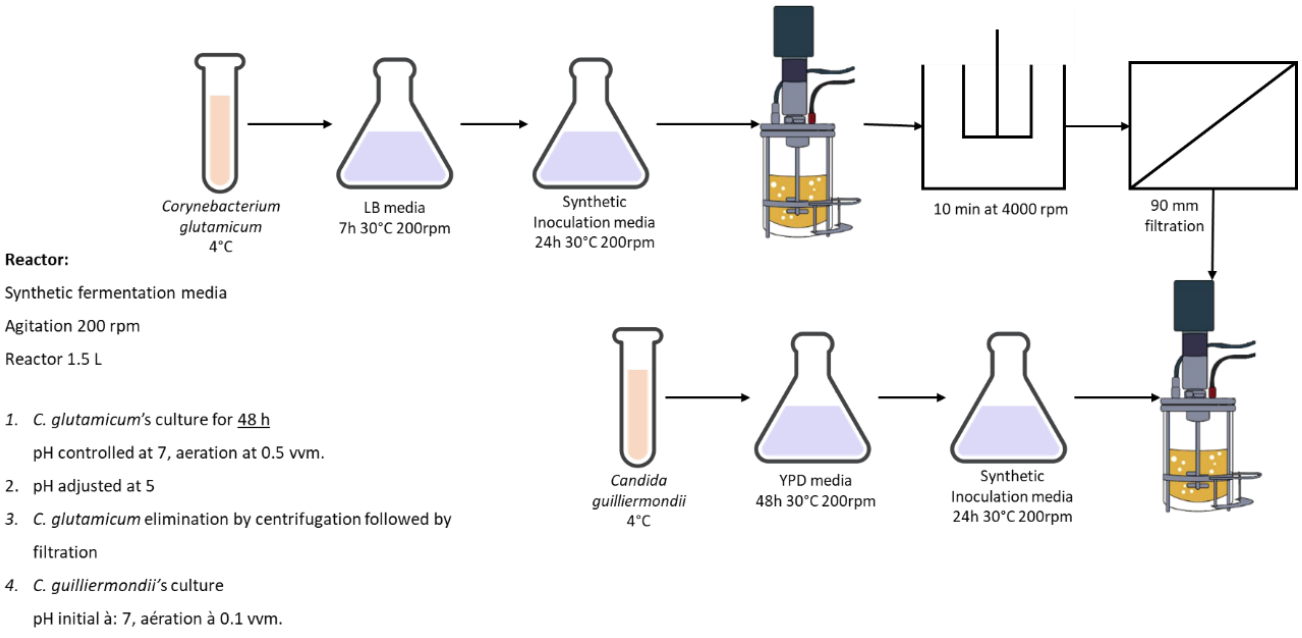


Figure II-6 Schema of sequential culture. *C. glutamicum* 's culture for 48h followed by *C. guilliermondii*'s culture in the recycled media.

II-C. Kinetics following methods

II-C.1 pH

In Erlenmeyer culture, pH was verified with a Eutech Instrument pH 700. Calibration was realized with standard solutions at pH 7 and pH 4. For bioreactor fermentation, pH probes were calibrated with standard solutions at pH 7 and pH 4 directly on the bioflow control towers.

II-C.2 Dissolved oxygen

Mettler Toledo dissolved oxygen probes were filled with the electrolyte solution and then connected and polarized for at least 6 hours on the bioflow control towers. The zero was realized with nitrogen injection in the reactor, and the 100 % was obtained with air injection into the reactor until it was kept constant.

kLa measurement

The 4 reactors with the Synthetic fermentation media were degassed with nitrogen for 30 min. After this, oxygen was introduced at 0.5 vvm (0.6 L/h for the 1.2 L of fermentation media) with agitation at 200 rpm until reaching 100 % saturation. The percentage of dissolved oxygen was measured every 2 minutes for reactors 2, 3, and 4 and every 30 seconds for reactor 1 (figure III-7)

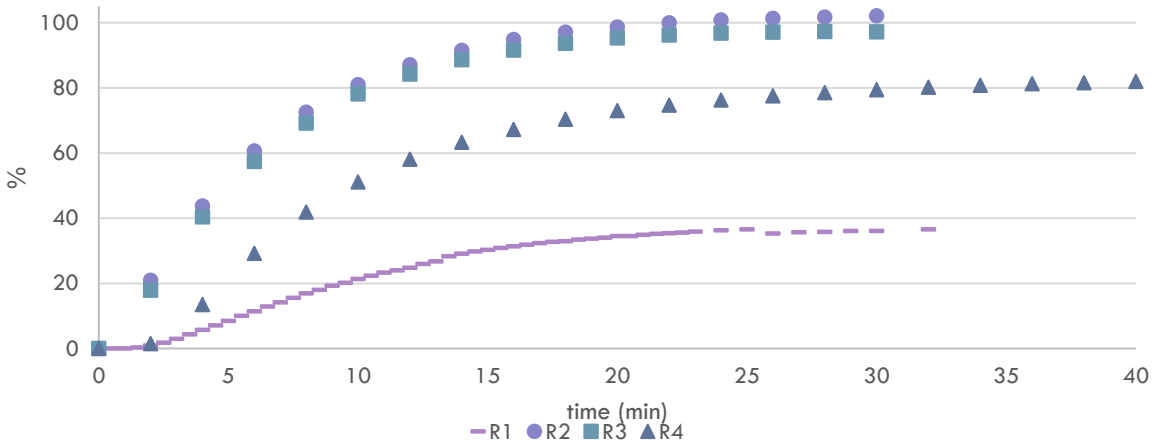


Figure II-7 Kinetics of dissolved oxygen for the 4 reactors at 0.5 vvm and 200 rpm

Oxygen concentration at different instants were determined and then the maximal concentration was used for calculation. k_La was calculated by the plot of natural logarithm of 1-saturations versus time as presented in Equation 1. Where k_La will be the slope obtained in the linear stage of the graphic (Najafpour, 2006).

$$\ln\left(1-\frac{saturation\ at\ time\ t}{maximum\ saturation\ value}\right)=-K_La*t \qquad \text{Equation 1}$$

kLa values obtained for the reactors are summarized in Table II-11.

Table II-11 KLa values for each reactor

Reactor	kLa (h^{-1})
R1	7.3
R2	10.2
R3	9.3
R4	7.4

II-C.3 Cell growth

a) Cell count

Cell count was realized on a Laborlux 12 Leitz microscope. The used strains have different sizes. Therefore, two different cell chambers were used.

C. guilliermondii FTI 20037 was followed in a Thoma cell chamber. At least 4 squares at 10 X were selected and then counted at 40 X, as seen in Figure II-8.

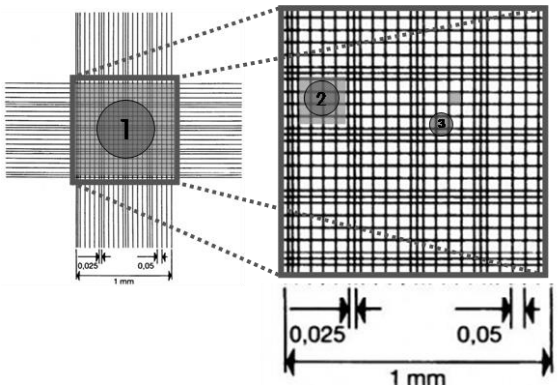


Figure II-8 Thoma cell counting chamber[8]

C. glutamicum ATCC 13032 was counted in a Neubauer cell chamber. At least five squares of the number 3 (as in Figure II-9) were counted at 65 X.

[8]http://www.celeromics.com/en/resources/Technical%20Notes/thoma-chamber-cell-concentration/thoma-chamber-cell-concentration.php

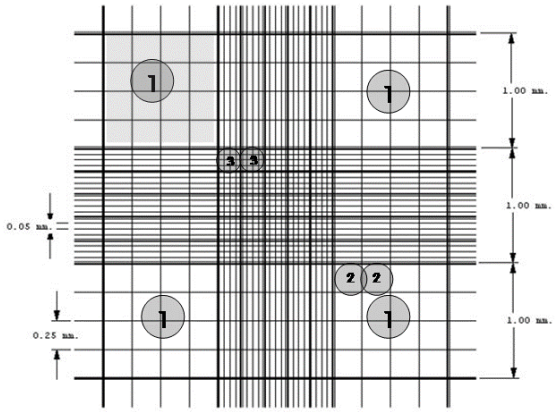


Figure II-9 Neubauer cell counting chamber[9]

Squares on both chambers were selected to be equivalent in size, and so the formula used for the conversion into concentration is the same:

Concentration in cell/ml was calculated as: $\frac{Cell}{ml} : \frac{\sum cell}{(N_{squares})} * \frac{250000}{ml}$

b) Optical density

Cell development was followed by measuring optical density on a Jenway 7315 spectrophotometer at 620 nm. Special cuvettes for spectrometer analysis in visible were used. The optical path length was 1cm. The sample was diluted with distilled water when absorbance was superior to 1, and the transmittance was under 20 %.

c) Dry weight

At the start and end of fermentation, 10 ml of the media were taken and were centrifuged on a Thermoscientific megafuge 40R centrifuge at 4700 rpm for 10 min. The supernatant was discarded. Samples were rinsed with 5 mL of distilled water and centrifuged a second time under the same conditions. The supernatant was discarded. The sample was resuspended in 1 mL of distilled water placed on aluminum cups. The dry weight measurement was realized on a Swiss Quality Previsa HAGO thermobalance.

No correlation between the cell count and other determinations was possible due to carbonate precipitation in the media.

[9]http://www.celeromics.com/fr/resources/Technical%20Notes/neubauer-chamber-cell-concentration/neubauer-cellule-numeration-concentration-cellulaire-1.php

II-C.4 Sugar and xylitol quantification

Samples were centrifugated at 9300 rpm for 4 minutes. The supernatant was recovered and diluted by a factor of 10 with distilled water and further filtered on a 0.4 µm filter.

Glucose, xylose, lactic acid, ethanol, and xylitol were analyzed on HPLC (Fisher) with a Rezex ROA H^+ 8 % 250 x 4.6 mm ion exclusion column thermostated at 30 °C. Sulfuric acid at 10 mM was used as a mobile phase at a flow of 170 µL/min. RI detector (Fisher) at 40 °C was used. Chromoleon 7 software was used to analyze the results.

II-C.5 Urea/ammonia

Megazyme Urea/Ammonia (Rapid) kit [K-URAMR 10/18] kit was used to verify available nitrogen from the urea and ammonia in the fermentations. The detection limit is 0.071 mg/L of ammonia and 0.1258 mg/L of urea.

a) Principle:

Urea was hydrolyzed to ammonia (NH_3) and carbon dioxide (CO_2) by the urease enzyme.

$$Urea + H_2O \xrightarrow{(urease)} 2NH_3 + CO_2$$

In the presence of glutamate dehydrogenase (GIDH) and reduced nicotinamide-adenine dinucleotide phosphate (NADPH), ammonia (as ammonium ions; NH_4) reacted with 2-oxoglutarate to form L-glutamic acid and NADP+ (2).

$$2-Oxoglutarate + NADPH + NH_4^+ \xrightarrow{(GIDH)} L-glutamic\ acid + NADP^+ + H_2O$$

The amount of NADP+ formed stoichiometric with the amount of ammonia. For each mole of urea reacted, two moles of NADPH were consumed.

The assay is specific for urea and ammonia. The detection limit is 0.071mg of ammonia/L.

b) Assay

The Urea/Ammonia (rapid) test came with 5 reagent solutions.

- Solution 1: Buffer (pH 8.0) plus 2-oxoglutarate and sodium azine (0.02 %) as a preservative.
- Solution 2: NADPH. Lyophilized powder.
- Solution 3: Glutamate dehydrogenase suspension.
- Solution 4: Urease solution
- Solution 5: Urea control powder. A solution of 70 mg/L of urea has to be prepared with distilled water to use it as a standard control.

The analysis was realized in UV cuves at 340 nm at 25 °C.

The method and volumes corresponding to each solution are presented in Table II-12

Table II-12 Procedure followed for Urea/Ammonia quantitative analysis

Pippete into cuves	Blank	Sample
Distilled water	1.05 mL	1 mL
Sample	-	0.05 mL
S1 Buffer	0.15 mL	0.15 mL
S2 NADPH	0.2 mL	0.1 mL
All was mixed, the absorbance of the solutions were readed (A1) after approximately 2minutes. The reaction was immediately started by the addition of:		
S3 GIDH	0.01 mL	0.01 mL
Solution was mixed and the absorbances of the solutions were read (A2) after approximately 5 minutes. The solution S4 was then added.		
S4 (Urease)	0.025 mL	0.025 mL
Solution was mixed, and the absorbances were read (A3) at the end of the reaction (5minutes). If after 5min the absorbance wasn't stable, it continued to be measured every minute until stabilization.		

c) Calculation

The absorbance differences were calculated (A1-A2) for the blank and the samples. Once the blank differences were subtracted from the samples differences, the $\Delta A_{ammonia}$ is obtained.

The difference between A2 and A3 is then obtained. The blank absorbances are also subtracted and ΔA_{urea} is obtained.

$\Delta A_{ammonia}$ and ΔA_{urea} the minimum value should be 0.1 to have accurate results.

To obtain ammonia and urea concentrations in g/L, the Δ values are multiplied as follows:

[Ammonia (g/L)]= 0.07082*$\Delta A_{ammonia}$

[Urea (g/L)]= 0.1273*ΔA_{urea}

II-C.6 Glutamic acid quantification

Megazyme Glutamic acid enzymatic kit (supplied by BIOSINTEC) was used for glutamic acid quantification on the samples.

a) Glutamic acid quantification principle

L-Glutamic acid is oxidized by nicotinamide-adenine dinucleotide (NAD+) in the presence of glutamate dehydrogenase (GlDH), leading to the formation of 2-oxoglutarate, reduced nicotinamide-adenine dinucleotide (NADH), and ammonium ions ($NH4^+$).

(I) L-Glutamic acid + NAD^+ + H_2O (GIDH)→ 2-oxoglutarate + NADH + NH_4^+

However, as the equilibrium of this deamination reaction lies markedly in favor of the reactants, a further reaction catalyzed by diaphorase is required, in which NADH reduces iodonitrotetrazolium chloride (INT) to an INT-formazan product, leading to a rapid and quantitative conversion of L-glutamic acid (II).

(II) INT + NADH + H^+ (diaphorase) → NAD^+ + INT-formazan

The amount of INT-formazan formed in this reaction is stoichiometric with the amount of L-glutamic acid. It is the INT- formazan, which is measured by the increase in absorbance at 492 nm.

b) Limits

The assay is linear in the range of 0.054mg/L and 0.21 mg/L.

c) Sample pre-treatment

As there are ions $NH4^+$ at a concentration superior to 5mM in the media, the sample has to be treated to remove them to avoid irregularities on the analysis.

100µL of the previously centrifugated sample were adjusted to 9 with no more than 10µL of NaOH at different concentrations. Then, samples were heated in a thermostated bath at 60°C for 30minutes to evaporate NH_4 produced.

d) Assay

The glutamic acid kit cames with 5 solutions as follow:

- Solution 1: Buffer at pH 8.6 plus sodium azide (0.02 % w/v) as a preservative
- Solution 2: NAD^+ plus INT
- Solution 3: Diaphorase suspension
- Solution 4: glutamate dehydrogenase
- Solution 5: L-glutamic acid standard solution at a concentration of 0.1 mg/L

The assay was made in microplates. First, a mix of solutions 1 to 3 was prepared as follows:

	µL
Water	200
Sample	10
S1	50
S2	20
S3	5

Then, the absorbances were read after 5min at 25°C. With this, the value A1 was obtained. To start the reaction, 5 µL of solution 4 (GIDH) were added- The absorbance was read after 10 minutes (the end of the reaction), this new value is A2.

e) Glutamic acid concentration calculation

To determine the concentration, the difference (A2-A1) was multiplied by the factor 0.2144. The final concentration is on g/L. The detection limit is 0.214 mg/L.

II-C.7 L-Lactic acid

Megazyme L/D-lactic acid enzymatic kit (supplied by BIOSINTEC) was used to verify the presence of lactic acid as a metabolite in the samples.

a) Principle:

Qualitative analysis of D-/L- Lactic acid required two enzyme reactions. The first is catalyzed by D-/L-lactate dehydrogenase (D-/L-LDH). D-/L-lactic acid (D-/L-lactate) is oxidized to pyruvate in the presence of nicotinamide-adenine dinucleotide (NAD^+) (1).

(1) $$D-/_L - \text{Lactate} + NAD^+ \xleftrightarrow{(D-/_L-\text{LDH})} \text{Pyruvate} + \text{NADH} + H^+$$

However, since the equilibrium of reaction (1) lies firmly in the favor of D-/L-lactic acid and NAD^+, a further reaction was required to "trap" the pyruvate product. This is achieved by the conversion of pyruvate to D-alanine and 2-oxoglutarate, with the enzyme D-glutamate-pyruvate transaminase (D-GPT) in the presence of a large excess of D-glutamate (2).

(2) $$Pyruvate + \text{D} - \text{glutamate} \xleftrightarrow{(\text{D}-\text{GPT})} \text{D} - \text{alanine} + 2 - \text{oxoglutarate}$$

The amount of NADH formed in the above-coupled reaction is stoichiometric with the amount of D-/L-lactic acid. It is the NADH, which is measured by the increase in absorbance at 340 nm.

b) Assay

The D-/L-Lactic acid came with 6 solutions as follows:

- Solution 1: Buffer at pH 10 plus D-glutamate and sodium azide (0.02% w/v) as a preservative
- Solution 2: NAD^+
- Solution 3: D-Glutamate-pyruvate transaminase suspension
- Solution 4: L-Lactate dehydrogenase suspension
- Solution 5: D-Lactate dehydrogenase suspension
- Solution 6: D-/L-Lactate dehydrogenase suspension

The analysis was realized on UV cuves at 340 nm. The cuves had 1 cm of the light path. The sample was read against water. The method used is presented in Table II-13.

Table II-13 Procedure followed for Lactic acid qualitative analysis

Pipette into cuvettes	**Blank**	**Sample**
Distilled water (~ 25°C)	1.60 mL	1.50 mL
Sample	-	0.10 mL
Solution 1 (buffer)	0.50 mL	0.50 mL
Solution 2 (NAD+)	0.10 mL	0.10 mL
Suspension 3 (D-GPT)	0.02 mL	0.02 mL
Cuves were mixed*, the absorbances of the solutions were read obtaining (A1) after approx. 3 min. Later the reaction was started by the addition of:		
Suspension 5 (D-LDH)	0.02ml	0.02 ml
The absorbances of the solutions were read obtaining (A2) at the end of the reaction (approx. 5 min). If the reaction didn't stop after 5 min, the absorbances continued to be read at 1 min intervals until they remain unchanged.		
Suspension 4 (L-LDH)	0.02 ml	0.02 ml
Cuves were mixed* At the ed of the reaction (approx. 10min) the absorbances were read obtaining (A3). If the reaction didn't stop after 10 min, the absorbances were read at 5 min intervals until the absorbances either remain the same, or increase constantly over 5 min		

c) Lactic acid concentration calculation

The values of A1, A2 and A3 are used to calculate the concentration.

For D-Lactic acid: 0.3204 x ΔAD-lactic acid (A2-A1)

For L-Lactic acid: 0.3232 x ΔAL-lactic acid (A3-A2)

Both concentrations are given on g/L. Detection limit is 0.214 mg/L

RESULTS

This is the beginning of the results part of the work, which is separated in two parts:

The first one is the choice and optimization of the culture medium in Erlenmeyer. In which the main focus was centered on cell growth for both strains in the same culture media. The culture media composition was chosen based in a glutamic acid production patent and sugar composition in agave bagasse hydrolysates.

The second part is focused on the culture conditions to produce the metabolites in reactors. Conditions like pH regulation, sugar concentration, media composition and aeration rate were discussed in this part. Several essays in co-culture were also realized with different process strategies for the co-production of the metabolites of interest.

Apart from the experiments presented in the results part, appendix at the end of this work are presented as complementary information to show the path followed in this work.

In "Appendix 1: Test realized in Erlenmeyer for culture medium optimization" the different hypothesis followed are presented: From the selection of CaCO3 as buffer, to the selection of nitrogen source. Each essay cleared some preferences in the culture media and the fermentation conditions for the cell development of *C. glutamicum* and *C. guilliermondii.*

In "Appendix 2: Test realized in Reactors for xylitol production optimization" the table presents several informations about the test realized: from the code of the experiment, along with the name of the strain tested and the variables changed in each essay. The influence of variables like: initial sugar concentration, aeration rate, pH regulation was observed. Values like, sugar consumed, maximal biomass obtained, production yield and productivity are specified for each test realized.

III. Choice of culture media in Erlenmeyer

The objective of this chapter is to determine a media-rich enough in nutrients for both strains. We will first work on the nutrient needs of *Corynebacterium glutamicum*. This decision was made because bacteria have, in general, more nutritional needs than yeasts. Once the fermentation media and culture conditions are selected for the bacteria, the same conditions will be tested on the yeast to verify the nutritional and conditions compatibility in the cell growth.

III-A. Sugar composition in agave bagasse hydrolysates

In the first chapter, we talked about the agave bagasse residues and the fact that for this research, this was the chosen lignocellulosic material to work with. A model media simulating sugar composition in agave bagasse hydrolysate will be proposed to be used in the work.

For this, bibliographical research was first realized to define the sugars contained in this media, their concentrations, and the glucose:xylose ratio. The values for the composition of different agave hydrolysates are compiled in Table III-1.

Table III-1 Composition of different agave tequilana bagasse hydrolysates.

Hydrolysis process	Glucose (g/L)	Xylose (g/L)	Total Sugars (g/L)	Reference
Washed, dried, and further acidic.	11	14	25	(Saucedo-Luna *et al.*, 2011)
Acid hydrolysis (Metzontetel)	5	22.18	27.18	(Hernández-Salas *et al.*, 2009)
Cooked bagasse			27.9	(Arreola-Vargas *et al.*, 2015)
Acid hydrolysis (Metzal)	24.37	6.25	30.62	(Hernández-Salas *et al.*, 2009)
Combination of steam explosion and enzymatic hydrolysis	26.25	23.75	50	(Arrizon *et al.*, 2012)
Enzymatic hydrolysis (Metzontetel)	48.125	10.62	58.745	(Hernández-Salas *et al.*, 2009)
Enzymatic hydrolysis (Metzal)	58.75	9.37	68.12	(Arrizon *et al.*, 2012)

To choose a representative ratio between glucose and xylose and a usual composition in total fermentable sugars, Table I-1 presents total sugar composition. The average values are: 41 g/L of total sugars with 28.9±20 g/L on glucose and 14±7.1 g/L on xylose. The standard deviation for the sugar composition is high because it will depend on the cultivation conditions of the agave, as well as the weather conditions of the year and, more specifically, the hydrolysis method used. The obtained glucose:xylose ratio is around 2.

III-B. Replicating the US patent protocol

In this part, we first wanted to reproduce *C. glutamicum* ATCC13032 glutamic acid production in the conditions previously reported by Kinoshita in the 1961 patent (Kinoshita, Tanaka and Akita, 1961) **that can be considered as a reference.**

III-B.1 Initial patent protocol

The composition and protocol used for glutamic acid production in the patent is explained in the chapter Material and methods**.** As a reminder, this media is mostly a mineral media with glucose as a sole carbon source for both inoculation and fermentation media. A volume of 0.5 to 1 mL of a solution of urea at 10 % v/v was used to adjust pH between 6-9 every 4 to 6 hours with a total addition between 3 to 6 mL. The urea has the double function of increasing the pH and providing nitrogen.

Results of *C. glutamicum* culture on patent fermentation media (PFM) are presented in Figure III-1. It can be observed that the maximal glucose consumption rate was obtained at 48 h of culture at 1.25 g/L.h. Cell attaint maximal concentration after 24 h of culture. This growth suggests that culture conditions were adequate for cell development. After 24 h cell concentration decreased and attaint a stationary phase, this may be due to pH decreased at that fermentation time. Once pH was adjusted, at 41 h, pH continued to augment, and cells had a second wave of growth. These cells develop changes according to pH value suggest that cell growth is linked to pH variation in the media.

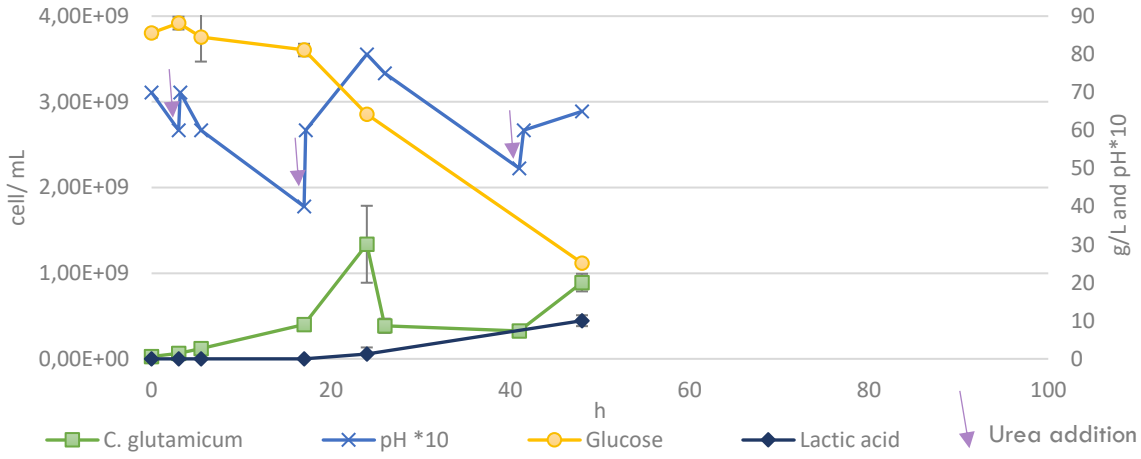


Figure III-1 *C. glutamicum's* kinetics on patent media, initial patent protocol.

In our experiment, pH was adjusted three times (at 3 h, 17 h, and 41 h) during the culture to keep it between 6 - 9; different volumes of urea were added each time for a total of 8.2 mL (Table III-2). It was observed that when more urea was added in the media, pH tended to augment. This may be due to the significant amount of urea added in a precise moment. For example, at 17 h, 5 mL of the urea solution were added, it seems that the concentration in the media was enough to switch *C. glutamicum*'s metabolism to hydrolyze urea into NH_3 and have a pH augmentation where it increased to 8. On the patent protocol, it was specified to add between 0.5 to 1 mL of the urea solution (Kinoshita, Tanaka and Akita, 1961). It seems that the volume was specified to prevent that the urea addition influenced the bacteria's metabolism.

When this protocol with supplementary urea is added, it's necessary to keep in mind the effect of this addition into pH value and consequently, in cell development. Some information that is important to emphasize, is the work that Follmann and his collaborators did (Follmann *et al.*, 2009); they research the effects of pH in the homeostasis of *C. glutamicum* ATCC13032, founding that the bacteria keeps a pH value around 7.5 when subject to pH values between 5.5 and 9.

Table III-2 pH at different times of the fermentation and the volume added to adjust it in the range from 6 to 9

Fermentation time (h)	Initial pH	Urea added volume (mL)	Final pH
3	6	0.7	7
17	4	5	6
41	5	2.5	6

In this assay, lactic acid production started at around 24 h of fermentation. After 48 h, 10 g/L of lactic acid was produced with a yield of 0.33 mol/mol and a productivity of 0.2 g/L.h. Glutamic acid wasn´t produced.

As seen on the subhead "Some products obtained from *Corynebacterium glutamicum* and their industrial interest**"** in page I-19, lactic acid is produced from pyruvate at the end of glycolysis before entering the Krebs cycle. Glutamic acid is produced when oxygen is not in excess (< 100 % saturation), but it has been reported (Wieschalka *et al.*, 2013; Kumar, Vikramachakravarthi and Pal, 2014) that under lower aeration than needed for glutamic acid production, *C. glutamicum* ATCCC13032 produces lactic acid as a primary metabolite as well as succinic and acetic acid. These other acids were not observed with the conditions we used. Lack of production of succinic acid could be due to the presence of Ca^{2+} ions in the media. It has been reported (Song and Lee, 2006; Shi *et al.*, 2014a) that the presence of the calcium ions is toxic for *C. glutamicum* in succinic acid production.

The lack of glutamic acid production even in the same experimental conditions reported in the L-glutamic acid production patent by (Kinoshita, Tanaka and Akita, 1961) could be due to the change in the material used. Indeed, in the patent, it was reported that 30 mL of the Patent fermentation media were placed in a 250 mL flask agitated at 220 rpm. In our experiments, because of the volume needed for analysis, we tried with 100 mL in a 500 mL flask agitated at 200 rpm. The change in the volume ratios and agitation could induce a different transfer of oxygen, leading to a lack of aeration, preventing the production of glutamic acid and having lactic acid as metabolite.

What to keep: Glutamic acid wasn't produced in this test, maybe due to the change in the liquid: volume ratios in the flasks used. From this experiment, it seemed that pH needed to be controlled. This regulation will be manual for the rest of the experiments realized in Erlenmeyer. The addition of a high concentration in urea seems to switch the bacteria's metabolism into urea's hydrolysis. When working with supplementary urea, the addition has to be controlled.

III-B.2 Urea's initial concentration on patent media

Previously we saw that urea influences the pH, which has effects on cell growth. Because of this, it was decided to verify the effect that the amount of urea in the medium would have directly on metabolite production. Inspired by the addition of urea proposed in the Kinoshita patent, two different conditions were compared. One in which the total amount of urea is directly added in the media and, on the second, a small quantity of urea is present in the media and controlled additions were realized until the same total urea concentration was attaint.

At the end of the previous part, the hypothesis that "The change in aeration ratio was the cause of the lack of glutamic acid production" was evoked. But, it was also proposed to carry out a controlled urea addition during cultivation. Therefore, based on the protocol reported by (Kinoshita, Tanaka and Akita, 1961), some conditions were changed to test the effect of the amount of urea present in the medium. The changed conditions and their reasons were the following:

1) Glucose concentration passed from 100 to 65 g/L. So sugar concentration was closer to a real glucose concentration in an agave bagasse hydrolysate.

The reader will see (Figure III-2) that even if this was what was envisaged in the media preparation, unfortunately, only 40 g/L of glucose were quantified by HPLC.

2) Two concentrations of urea were tested to determine if the initial concentration of urea had an impact on the overall kinetics: "higher initial urea," with 12.5 g/L of initial urea, was compared to "low initial urea" with 5 g/L of initial urea.

Theoretically, with 65 g/L of glucose in the media, with 80% of conversion yield, a maximum of 51.2 g/L of glutamic acid should be produced. Therefore, at least 5.2 g/L of nitrogen would be needed. For this, a concentration of 11.3 g/L of urea has to be in the media.

3) In the second condition, to obtain a final concentration of 12 g/L, 1 mL of urea 10 % w/v was added every 6 hours until 40 h of fermentation. This punctual addition was inspired by the "patent protocol" previously tested.

For higher and lower initial urea conditions at the beginning of the culture pH was adjusted at 7. Later in the culture, pH was manually regulated at 7±0.3 with NaOH 4 M and HCl 2 M.

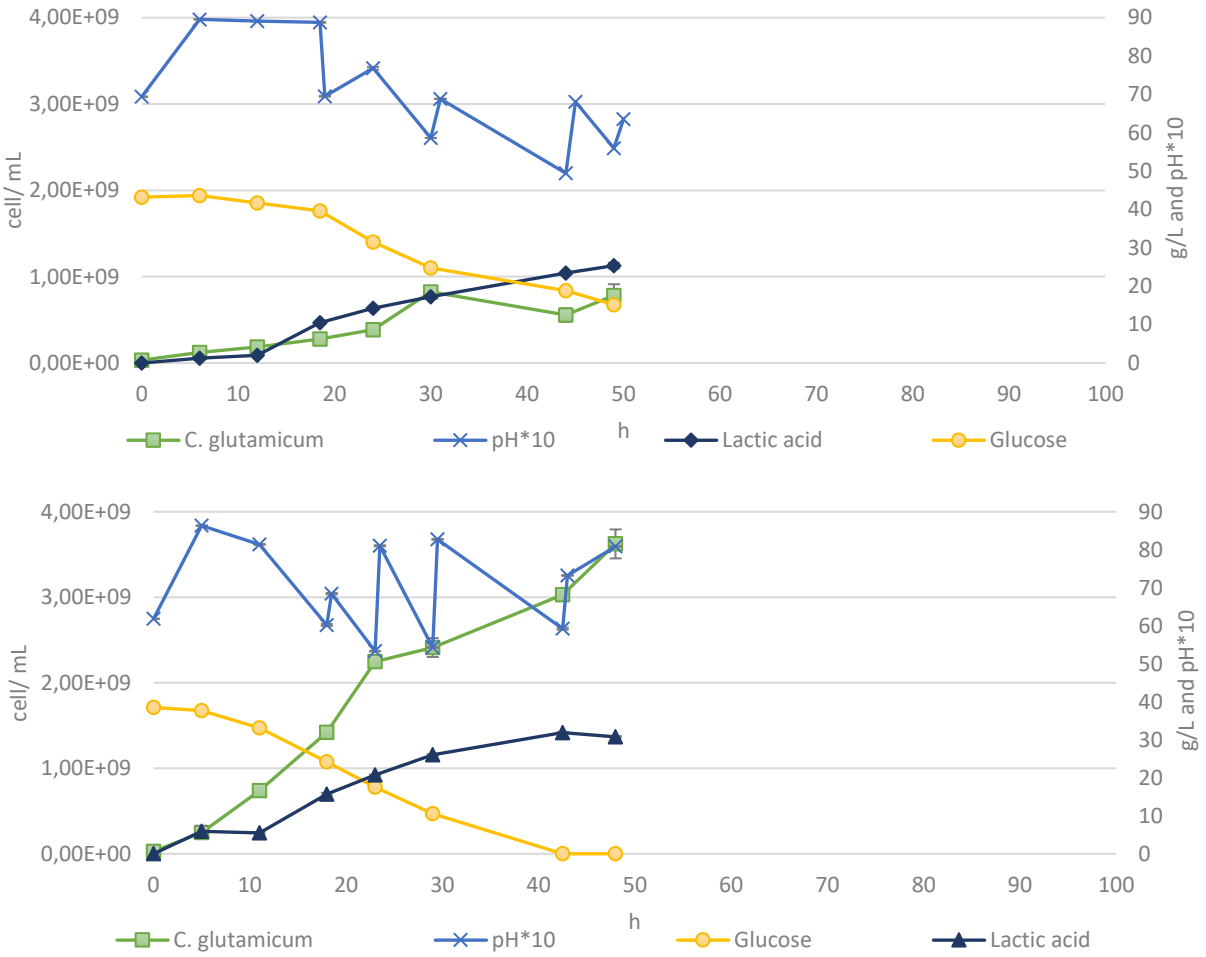


Figure III-2 *C. glutamicum* kinetics on patent media to compare the effect on the initial nitrogen concentration

Up: 12.5 g/L of initial Urea. Down: 5 g/L of initial Urea with the addition of a urea solution at 10 %.

It can be observed (Figure III-2) that cell growth has different rates in both conditions. *C. glutamicum* attained exponential phase after 6 h of culture in the "lower initial urea" condition. In contrast, on the condition with 12.5 g/L of initial urea, cells seemed to slowly develop, with a growth phase starting after 20 h of culture. After 50 h of culture, cell concentration was almost five times higher in the media with lower initial urea concentration.

During the first 5 hours, the pH value raised, the same tendency was observed in both conditions. During the first 18 hours, pH wasn't adjusted to be able to observe the variation after the addition of urea. At 12 h of fermentation, pH started to differ; the condition with 5 g/L of initial urea passed through another metabolic path and started to acidify the media. This acidification seemed to correspond with lactic acid production.

Glucose consumption kinetics varied with initial urea concentration. Glucose was fully consumed after 40 h of fermentation in the media with 5 g/L of initial urea. In the media with higher initial urea, glucose wasn't entirely consumed after 50 h: indeed, 16 g/L of residual glucose remained.

In terms of yield, the media with lower initial urea had 1.65 $mol_{lactic\ acid}/mol_{glucose}$ versus 1.8 mol /mol when 12 g/L of initial urea was used in the formulation. Lactic acid productivity was

0.5 g/L.h for higher initial urea and 0.7 g/L.h for lower initial urea. These values are no so different from each other, but the lower initial urea was chosen because productivity was slightly higher, and fermentation end at around 40 h of culture.

Once again, no glutamic acid production was observed in the experiment realized. Two hypotheses seem to appear: 1) Biotin concentration isn't optimal for glutamic acid excretion or 2) Oxygen transfer isn't enough in the culture conditions on Erlenmeyers to produce glutamic acid. Indeed, after an adequate pH adjustment in this assay, it seemed that, as said before, it was the change of scale in the Erlenmeyer was not adequate to have sufficient aeration for the production of glutamic acid.

It was concluded that 5 g/L of initial urea followed by controlled urea addition until 12 g/L; is a better condition to have higher lactic acid production and use of the substrate for C. glutamicum. It is to notice that liquid-flask proportion and agitation conditions weren't probably sufficient for glutamic acid production.

III-B.3 Patent media with Xylose for C. glutamicum

This part aims to test the fermentation kinetics for *C. glutamicum* in the patent media protocol proposed, when a mix of sugars is present in the media as bagasse hydrolyzates contain both glucose and xylose. For this, xylose was added to the formulation in both inoculation and fermentation media. These new medias were called "Modified patent inoculation media" (MPI) and "Modified patent fermentation media" (MPF).

When the xylose was added into the inoculation media to start a culture, the preparation time[10] passed from 24 h to 72 h. It seemed that the addition of xylose in the media had a negative effect in cell development, because the presence of xylose was the only variable. All reactivation times and culture conditions were the same as defined in "Material and methods**"**. The cells grown on the "MPI" (glucose:xylose 32:17) were inoculated in two different fermentation medias:

- Modified patent fermentation media (glucose:xylose 65:35). According to Table III-1
- Patent fermentation media (only glucose as substrate).

For each condition: pH was manually adjusted between 6 and 9 with NaOH or HCl 4 M. Also, 1 mL of urea 10 % w/v was added every 6 hours until 40 h, to obtain a total of 12 g/L of urea in the media. Initial nitrogen concentrations were 5 g/L of urea plus 2 g/L of $(NH_4)_2SO_4$.

The curves in Figure III-3 represent the average value of two duplicates; their standard deviation is also represented.

[10] The culture time for the inoculation media to attaint cell concentration adequate to be inoculated.

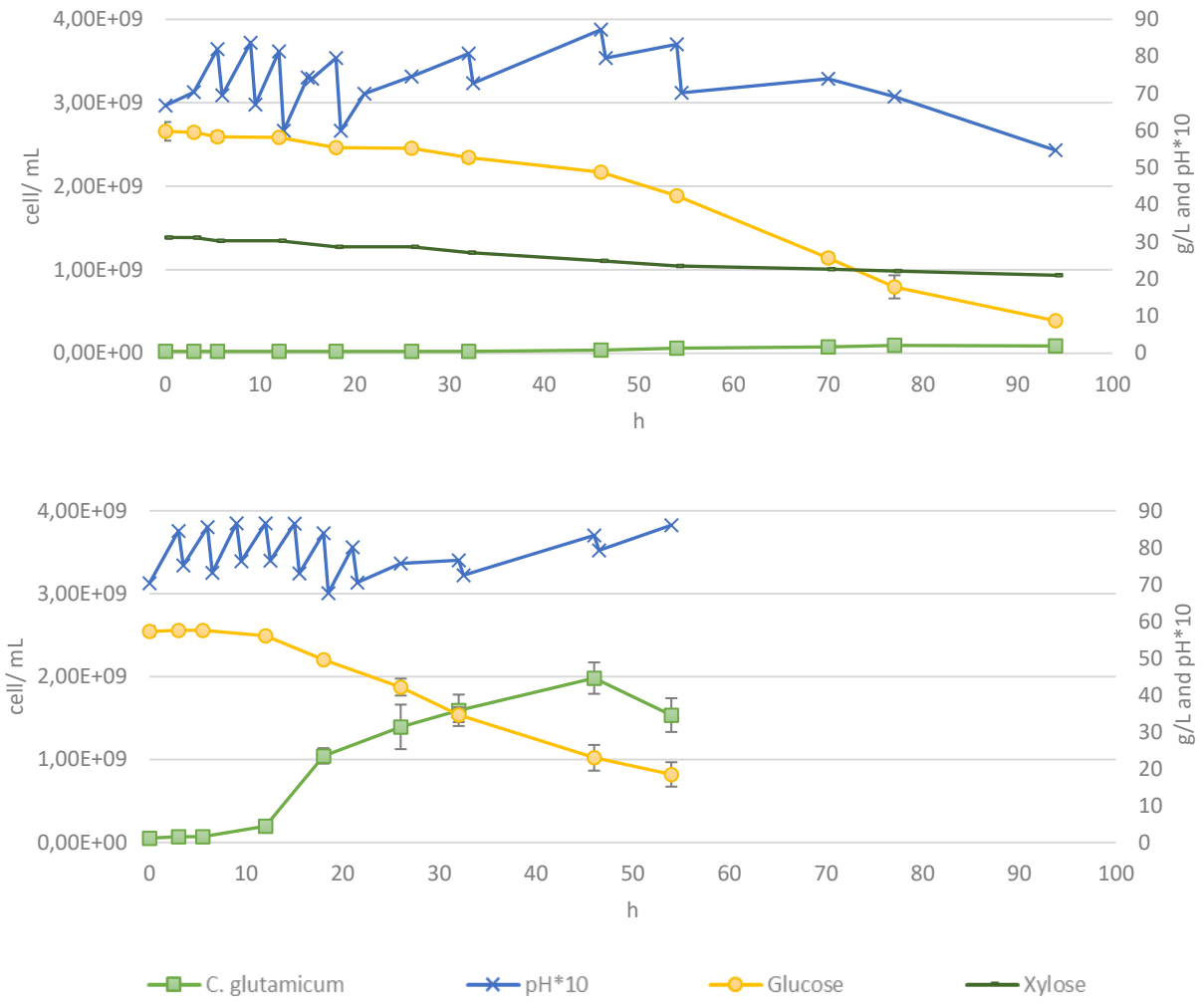


Figure III-3 Up: *C. glutamicum* on the Modified patent fermentation media with glucose and xylose as sustrate
Down: *C. glutamicum* on the Patent fermentation media with glucose as only substrate.

After the 100 h of fermentation for the modified patent fermentation media, no cell growth was observed in Figure III-3. Indeed, *C. glutamicum* consumed 60 g/L of total sugars, but the strain didn't' reach an exponential phase. Lactic acid and glutamic acid were not detected. Suggesting that glutamic acid was either not produced by this culture, or, the conditions weren't adequate for the cell to excrete the amino acid. Just to remember, glutamic acid is excreted by the cell under different conditions such as biotin limitation, the addition of surfactants, the addition of penicillin, and thermal triggered (Delaunay *et al.*, 1999b).

As stated before, the inoculation media was modified by adding xylose in its composition. This change makes the adaptation time three times longer than when only glucose was in the media. This change in the culture time can be explained because microorganisms have a preference over a substrate. The phenomenon exists by a process called "carbon catabolite repression (CCR)," and this causes an inefficient co-utilization of glucose and xylose (Khunnonkwao *et al.*, 2018). These phenomena would make sugars to be consumed sequentially one after another. It has been reported (Ikeda, 2012) that the *C. glutamicum* ATCC 13032 doesn't have one dependent sugar system. Indeed, the strain is capable of consuming

several sugars in C5 and C6, but glucose is its preferred substrate (D'Este, Alvarado-Morales and Angelidaki, 2018).

pH allure in both cases was similar; it increased in both fermentations. Suggesting that *C. glutamicum* keeps the same metabolic pathway under both conditions. However, there are many differences in substrate consumption. Indeed, as observed in Figure III-3 glucose consumption rate was higher in the Patent media, whereas in the Modified patent media, after 30 h of fermentation, glucose, and xylose were consumed at the same time.

For instance, the cell concentration curve is different between both. On the patent media, the cell attains the stationary phase at a concentration of 10^9 cell/mL. Whereas in the modified patent media, the bacteria didn't attaint the exponential phase even after 90 h of fermentation.

As previously mentioned, *C. glutamicum* doesn't have a predominant interest in glucose over xylose. Nevertheless, it has been reported that when *C. glutamicum* grows on a mixture of glucose and acetate, glucose uptake rate is reduced by half compared to the glucose rate with glucose alone (Graaf *et al.*, 2000). In another study, the glucose consumption rate was enhanced by the presence of maltose, increasing the growth rate (Krause *et al.*, 2010). The different behavior of the strain under different sugar mixes could explain why *C. glutamicum* needed more fermentation time on the "Modified patent media" composition.

No lactic or glutamic acid production was observed in either case. It seems that the inoculation media used had a significant impact on the production of the metabolites. Indeed, the use of a mix of sugars in the MPI seemed to prevent lactic or glutamic acid production by *C. glutamicum*.

Comparing the results obtained for the patent media with 5 g/L of initial urea (Figure III-2 Down and Figure III-3 down), it's possible to compare the effect of the inoculation media on the culture. Indeed, the mix of sugars in the inoculation media changed the kinetics on *C. glutamicum*. As stated before, when only glucose is used as a substrate, cell concentration in the inoculum is attaint after 24 h. Instead, when a mix of glucose and xylose is used, cell concentration is attainted after 72 h.

The cell growth profile was different in both cases. When the inoculum had only glucose, *Corynebaterium* passed directly to the exponential growth phase. In the other case, a latency phase of 12 h was observed. This seemed linked to sugar consumption, which was faster (0.9 g/L.h vs. 0.7 g/L.h) when only glucose was used in the inoculum. Lactic acid was only produced in the patent media inoculated with a media with glucose as the only substrate.

After the different assays on C. *glutamicum*, it was decided that a new media had to be tested to optimize culture times in both strains when a mix of sugars exists. Inoculum and fermentation media should be a complement to have the best fermentation conditions. pH control is suggested to maintain homeostasis in C. *glutamicum* and continue with metabolical functions.

III-C. Determination of a synthetic fermentation media composition

In this part, the nutrients needs for both strains are selected.

In the last series, it was concluded that a new media composition had to be presented for *C. glutamicum* cell growth. The agave bagasse model media defined at the beginning of this chapter, glucose:xylose ratio (65:35), was chosen to be the composition in the proposed culture media.

For the choice of nutrients content of the synthetic media, a comparative analysis between different references in the literature was done to determine the micronutrients necessary for both strains.

In Table III-3 it can be observed that the main compounds found in the different fermentation media for both strains were: $MnSO_4 \cdot H_2O$, $FeSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$; Biotin, nitrogen source (urea or $(NH_4)_2SO_4$), and KH_2PO_4.

Table III-3 Fermentation conditions and media compositions used to obtain chemical building blocks by different authors with *C. glutamicum* and *C. guilliermondii*.

		Initial (g/L)					Conditions				Products and residual sugars (g/L)				Material
Strain	**Ref.**	**Carbon source**		**Nitrogen**	**Salts**	**Vitamins**	**pH**	**Aeration conditions**	**Time (h)**	**T (°C)**	**Xylose**	**Xylitol**	**Glucose**	**Glutamic Acid**	**Reactor/ Erlenmeyer**
***C. glutamicum* ATCC No. 13058**	(Amin, 1994)	100 Glucose	2 Corn Steep Liquor	1 Urea	0.5 $MgSO_4$ •$7H_2O$, 0.1$MnSO_4$	Biotin 4µg /L	7.5	Oxygen transfer rates between 20.4- 90.2 - 200 mM O_2/L.H	120		-	-	3.2	50.7	Vertical rotating immobilized cell reactor (VRICR)
***C. glutamicum* NIAB SS-27**	(Niaz *et al.*, 2017)	10 Glucose	-	3 $(NH_4)_2SO_4$	KH_2PO_4, 0.07; K_2HPO_4, 0.4; $CaCO_3$, 2.0; $(NH_4)_2SO_4$, 3.0; $MgSO_4.7H_2O$, 0.03;	Thiamine -HCl, 5 m g/L. Biotin 60µg /L	7	150 rpm 50 mL of media in a 250 mL flask	96	30	-	-	10	25	Erlenmeyer flask
***C. glutamicum* NCIM 2168**	(Shyamkumar *et al.*, 2014)	50 Glucose	-	8 Urea	K_2HPO_4-1.0, $MgSO_4$•$7H_2O$-2.5, $MnSO_4$•$7H_2O$ 0.1, $CaCO_3$ - 1.6	0.002 g/L Biotin	7	120 rpm 100 mL of media in 250 mL flask	72	30	-	-	-	5.42	Erlenmeyer flask
***C. guilliermondi* FTI 20037**	(Silva, Quesada-Chanto and Vitolo, 1997)	70 Xylose	-	5 $(NH_4)2SO_4$	1.0 Yeast Extract, 0.5 $Mgs0_4$•$7H_20$, 0.1 $CaCl_2$•$2H_20$, 1.0 KH_2PO_4	-	2.5	300 rpm aeration at 20 mL/min	40	30	-	48.6	-	-	2.5L Bench-fermenter containing 1.5 1 of the media.
***C. guilliermondi* FTI 20037**	(Gurgel *et al.*, 1998)	10 Glucose	70 Xylose	5 $(NH_4)_2SO_4$	1.0 Yeast Extract, 0.5 $Mgs0_4$•$7H_20$, 0.1 $CaCl_2$•$2H_20$, 1.0 KH_2PO_4	-	2.5	300 rpm 200 mL of media in a 500 mL flask	40	30	-	25.4	-	-	Erlenmeyer flask
C. guilliermondi **FTI 20037**	(Silva and Roberto, 2001)	85 Xylose	-	3 $(NH_4)_2SO_4$	$CaCl_2$•$2H_2$ O 0.1 Rice Bran Extract 20.0	-	-	250 rpm 50 mL of media in a 125 mL flask	25	30	8	58	-	-	Erlenmeyer flask

Based on this observation, we proposed a composition for the synthetic fermentation media (SFM); presented in Table III-4. Because the average value of total sugars in the hydrolysate was low to have a fair observation of the kinetics, it was decided to increase the total concentration of sugars up to 100 g/L. The concentrations proposed were then 65 g/L of glucose and 35 g/L of xylose, to keep similar glucose:xylose ratio (2:1). YE was chosen because it contributes as a source of micronutrients for both strains.

Table III-4.Synthetic fermentation media

	(g/L)
Glucose	65
Xylose	35
K_2HPO_4	1
KH_2PO_4	1.7
$MgSO_4•7H_2O$	3
$CaCO_3$	1.6
$(NH_4)_2(SO_4)$	3
$MnSO_4*H_2O$	0.1
$FeSO_4•7H_2O$	0.01
$CaCl_2•2H_2O$	0.1
Biotin	5.10^{-6}

As previously stated in the Coupling the microorganisms part in the bibliographical chapter, the choice of the culture pH was realized by a compromise between the maximal production pH in both strains. The optimal pH for glutamic acid production is 7, but, as stated by Follmann and collaborators (Follmann *et al.*, 2009), internal homeostasis is kept in a range between 6 and 9. In the case of *Candida guilliermondii,* the optimal production pH value is 5.5. Other pH values were tested by Rodrigues and collaborators (Rodrigues *et al.*, 2003), and they found that at pH 7.5, xylitol was produced but disadvantaged behind cell growth. Because of this, it seemed that 7 was a good compromise value for the essays.

In the bibliographic research, $CaCO_3$ presence wasn't reported in the culture media of *Candida guilliermondii*. The salt was added because its presence has a positive effect on glutamic acid production by *C. glutamicum* (Abdenacer *et al.*, 2012; Kahina, 2012). Other authors reported that calcium carbonate could also be used as a buffer for the media (Strub *et al.*, 2008; Ilmén *et al.*, 2013).

III-D. Nitrogen source

In this series, Urea and $(NH_4)_2SO_4$ are compared as nitrogen sources for *C. glutamicum* development and glutamic acid production.

A more specific bibliographic research for the composition of the production media for *C. glutamicum* to produce glutamic acid was realized. As a reminder, we decided to focus primarily on the bacteria's fermentation needs because yeast nutrient needs are not so complex as *Corynebacterium*'s culture ones. Table III-5 synthesized the composition of the media were glutamic acid was being produced by *C. glutamicum* ATCC13032.

Table III-5 Culture media used in literature for *C. glutamicum* (units are on g/L)

	SFM	(Sekine *et al.*, 2001)	(Amin and Al-Talhi, 2007)	MCGC media[11]	(Shyamkumar *et al.*, 2014)	(Kumar *et al.*, 2013)	(Kahina, 2012)	Patent media (Kinoshita, Tanaka and Akita, 1961)
Glucose	65	100	100	34-60	50	50	-	100
Xylose	35	-	-	-	-	-	-	-
K_2HPO_4	1	1	1.2	K_2HPO_4 Na_2HPO_4	1	1	-	1
$CaCl_2$•2H_2O	0.1	-	-	*	-	-	-	-
$FeSO_4$•7 H_2O	0.01	0.01	0.006	*	-	-	-	0.01
Yeast Extract	1	-	-	-	-	-	-	-
$CaCO_3$	0.24	-	-	-	1.6	1.6	5	-
Nitrogen source	3 $(NH_4)_2SO_4$	4 Urea	-	*	8 Urea	10 Urea	15 Urea	2 $(NH_4)_2SO_4$+ Urea addition
$MgSO_4$•7H_2O	2.5	1	6.2	*	2.5	2.5	0.6	0.25
$MnSO_4$•7H_2O	0.1	0.01	0.006	*	0.1	0.1	2.5	0.01
Biotin	$5•10^{-6}$	3.10^{-6}		Mix of vitamins	0.002	2.10^{-6}	5.10^{-3}	$2.5.10^{-6}$
pH	7	7	7.8	7.6	7	7	7.3	6-9

*Present but not specified.

First, it's interesting to emphasize that, in literature, pH is preferably controlled during the fermentation. From the research presented in the Culture media composition part of the bibliographic chapter, it was observed that urea and ammonium sulfate had been used as a nitrogen source. As a reminder: None of the authors have compared the effects of the nitrogen source in the ATCC13032 strain. And, the only author who has reported a comparison between nitrogen sources in a *C. glutamicum* strain were Niaz and collaborators (Niaz *et al.*, 2009). They worked with the strain *C. glutamicum* NIAB BNS-14. They found that ammonium nitrate led

[11] Used by several authors not only for 13032 but for various of the others *Corynebaacterium glutamicum* strains and GMO strain (Delaunay *et al.*, 1999b; Kahina, 2012; Kaboré *et al.*, 2017; Briki *et al.*, 2020).

the maximal glutamic acid production (5.5 g/L), followed by 4.9 g/L produced with ammonium sulfate. Urea, however, wasn't chosen as one of the nitrogen sources in their work.

Because both (urea and ammonium sulfate) had been used before in the ATCC13032 strain, an experiment was realized to observe which one was a better nitrogen source to produce glutamic acid.

As a reminder, with 65 g/L of glucose in the media at 80 % of conversion yield, maximum of 51.2 g/L of glutamic acid should be produced. 5.2 g/L of nitrogen would be needed for this production. These values correspond to a concentration of 11.3 g/L of urea. Because we want to compare with ammonium sulfate, to obtain the same nitrogen concentration in the culture media, 25 g/L would be needed in the case of ammonium sulfate.

Tests were carried out to choose a nitrogen source to enhance *C. glutamicum*'s development and glutamic acid production. This comparison was between urea and ammonium sulfate. The media used for this series is the Synthetic fermentation media presented previously in Table III-4.

Ammonium sulfate at 3 g/L was added in the media, because as reported in the first chapter, this organic source of nitrogen is better for xylitol production by *C. guilliermondii*. For the comparison 25 g/L of $(NH_4)_2SO_4$ were added to obtain a total of 28 g/L in one condition, and 12 g/L of urea were added directly to the media. pH was adjusted at 7 without followed regulation.

Table III-6. Nitrogen source tested in SFM

Condition 1	**(g/L)**	**Condition 2**	**(g/L)**
Nitrogen source	$(NH_4)_2SO_4$ 3 + Urea 12	**Nitrogen source**	$(NH_4)_2SO_4$ 28

Kinetics for both conditions are shown in Figure III-4. The curves are the mean value of duplicates. Xylose was present in the media, but it is not shown in the figure because its concentration didn't vary during the fermentation.

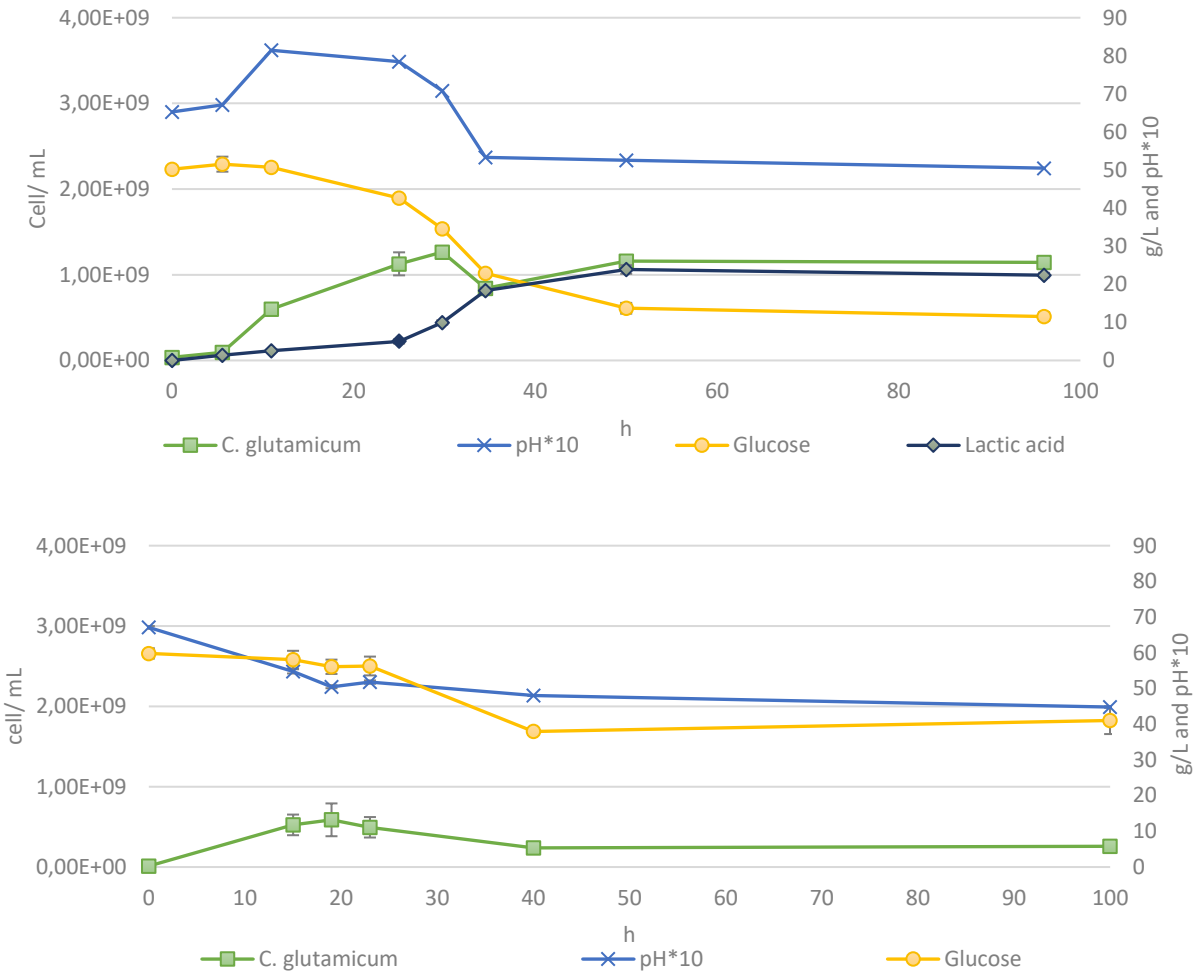


Figure III-4 Kinetics of *C. glutamicum* fermentation for different nitrogen sources in SFM. Up: Urea + ammonium sulfate. Down: ammonium sulfate only.

It was observed that more cells were produced on the media with urea. The nitrogen source on the fermentation had an impact on sugar consumption. Indeed, even if sugar consumption reached a constant value after 50 h and 40 h for both fermentation, kinetics were different, with 36 g/L of glucose consumed in the media with urea, while only 19 g/L were consumed in the media with ammonium sulfate.

pH had a different behavior depending on the nitrogen source. Indeed, *C. glutamicum* has ureases that produce NH_3 in the presence of urea (Clement, 1988), and this caused an increase in the pH. With the urea, cells consume the substrate, even at an elevated pH (9) at 20 h of culture. In the fermentation media with ammonium sulfate, pH decreased until it reached a value of 4.5, and cells stopped growth.

In terms of metabolites production, lactic acid was the only product obtained and only in urea's presence. The yield was 1.3 mol/mol, with a productivity of 0.47 g/L.h. The maximal theoretical lactic acid yield value on glucose is 2 mol/mol.

Lactic acid production suggests that again, the media volume in the Erlenmeyer flask is not adequate for glutamic acid production (lower than needed) by *C. glutamicum*. It was said before when patent media was tested that usually (Wieschalka *et al.*, 2013; Kumar, Vikramachakravarthi and Pal, 2014), lactic acid is produced as a primary metabolite, and

succinic and acetic acids are supposed to be also produced. It was stated before that probably the presence of Ca^{2+} ions prevented succinic acid production by the bacteria (Song and Lee, 2006; Shi *et al.*, 2014a).

Urea will be used as a nitrogen source for the strains because lactic acid production was obtained. The 3 g/L of ammonium sulfate will be kept in the formulation for a supplementary nitrogen source.

III-E. Culture of *C. glutamicum and C. guillermondii* in synthetic media, with the chosen conditions

As stated before, the SFM will be used for the culture of both microorganisms. The conditions previously tested and selected for the PFM (initial urea concentration and its regular addition and pH regulation) were taken over because they show to be adequated for *C. glutamicum* development and lactic acid production.

The next experiment was realized to test the chosen conditions on the selected media for each of the strains. The composition used was the SFM presented previously in Table III-4.

To remember:

- There are two nitrogen sources in the media: 3 g/L of $(NH_4)_2SO_4$ and 5 g/L of urea.
- pH is manually regulated to be between 6 and 9 with NaOH or HCl 4M, to maintain homeostasis in *Corynebacterium*'s cell.
- 1 mL of urea 10% w/v is added every 6 hours until total urea concentration attaint 12 g/L.

III-E.1 C. glutamicum ATCC13032

Results show (Figure III-5) a proper cell development of *C glutamicum* on the chosen conditions. The latency phase finished around 5 h of culture, and the exponential phase arrived at the end before 30 h.

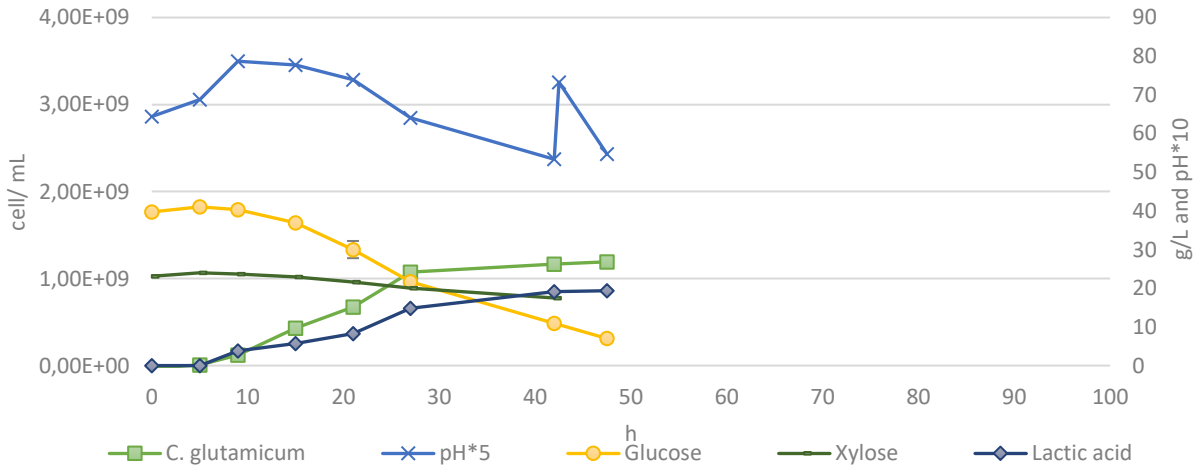


Figure III-5 Kinetics of the culture of *C. glutamicum* on SFM with urea addition and manual pH adjustment.

With the urea addition, pH variation kept the same allure seen on the patent media Figure III-2. pH started to augment, after the last urea addition and between sample taking (between 27 and 42h of culture), it slowly came down to stabilize at around 5.3 The urea addition prevented the need to adjust pH manually for the first part of the culture. It was only after 40 h that pH needs to be adjusted with the base to continue cell development.

Speaking about sugar consumption, in the case of xylose, only 5 g/L was consumed, which was expected due to the first adaptation on the media with the "Synthetic inoculation media". In the case of glucose, even if at 48 h, it wasn't wholly consumed, the glucose consumption rate attains a mean of 0.69 g/L.h. It is observable that even if cell growth attains the stationary phase, sugars continued to be consumed to produce lactic acid.

Even if there was residual glucose in the media, *C. glutamicum* produced around 20 g/L of lactic acid. This represents a production yield of 1.18 mol/mol with a productivity of 0.4 g/L.h. Being the optimal culture in a mixed sugar substrate, this condition tested in the synthetic media is yet the best for *C. glutamicum* development.

III-E.2 Candida guilliermondii FTI20037

Once the cell growth conditions were defined and validated for *Corynebacterium*, the same growth and production conditions were tested for *Candida guilliermondii*.

Candida guilliermondii was cultivated under the same conditions as *C. glutamicum.* Kinetics of this experiment are presented in Figure III-6. These results are the average value of two assays.

The lag phase wasn't observed in *Candida*, and the stationary phase started after 30h of fermentation. Results show a similar growth, like the one obtained by *Corynebacterium* (cell concentration around 10^9cell/ mL). This cell development suggested that fermentation conditions (pH adjusted between 6 and 9 and urea addition), didn't have a negative effect on the yeast development.

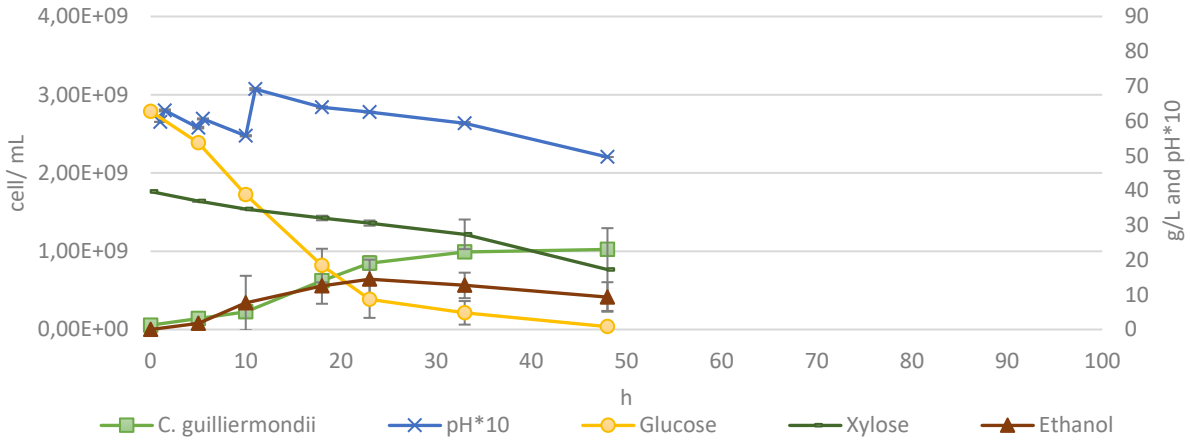


Figure III-6 *Candida guilliermondii's* kinetics on synthetic media with urea addition and manual pH adjustment.

It can be observed that glucose was consumed first. As explained in part "Products obtained from Candida guilliermondii FTI 20037" in the first chapter, the yeast can produce ethanol under oxygen limitation via fermentation in the presence of glucose and xylose also under oxygen limitation conditions in the presence of xylose. It seems that the glycolysis pathway was the one used by the yeast, and ethanol was produced. Once glucose was almost no longer available, the yeast started to consume xylose via the isomerase pathway to continue to the Krebs cycle via the pentose phosphate pathway. The average glucose consumption rate was 1.2 g/L.h, while the average xylose consumption rate was 0.409 g/L.h.

The yeast culture was stopped after 50 h of fermentation. Xylose consumption may have continued, but ethanol present in the media could affect xylitol production. In this first experiment, cell development under the chosen conditions was the priority, since aeration optimization for xylitol production will be studied in detail in the next chapter.

In this culture, ethanol productivity was 0.628 g/L.h, with a starting production after 24 h of culture. Ethanol production by *Candida guilliermondii* FTI20037 has been previously reported (Wen, 2014). According to Wen's work, the yeast was able to metabolize C6 sugars like glucose, mannose or galactose. It produced ethanol concentrations from 6 to 9.3 % w/v. In our work, ethanol yield at 24 h was of $1mol_{ethanol}/mol_{glucose}$, which corresponds to the 50 % of the theoretical value. Nor xylitol or other metabolites were detected.

It has been reported (Gurgel *et al.*, 1998; Kastner, Eiteman and Lee, 2001) that *Candida guilliermondii* delays the use of xylose until no more glucose is present in the media. Indeed, it seems that this occurs with most of the pentose consuming yeasts (Arrizon *et al.*, 2012). Roberto (Mussatto, Silva and Roberto, 2006) compared xylitol production performance on several *Candida* strains, and found that the presence of glucose reduced the xylose consumption rate by 30%.

Silva and collaborators (Silva, Quesada-Chanto and Vitolo, 1997) also reported the inhibition in xylitol production in the presence of glucose. Nevertheless, as glucose is always present in lignocellulosic hydrolyzates, they found that the best xylitol production was obtained when a ratio of 1: 6 glucose:xylose was used. Indeed, in the first part, glucose was used in cell development, and then xylose can be used for xylitol production. Because of this, even if no xylitol was produced, it was confirmed that the fermentation media composition and the fermentation conditions are adequate for *Candida guilliermondii* FTI20037 development. An adequate sugar proportion will be tested later to obtain xylitol with the yeast.

III-F. Conclusion

In this chapter, three critical aspects of the research were defined: 1) The sugar composition of the synthetic agave bagasse media; 2) the nutrients for both microorganisms and, 3) the optimization of cell development with the selected fermentation conditions.

- Chosen glucose: xylose ratio was 65:35. Salts on the media were chosen with the cross-linked nutrients for each strain.
- For the inoculation media, final composition was: K_2HPO_4 1 g/L; KH_2PO_4 1.49 g/L; $MgSO_4 \cdot 7H_2O$ 3 g/L, $CaCO_3$ 1.6 g/L $(NH_4)_2(SO_4)$ 3 g/L; $MnSO_4$*H2O 0.1 g/L, $FeSO_4 \cdot 7H_2O$ 0.01 g/L, $CaCl_2 \cdot 2H_2O$ 0.1 g/L and Yeast extract 1 g/L.
- Fermentation media was the same with the addition of Biotin 5µ g/L and urea 5 g/L with a supplementary 1 mL addition of a 10%v/w solution every 6 hours until a total urea concentration at 12 g/L.

Fermentation culture composition and conditions, which have been tested, were adequate for the cell development of both strains. They were: pH adjustment during fermentation and addition of a solution of urea at 10% w/v at a rate of 1 mL every 6 hours until 40h of culture. In these conditions, *C. glutamicum* produced 1.18 mol/mol of lactic acid, possibly because aeration wasn't enough for glutamic acid production. And, even if xylitol wasn't produced by *Candida guilliermondii*, ethanol was produced at a yield of 1 mol/mol. This because, as explained in the bibliographic research, the yeast preferred glucose as the first substrate.

In the next chapter, the tested conditions in Erlenmeyer were replicated in bioreactors to verify if aeration conditions limit glucose bioconversion into glutamic acid and xylose conversion into ethanol. Also, mixed culture strategies were tested to verify the production of the target metabolites.

IV. Optimization of fermentation conditions in reactors

The objective of this chapter is to determine the best fermentation conditions in reactor for *Corynebacterium glutamicum* and *Candida guilliermondii* for lactic acid and xylitol production

This chapter is organized into two parts. In the first one, preliminary experiments were realized to determine the effects of urea addition and calcium carbonate in bioreactor fermentation. The second part is formed by a series of experiments presented in the form of an article submitted at the "Journal of industrial microbiology and biotechnology".

IV-A. First part : preliminary experiments

IV-A.1 Tests with urea addition

In the following tests, the conditions proposed in the last chapter were tested in bioreactor. At the end of the chapter Choice of culture media in Erlenmeyer, the sugar ratio in the fermentation media was chosen (glucose:xylose ratio 65:35), pH had to be regulated at 7 and, at the beginning of the fermentation 3 g/L of $(NH_4)_2SO_4$ and 5 g/L of urea were present in the media. Once the fermentation started, a solution of urea at 10 % w/v was added until a total of 12 g/L of urea were in the media.

In the bioreactors used, pH was automatically regulated at 7with two pumps and NaOH and HCl 5 M. An external peristaltic pump was used to realize the constant urea addition at a flowrate of 0.175 g/L.h until 40 h of fermentation. The tested aeration rate was 0.5 vvm as suggested at the end of the bibliographic chapter in the part "Oxygen consumption".

a) Single fermentation

Corynebacterium glutamicum

Kinetics of the bacteria are shown in Figure IV-1, 0.1 mol/mol of lactic acid were obtained at productivity of 0.048 g/L.h. Lactic acid was the majoritarian metabolite, however, three un-identified co-metabolites were observed with the RI detector and one at 192 nm in UV detector. Either of the co-metabolites could be determined. However, aspartic acid, fumaric acid, glycerol, isocitric acid, malic acid, fumaric acid, pyruvate, succinic acid, tartaric acid, and valine, were dismissed.

This was the first time that the production of other metabolites was observed in our experiments. It seemed that the aeration conditions in the bioreactor (200 rpm and 0.5 vvm) were the main reason for the obtention of by-products. Dissolved oxygen data is not available for this round of experiments.

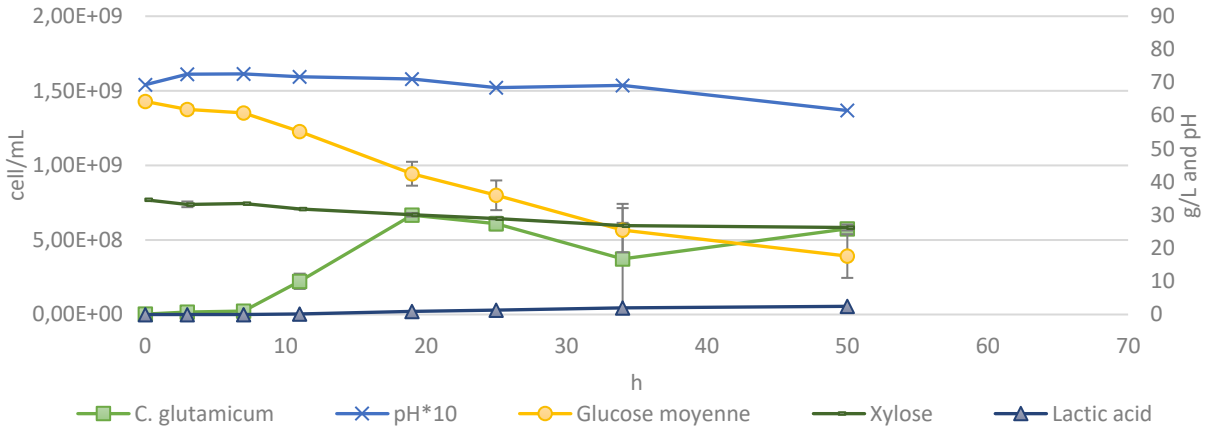


Figure IV-1. ***C. glutamicum kinetics at a glucose:xylose ratio 65:35 0.5 vvm, 200 rpm, 30°C, pH regulation at 7, initial urea at 5 g/L, followed by addition of a urea solution at 10% w/v for 40h at a flow 0.17 g/L.h.***

Glutamic acid enzymatic kit was used to verify if amino acids were produced. For the experiment, less than 0.1 g/L were detected. Which confirms that the metabolite produced wasn't glutamic acid either. It's probable that other organic or amino acids were produced and weren't able to be observed in the method used. Indeed, it has been reported that several organic acids are produced by *C. glutamicum*, Okino and collaborators (Okino, Inui and Yukawa, 2005) reported that bicarbonate presence and aeration rate changed the ratio of the molecules obtained.

Candida guilliermondii

The yeast kinetics is presented in Figure IV-2. Even if there was sugar consumption, it seems that the yeast only used the metabolite to maintain it's internal functions. Limited cell growth was observed (from 2.10^6 to 2.10^8 cell/mL) and, either xylitol or ethanol were obtained in the conditions tested. Indeed, in the last chapter ethanol was produced when the experiment was realized in Erlenmeyer flask. These results suggest that the scale change wasn't adequate for metabolite production of the yeast. Also, it has been reported that glucose:xylose ratio has an effect on xylitol production by *Candida* species. The sugar ratio effect for xylitol production will be discussed in more detail in the second part of this chapter.

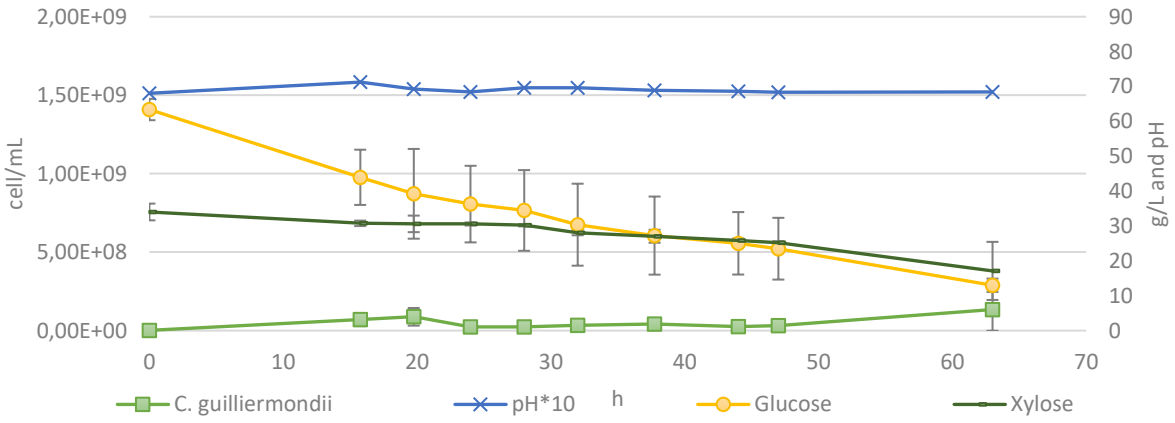


Figure IV-2 ***C. guilliermondii's kinetics at a glucose:xylose ratio 65:35, 0.5 vvm, 200 rpm, 30°C, pH regulation at 7, initial urea at 5 g/L, followed by addition of a urea solution at 10% w/v for 40 h at a flow 0.17 g/L.h.***

b) Co- fermentation

Mixed fermentation

For the mixed fermentation (Figure IV-3), the same fermentation conditions were tested (pH regulation at 7, the constant addition of urea by an external pump, 200 rpm, and aeration at 0.5 vvm).

Each strain started at a concentration of 3.10^6 cell/mL. In this culture, sugars were consumed at a rate of 0.77 g_{sugar}/L.h. Even if 55 g/L of sugars were consumed, cell development for each species was limited. It can be observed (Figure IV-3 Down) that *C. glutamicum* arrived at the exponential phase. At the end of 3 days, only lactic acid was produced with a yield of 0.05mol/mol. This yield is obtained with the hypothesis that glucose was consumed only by the bacteria.

Dissolved oxygen consumption continued for the first 24 h of culture. This could be due to the cell adaptation at the beginning of fermentation. At around 30 h of culture, dissolved oxygen percentage augmented, to around 70 %, this consumption was mostly due to *C. glutamicum*'s growth and the continuity of the bacteria's metabolism to survive in the fermentation media.

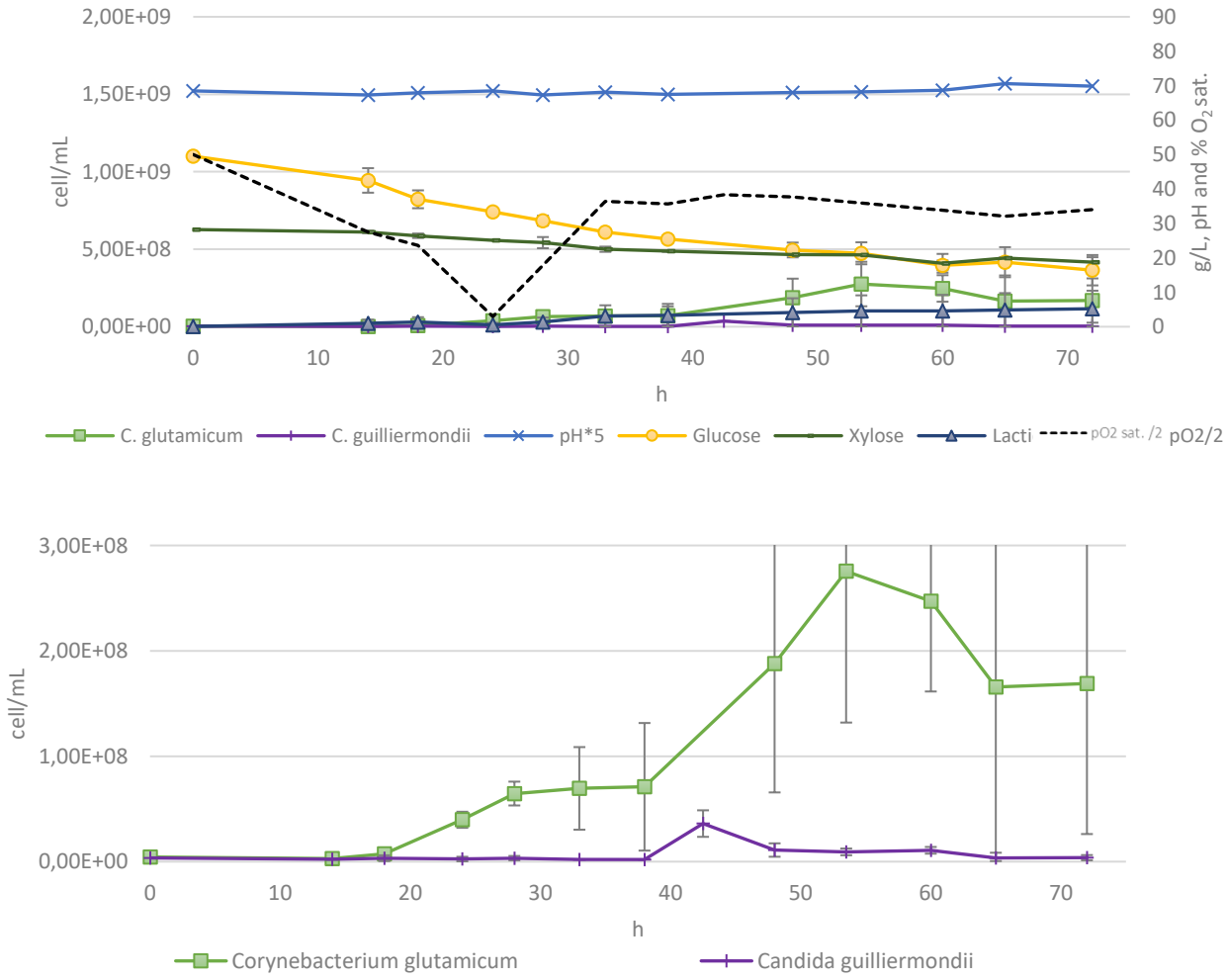


Figure IV-3. Up: Kinetics on the mix fermentation with added urea. Down: cell growth for both strains.

Sequential fermentation

Sequential fermentation was realized with two different fermentation conditions. On the first 48 h, pH was controlled at 7. When glucose concentration reached 15 g/L, pH was changed to 5. This pH was selected, to surpass the homeostasis in *Corynebacterium glutamicum* and prevent it from developing (Follmann *et al.*, 2009). The acidic pH was chosen for *Candida guilliermondii* because it had been reported (Rodrigues *et al.*, 2003) that this was the optimal xylitol production pH.

A media of the kinetics of both the experiments is shown in Figure IV-4. Lactic acid was produced at a concentration of 35 g/L. *C. guilliermondii's* concentration can't be shown on the graphic because of its low concentration. The yeast concentration at the end of the fermentation was 3.7 10^5 ± 1.3 10^5 cell/mL.

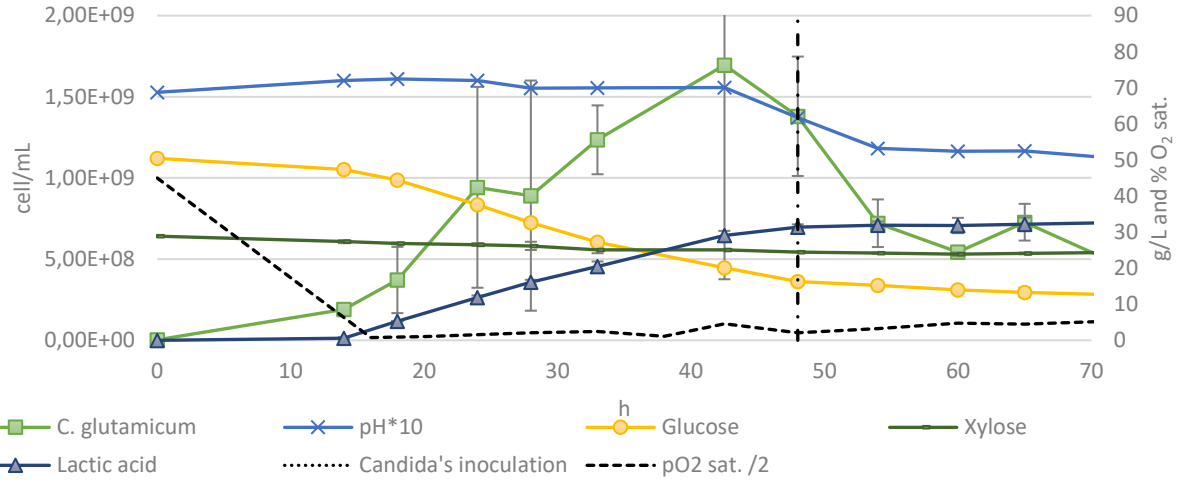


Figure IV-4 Media of the kinetics on both reactors for sequential fermentation with the addition of urea at 0.5vvm with initial pH adjusted at 7, followed by pH adjustment at 5.

At an aeration rate of 0.5 vvm (kLa 9 h^{-1}), only lactic acid was produced. In the entire culture, glucose was the main consumed sugar at an average consumption rate of 0.52 g/L.h. The maximal consumption was during *C. glutamicum*'s exponential phase. Which also corresponds with the maximal production rate of lactic acid in the culture. After 32 h of sequential fermentation, 32 g/L of lactic acid were produced. This corresponds to a yield of 1.69 $mol_{lactic\ acid}/mol_{glucose.}$ In the hypothesis of glucose was only consumed by *C. glutamicum*.

pH effect is only observable on *Corynebacterium* because bacteria's concentration decreased with the pH decrease at the beginning of the second part. For *Candida*, on the contrary, xylose wasn't consumed. So, no xylitol was obtained under these fermentation conditions.

Better results were obtained in sequential fermentation than in mixed fermentation. Indeed, 31.4 g/L of lactic acid were produced on the sequential fermentation vs. 2.7 g/L on the mixed fermentation. Because in the first 48 h of the sequential fermentation, the only difference between the fermentation was *Candida* in the media, it seems fair to say that the competition for the substrate is the main reason to avoid this approach.

It was observed that for the cultures at 0.5 vvm flowrate, lactic acid was the main metabolite, because 1.6 mol/mol of were produced as main product with 3 un-identified co-metabolites observed with the RI detector and one at 192 nm in UV.

c) Effect of urea addition in reactor for C. glutamicum culture in reactor

Because the additional urea was first chosen to have enough nitrogen source to produce glutamic acid with *C. glutamicum*, and at the aeration conditions in reactor lactic acid is the main metabolite, a test was realized to verify if no additional urea had to be added during culture. Fermentation conditions were replicated (30°C, 0.5 vvm and 200 rpm) the only difference was the regular addition of urea. The first 48 h of the sequential fermentation (Figure IV-4) were compared against the first 48 h of the kinetics presented in Figure IV-5.

Cell concentration was two times higher in the media with supplementary urea. However, the sugar consumption rate was around the same in both cases (0.7 g/L.h). Lactic acid was the main metabolite obtained under both conditions. Production yield were: 1.8 mol/mol with urea supplementary addition and 1.3 mol/mol without it. Lactic acid yield is slightly higher in the condition with urea, however, the use of external pumps is required which in terms of a larger process would mean more energy and complication in the process.

It is proposed to eliminate the urea supplementary addition. This seems to be a good compromise between process simplicity and energy requirements for industrial applications versus production yield.

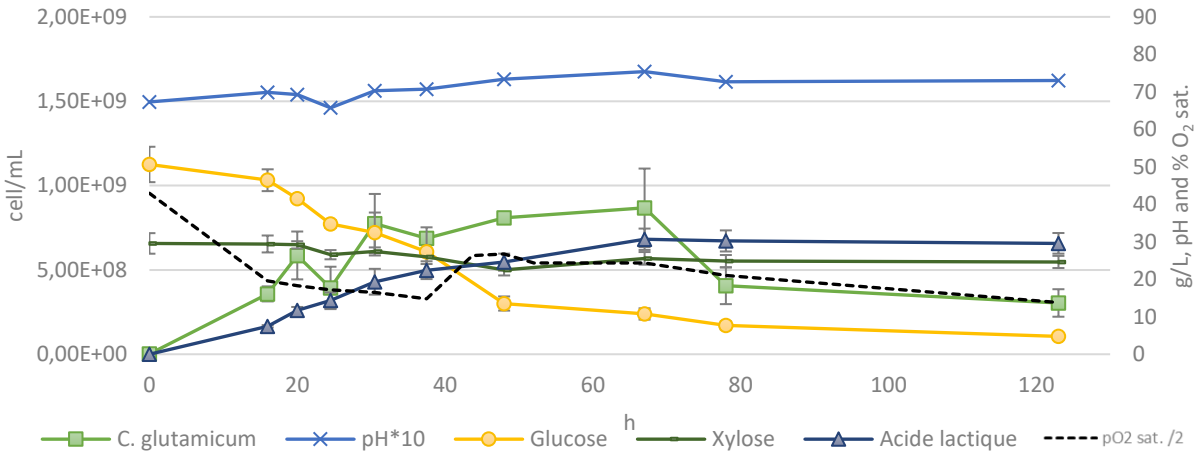


Figure IV-5 ***C. glutamicum kinetics at a glucose:xylose ratio 65:35 0.5 vvm, 200 rpm, 30°C, pH regulation at 7, initial urea at 5 g/L.***

Mixed fermentation is not a good strategy to obtain metabolites from a mix of sugars with C. *glutamicum* and C. *guilliermondii*. Urea supplementary addition was eliminated from the fermentation conditions. More studies have to be realized to find the fermentation conditions for xylitol production by C. *guilliermondii* after the first stage of the fermentation.

IV-A.2 Effect of calcium carbonate in the strains

Now that urea addition was eliminated from the fermentation conditions, the presence of calcium carbonate was analyzed. Tests in reactor were performed on both species. The objective was to determine the effect of salt on the production of the desired metabolite.

a) Corynebacterium glutamicum

Results of the *Corynebacterium glutamicum's* culture without carbonate are present in Figure IV-6, $CaCO_3$ effect can be obtained comparing with Figure IV-5. Results show that glucose consumption is slightly lower in the presence of the salt (0.37 g/L.h vs. 0.45 g/L.h). However, regarding lactic acid yield, calcium carbonate increased production by 30 % (1.3 mol/mol versus 0.98 mol/mol). Indeed, *C. glutamicum* has the advantage of having anaplerotic pathways that help the production of cells, and the glucose consumed can be oriented into lactic acid production.

Dissolved oxygen profile in the fermentation varied without calcium carbonate in the culture media. When the salt is eliminated from the media, it is observed that dissolved oxygen in the reactor is completely consumed, whereas in the presence of carbonate, it was slightly consumed, confirming that *C. glutamicum* passed for the anaplerotic pathway for cell development.

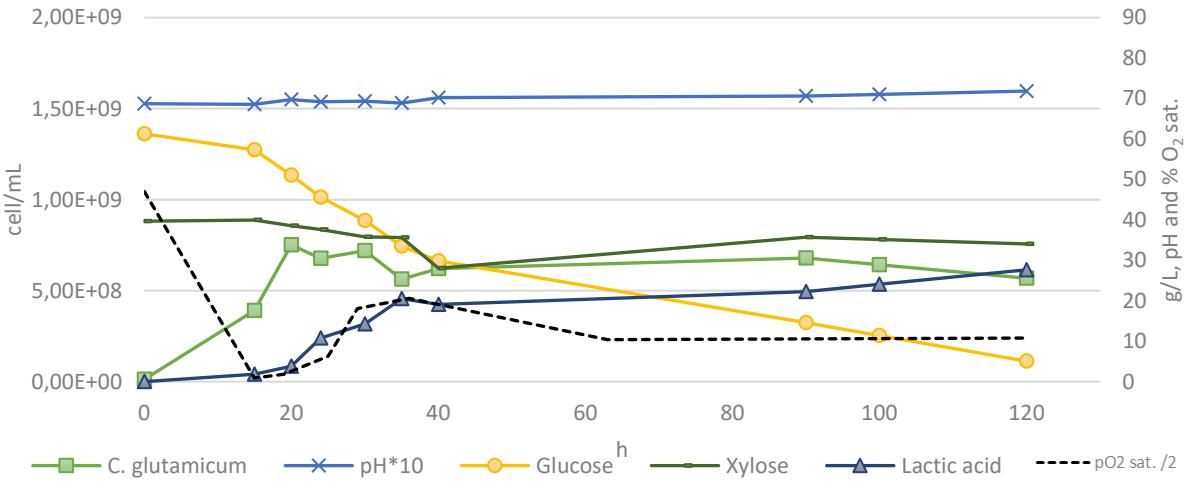


Figure IV-6 Culture of *Corynebacterium glutamicum at* 0.5vvm, 30°C, pH automatically adjusted at 7. Without $CaCO_3$.

In conclusion, for the bacteria, it is possible to dismiss carbonate in the culture. This could simplify the culture medium. Indeed, at pH regulated at 7 and with calcium carbonate, the culture medium precipitates. The 30 % less production of lactic acid could be gain in terms of energy and costs savings in the process.

b) Candida guilliermondii

To probe the calcium carbonate effect on *C. guilliermondii*, the test was realized on a media with less glucose:xylose ratio. Several authors (Silva, Quesada-Chanto and Vitolo, 1997; Kastner, Eiteman and Lee, 2001; Kwon, Park and Oh, 2006) reported that the yeast preferred a smaller proportion to produce xylitol. The reason is that a high concentration in glucose inhibits xylose consumption in the yeast by carbon catabolite repression (CCR) since most microbes possess it (Kim, Block and Mills, 2010). But, a low concentration of glucose is recommended, as this be used in cell production, and once it is fully consumed, then the yeast can metabolize xylose.

Mix fermentation couldn't be realized because of the competition between the species. Therefore, *Candida*'s inoculation could be realized once *Corynebacterium* had finish lactic acid production.

Results show (Figure IV-7), that $CaCO_3$ had a significant impact on xylitol production in the tested culture conditions. Indeed, xylitol was produced at a yield of 0.71 mol/mol**.** On the contrary, when no calcium carbonate is used, the cell wasn't even able to attain the exponential phase. Suggesting that *C. guilliermondii* has anaplerotic pathways. In the anaplerotic pathway, If the strain has the enzyme "pyruvate carboxylase", pyruvate can be metabolized into oxaloacetate in the presence of calcium carbonate, biotin, and glucose. As, it has been reported that anaplerotic pathways are present in some *Candida* species (Ermakova *et al.*, 1986). This means that it could be possible that this strain has the enzyme "pyruvate carboxylase" leading cell development, and have an influence in xylitol production.

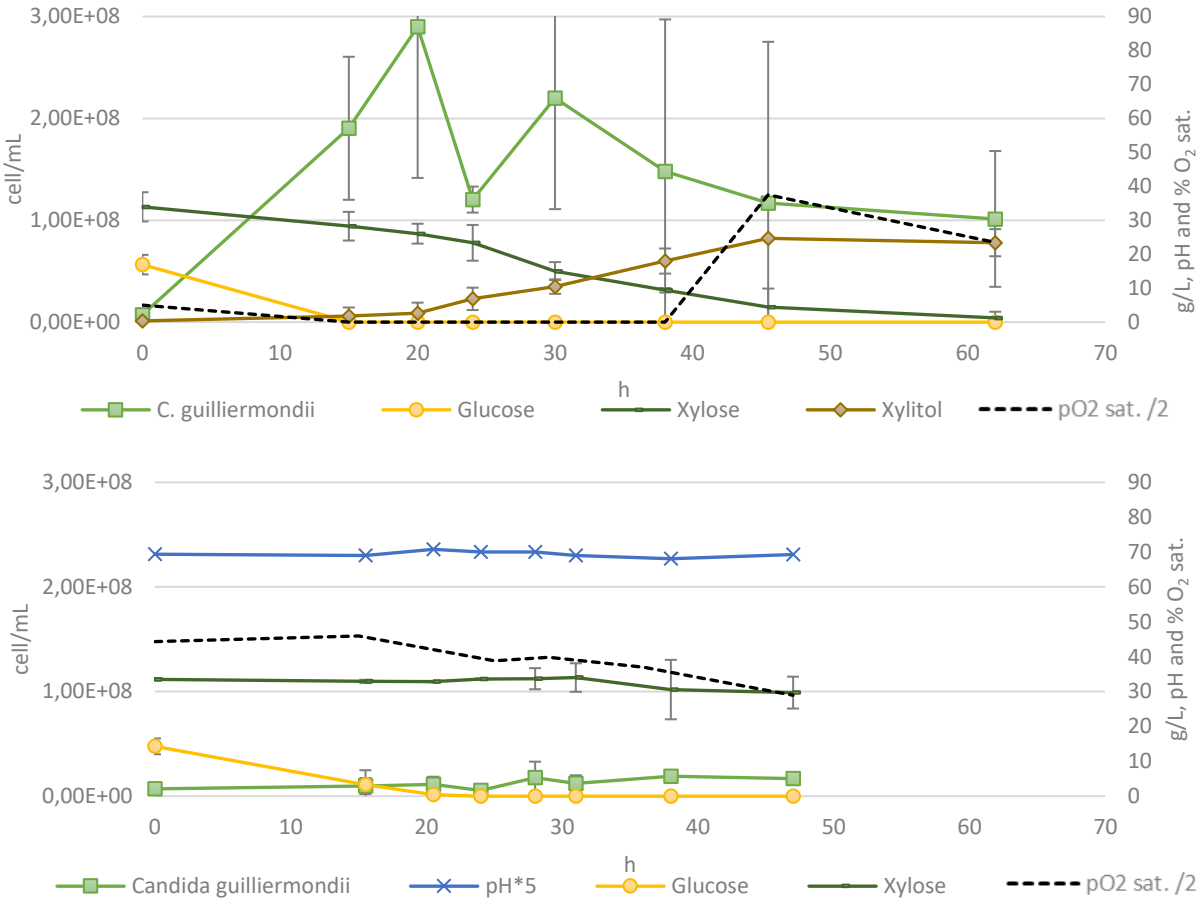


Figure IV-7 Culture of *Candida guilliermondii at* 0.5vvm, 30°C, pH automatically adjusted at 7. Up: in the presence of $CaCO_3$. Down: Without $CaCO_3$.

The results obtained in this part showed that under the fermentation conditions proposed in this work, $CaCO_3$ in the culture media is essential for xylitol production by C. *guilliermondii*.
Even if the presence of the salt for Lactic acid production could be neglected, we propose to have it since the beginning of the culture.

IV-B. Article submitted at "Journal of Industrial Microbiology and Biotechnology"

Co-fermentation to produce chemical building blocks from lignocellulosic hydrolysate.

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Abstract

Co-production of value-added molecules in biorefining and simplification of hydrolysate preparation before fermentation are the principal strategies that make biorefineries competitive on the market. The model sugar composition proposed simulates sugar ratio of agave bagasse hydrolysates. Three co-fermentation strategies were tested for lactic acid and xylitol production from the sugar mix. No cell growth was observed in mixed fermentation. *Corynebacterium glutamicum* produced $25g_{lactic\ acid}/L$ in 48h, in a medium with a glucose:xylose ratio of 65:35, 0.5vvm, and pH regulated at 7. *Candida guilliermondii* was tested in different conditions of aeration and pH regulation to produce xylitol in a medium composed of glucose:xylose (15:35) and $25g_{lactic\ acid}/L$ to simulate the medium pre-fermented by *C. glutamicum*. Xylitol was produced at 0.63 mol/mol after 60h in fermentation conditions: 0.1vvm, pH 7 without regulation. Strain interactions prevented xylitol production. Bacteria had to be eliminated in sequential fermentation. However, in real sequential fermentation, no xylitol was produced.

Keywords

Co-fermentations, biorefineries, lactic acid, xylitol, mixed sugars.

Introduction

The world of biorefining is mainly centered on biofuel production, which is still not an attractive investment because production costs are too high, given the low selling prices. However, biorefining offers a sustainable green option that uses lignocellulosic waste fermentation to produce a wide range of marketable bioproducts and bioenergy to compete with petrochemical refining (Choi *et al.*, 2015). One of the main ways to make biofuels profitable would be to invest in multi-product biorefineries (Sacramento-Rivero *et al.*, 2010), producing biofuels and value-added molecules, and completely using all the lignocellulosic waste. This would require prior hydrolysis of the biomass to obtain a mix of sugars, the composition of which would depend on the proportions of cellulose, hemicellulose, and lignin in the original biomass. In lignocellulosic biomass, the main components are C5 and C6 sugars.

Nevertheless, there are several issues in residue valorization by microbial fermentation. First, not all microorganisms can metabolize C5 sugars. This limits the choice of microorganisms that can be used to valorize lignocellulosic residues. Then, in a mix of sugars, the microorganism usually prefers the simplest carbon source and does not metabolize the more complex ones (Suryadi *et al.*, 2000; Chen, 2011). Moreover, carbon catabolite repression could limit sugar consumption in a sugar mix (Khunnonkwao *et al.*, 2018). Because of this repression, a supplementary stage of sugar separation has to be added to the process before the transformation. Since sugars

are close in size and structure, their separation requires a lot of energy. This stage causes a longer, more energy demanding and more expensive process.

Several strategies have been studied to overcome this mixed sugars issue. The first one is the genetic modification of the strains (Li *et al.*, 2010; Gonçalves *et al.*, 2014; Argyros *et al.*, 2017) or adaptive evolution (Li *et al.*, 2019). In this work, genetic modification of strains was set aside because of the risk of low stability over time. The second strategy is to find a pair of microorganisms (genetically modified or not) to maximize the production of one molecule. Several authors (Bader *et al.*, 2010; Chen, 2011; Jiang *et al.*, 2017) list different pairs of microorganisms that have been proposed so that glucose can be metabolized by one microorganism and xylose by the other, mostly for ethanol production. A third strategy is to use different microorganisms to produce different products from the same biomass. This method has been studied mainly for xylitol and ethanol production (Arrizon *et al.*, 2012; Castañón-Rodríguez *et al.*, 2015; Zahed *et al.*, 2016). Arrison and his team (Arrizon *et al.*, 2012) used sugarcane bagasse as the substrate and, in sequential co-fermentation, they obtained yields of 0.22 $mol_{bioethanol}/mol_{glucose}$ and 0.4 $mol_{xylitol}/mol_{xylose}$. Castañón and collaborators (Castañón-Rodríguez *et al.*, 2015) reported that the use of the co-culture strategy by sequential fermentation, in continuous multi-step cultures of ethanol and xylitol, increased ethanol production by 50 %. The results obtained by the authors suggest that the method can work when the strategy is adapted to the strains used.

In the present work, this last strategy was investigated, as it can achieve the objective of producing several molecules. Concerning the products, different molecules with added value to the industry will be proposed instead of bioethanol, which has been largely studied.

The strategy proposed to diversify products and save costs, time and energy is thus a co-fermentation (either mixed or sequential) by a pair of microorganisms. Mixed fermentation occurs when both strains are inoculated at the same time. In sequential fermentation, one strain is inoculated and cultivated, and the second one is added afterwards (García *et al.*, 2017). In our work, one microorganism will be responsible for producing a molecule of added value from glucose, and another microorganism will produce a second molecule from xylose. For this to be achieved, several limitations have to be overcome, discussed, and tested.

All of them are important, but the first one to overcome is the inability of microorganisms to consume xylose. It is followed by the compatibility of production conditions (temperature, pH, aeration), which are specific to each microorganism. Next, would be the possible inhibitions caused by the products or co-products obtained and, no less important, the interactions between the chosen species.

For this work, *Corynebacterium glutamicum* was chosen to produce lactic acid from glucose and *Candida guilliermondii* for the production of xylitol from xylose. These microorganisms were selected for their compatibility of culture conditions. It has been reported that the maximum production temperature for each strain is 30°C, and both have to be in limitation of oxygen to produce the metabolite of interest (Amin, 1994; Soleimani and Tabil, 2014). In addition, the pH range is flexible in the case of *C. glutamicum* (Follmann *et al.*, 2009) and this microorganism was chosen because, according to the nutrients and the aeration, it can produce a wide variety of molecules (organic acids and amino acids) (Hermann, 2003; Becker and Wittmann, 2012).

The target molecules have different commercial applications. Lactic acid is used in a wide variety of industries. It is much used in the food industry as a preservative and to give flavor because of its acidity, and its water-retaining and moisturizing properties make it interesting in pharmaceutics. Also, in the plastics industry, it is interesting because the L-form can be polymerized into polylactic acid, a biodegradable and biocompatible polymer (Komesu *et al.*, 2017; Krishna *et al.*, 2018). Xylitol is a polyalcohol, mainly found in fruits and vegetables. Its main application area is in the food industry because of its sweetening and anti-cariogenic properties. It is useful for people with diabetes because it does not need insulin to be metabolized in the body (Silva, Quesada-Chanto and Vitolo, 1997; Rodrigues *et al.*, 2003).

In this work, a model culture medium simulating the sugar ratio (glucose:xylose) in an agave bagasse hydrolysate composition (Hernández-Salas *et al.*, 2009; Saucedo-Luna *et al.*, 2011; Arrizon *et al.*, 2012; Arreola-Vargas *et al.*, 2015, 2016) was used. This lignocellulosic residue was proposed because Mexico's production of beverages derived from agave is increasing year by year, generating tons of residues. For Tequila production alone, 418 000 tons of bagasse were discarded as waste in 2019 (Tequila-Regulatory-Council, 2020).

In this work, we aim to propose a co-fermentation process to produce lactic acid and xylitol from mixed sugars. Three strategies will be tested to determine the conditions for lactic acid and xylitol production: mixed fermentation (direct inoculation of both strains), sequential fermentation, and sequential fermentation with the elimination of the first cultivated strain. Different culture conditions will be tested to determine the best for the production of lactic acid and xylitol from a mix of sugars.

Materials and methods

The strains used for this experiment were *Corynebacterium glutamicum* (DSM20300) and *Candida guilliermondii* (FTI20037). The strains were conserved on LB and YPD agar slants, respectively, at 4 °C.

Inoculation preparation

Corynebacterium glutamicum was reactivated on LB medium at 30°C and 200 rpm for 7 h. Ten milliliters of the reactivation culture was added to 100 mL of the inoculation medium, composed of (g/L): glucose (32.5), xylose (17.5), K_2HPO_4 (1.5), KH_2PO_4 (1.7), $CaCl_2 \cdot 2H_2O$ (0.1), $FeSO_4 \cdot 7\ H_2O$ (0.01), yeast extract (1), $CaCO_3$ (1.6), $(NH_4)_2SO_4$ (3), $MgSO_4 \cdot 7H_2O$, (2.5), $MnSO_4 \cdot H_2O$ (2.5). To prepare the medium, sugars and yeast extract were autoclaved together and the salts were added by the preparation of concentrated solutions, which were sterilized and added after autoclaving. To prepare the concentrated solution of $CaCO_3$, HCl was added to dissolve it in water. The cultures were carried out at 30°C and 200 rpm on an orbital shaker for 24 h.

Candida guilliermondii was reactivated on YPD medium at 30°C and 200 rpm for 48 h. Five milliliters of the reactivation culture was added to 100 mL of the inoculation medium. Incubation was performed for 24 h at 30°C and 200 rpm on an orbital shaker.

Fermentation

Four bioreactors (Eppendorf Bioflo 110 and 120) were used for the experiments. Dissolved oxygen and pH were measured with Mettler Toledo probes. Automatic pH regulation was performed with NaOH, and HCl 5 M. pH was maintained at 7 when the test required it. Results shown are the average value of two fermentations, with their standard deviation.

The fermentation medium was composed of (g/L): glucose (65), xylose (35), K_2HPO_4 (1.5), KH_2PO_4 (1.7), $CaCl_2 \cdot 2H_2O$ (0.1), $FeSO_4 \cdot 7\ H_2O$ (0.01), yeast extract (1), $CaCO_3$ (1.6), $(NH_4)_2SO_4$ (3), $MgSO_4 \cdot 7H_2O$, (2.5), $MnSO_4 \cdot H_2O$ (2.5), Urea (5) and Biotin (5E-6). Constant fermentation conditions were 1.2 L of fermentation media, with pH regulated at 7 (when the experiment required it) by the internal pumps in the bioreactors, agitation at 200 rpm, and temperature at 30°C. Aeration was tested at 0.5 (kLa: 9 h^{-1}) and 0.1 vvm (kLa: 5 h^{-1}). An initial concentration of $5 \cdot 10^6$ cell/mL was inoculated in a sterile way to start the fermentation.

Single fermentations were performed for each strain in the bioreactor in a medium with a glucose:xylose ratio of 65:35, pH regulation at 7, at 200 rpm agitation, and 0.5 vvm aeration.

Two types of sequential fermentation were performed. For the first one, *C. glutamicum* was cultivated at 0.5 vvm, with pH regulated at 7, temperature at 30°C, and agitation at 200 rpm. Once the glucose concentration reached around 15 g/L, aeration was changed to 0.1vvm, pH was no longer regulated, and *Candida guilliermondii* was inoculated into the fermentor. For the second sequential fermentation, the first part remained the same but, when glucose reached 15 g/L, the pH was lowered to 5.5 with HCl 5 M to dissolve calcium carbonate in the medium. To eliminate *Corynebacterium glutamicum* cells, the culture medium was centrifuged for 10 min at 4000 rpm in a Thermo scientific Megafuge 40 R centrifuge, followed by sterile filtration in a Thermofisher Nalgene rapid flow 90 mm filtration unit. The recycled culture medium was added to a previously sterilized reactor, pH was adjusted to 7, temperature was regulated at 30°C, aeration at 0.1 vvm, and agitation at 200 rpm. The addition of *C. guilliermondii* determined the beginning of the second part of the fermentation.

Cell concentration

Cell concentration was determined by microscopic cell count. *Corynebacterium glutamicum* was counted on a Neubauer chamber on a 65 X objective. *Candida guilliermondii* concentration was determined with a Thoma chamber on 40 X objective.

Sugar analysis

Sugars (glucose and xylose) and metabolites (lactic acid and xylitol) were determined by HPLC (Thermo Scientific, Dardilly, France). Samples were centrifuged at 4300 rpm for 4 min and the supernatant was filtered with a 0.2 µm filter. Analyses were performed with a Rezex ROA H+ 8%, 250 x 4.6 mm ion exclusion column (Phenomenex, Le Pecq, France) thermostated at 30°C. Sulfuric acid at 10 mM was used as a mobile phase at a flow rate of 170 µL/min. An RI detector at 40°C and a UV detector at 210 nm were used.

Nitrogen concentration

The available nitrogen source was analyzed with a Megazyme Urea/Ammonia (Rapid) kit [K-URAMR 10/18].

Results and discussion

In single fermentation, *Corynebacterium glutamicum* (Fig. 1) cells attained the stationary phase at around 48 h, followed by a decrease in cell numbers from 78 h. The glucose consumption rate was maximum (0.77 g/L.h) at 48 h. Under the fermentation conditions tested, 30 g/L of lactic acid (1.3 $mol_{lactic\ acid}/mol_{glucose}$) was produced in 78 h. After this time, sugar consumption was mostly limited by the death of the cells in the medium. Okino and Yukawa (Okino, Inui and Yukawa, 2005) reported a yield of 1.8 mol/mol on *C. glutamicum* R. in a medium with glucose as the only carbon source. In our results, it was observed that, even if the bacteria were cultivated in a mix of

sugars, and although a small quantity of xylose was consumed (7 g/L in 48 h), the yield obtained was comparable with those reported in Okino's work in a glucose medium.

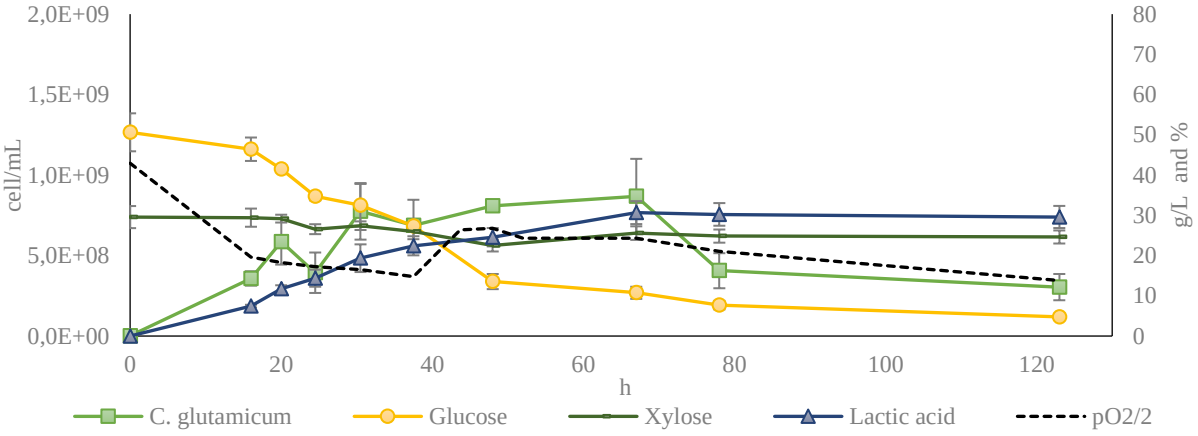


Fig. 1 *C. glutamicum kinetics for lactic acid production at 0.5 vvm 200 rpm 30 °C and pH regulation at 7*

For *Candida guilliermondii* in the same conditions as those tested for *C. glutamicum*, no glucose or xylose was consumed, and no cell growth was observed with a glucose:xylose ratio of 65:35 (data not shown). These results could be due to the glucose:xylose ratio tested, which was itself the result of catabolite repression (Khunnonkwao *et al.*, 2018). Da Silva and collaborators (Da Silva *et al.*, 2007) reported that the effect of glucose:xylose ratio was possibly due to catabolite repression. The sugar composition in the medium interferes with the production of the enzyme xylose reductase, which is responsible for xylose assimilation. It has been reported (Silva, Quesada-Chanto and Vitolo, 1997; Kastner, Eiteman and Lee, 2001; Kwon, Park and Oh, 2006) that the glucose:xylose ratio has an influence on xylitol production by the *Candida* species. The authors suggested that a low concentration of glucose helped cell development and retarded xylose consumption. They consistently state that, once glucose is fully consumed, xylitol is produced from the five-carbon sugar in a higher yield than when xylose alone was the primary source of carbon.

A sugar ratio of 15:35 was therefore tested (Fig. 2a) under the same fermentation conditions (30°C, 200 rpm, 0.5 vvm, pH regulated at 7). With this new ratio, glucose and xylose were consumed during this first growth phase. The cell attained its maximum growth at 20 h of culture (which corresponds to glucose depletion), and the yeast passed to metabolize xylose. Xylitol was produced at a yield of 0.7 $mol_{xylitol}/mol_{xylose}$ with a productivity of 0.54 g/L.h. This corresponds to the influence of the glucose:xylose ratio reported previously by various authors (Silva, Quesada-Chanto and Vitolo, 1997; Kastner, Eiteman and Lee, 2001; Kwon, Park and Oh, 2006). Glucose was indeed consumed first, improving cell development, and was then followed by xylose metabolization. The xylitol production yield obtained was comparable to the result obtained by Felipe and collaborators (Felipe, Vitolo and Mancilha, 1996). In their work, the yeast produced xylitol at a yield of 0.88 mol/mol (productivity of 0.28 g/L.h) when it was cultivated in a medium containing 30 g/L of xylose and 7 g/L of glucose, at 30°C with an aeration rate of 0.6 vvm. The productivity obtained in our research was twice as high, the reason probably being the glucose concentration present in our medium: more substrate was available, which means that more cells would have been produced to metabolize xylose.

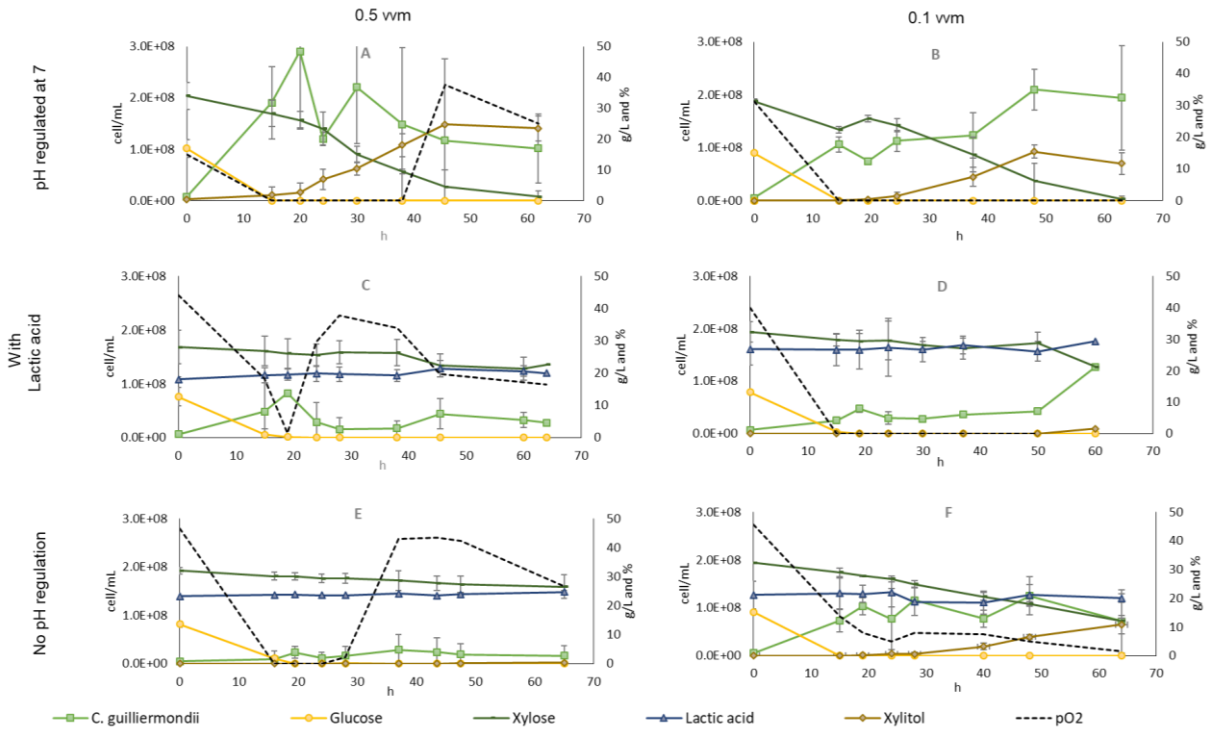


Fig. 2 *C. guilliermondii's kinetics at 30°C, 200 rpm on a medium with 15 g/L glucose: 35 g/L xylose under different conditions: a: pH 7, 0.5 vvm; b: pH 7, 0.1 vvm; c: pH 7, 0.5 vvm and 25 g/L of lactic acid; d pH 7, 0.1 vvm and 25 g/L of lactic acid; e: pH not automatically adjusted, 0.5 vvm and 25 g/L of lactic acid; f: pH not automatically regulated, 0.1 vvm and 25 g/L of lactic acid*

Nitrogen availability for Candida

Before carrying out the mixed or sequential fermentation, it was necessary to verify the amount of nitrogen available in the form of urea and ammonium to confirm whether, at the end of the lactic acid production, there was still enough nitrogen available for *Candida*. Urea and ammonia concentrations were analyzed for each strain at the beginning and the end of the single fermentation. The results are shown in Table 1. It can be observed that, in the first 48 h, *C. glutamicum* consumed all the urea present in the media, and excreted ammonium by hydrolyzation of urea (Sigurdarson, Svane and Karring, 2018). For *Candida*, results show that the yeast does not use urea. Instead, it prefers ammonia as a nitrogen source. With these results, it is sure that no additional nitrogen is needed to proceed with the sequential fermentation.

Table 7 Urea and ammonia content at the beginning and after 48 h of fermentation in single fermentation for each strain. For C. glutamicum, a sugar concentration of 65 g/L of glucose and 35 g/L of xylose. For C. guilliermondii at a sugar concentration of 15 g/L of glucose and 35 g/L of xylose

	Urea (g/L)		NH_4 (g/L)	
Fermentation time	0 h	48 h	0 h	48 h
C. glutamicum	4.27±0.07	0	0.70±0.05	1.98±0.7
C. guilliermondii	4.42±0.26	4.93±0.24	0.74±0.04	0.12±0.03

Co-fermentation

A mixed fermentation (0.5 vvm, 200 rpm, pH regulation at 7, temperature of 30°C) was then carried out to determine the feasibility of simultaneous cell development, lactic acid, and xylitol production. Although an earlier test had shown that the sugar composition repressed the yeast in the media, the condition was evaluated because, technically speaking, it was the best process for obtaining a one-step fermentation because, in the same conditions,

no growth was observed with either of the strains (data not shown). The principal reasons for these results may be the interaction between the microorganisms, competition between microorganisms for the sugar, and/or sugar repression on *C. guilliermondii* caused by the sugar composition in the medium. As the mixed fermentation strategy was not adequate, a strategy of sequential fermentations was investigated.

It was found that a sugar ratio of 15:35 was adequate for xylitol production by *C. guilliermondii*. This sugar ratio was obtained after 48 h of *C. glutamicum* culture. At that fermentation time, 25 g/L of lactic acid had been produced in the medium. In a sequential fermentation, the medium in which *C. guilliermondii* will transform xylose into xylitol would have the following composition: glucose:xylose ratio of 15:35 and also 25 g/L of lactic acid. Although *C. guilliermondii* could produce xylitol with a glucose:xylose ratio of 15:35 (Fig. 2: a), the presence of a high concentration of lactic acid could influence *C. guilliermondii*'s growth and xylitol production, even more so if this concentration was combined with a pH or aeration effect. The pH value is especially linked to lactic acid dissociation in the medium (Rodrigues *et al.*, 2003). As, in sequential fermentation, the conditions could change for each species, the effect of different aeration rates, lactic acid concentrations, and pH regulations were tested for xylitol production by *C. guilliermondii* for a glucose:xylose ratio of 15:35.

Aeration effect on Candida guilliermondii

Two aeration rates were compared with the low glucose:xylose ratio (15:35): 0.5 and 0.1 vvm (kla of 9 h^{-1} and 5 h^{-1} respectively) (Fig. 2: a and b).

It was observed that, even though the same xylose concentration was consumed (32 g/L), a higher xylitol yield (0.7 mol/mol vs. 0.36 mol/mol) was achieved at 0.5 vvm, giving productivities of 0.54 g/L.h at 0.5 vvm and 0.32 g/L.h at 0.1 vvm. The yeast growth was also faster with the highest aeration. In this condition, the dissolved oxygen in the medium was zero during the exponential phase, followed by an increment once the stationary phase was attained. In contrast, at 0.1 vvm, no dissolved oxygen was detected along with the fermentation. This suggests that, at 0.1 vvm, the dissolved oxygen was much more limiting in the medium and the substrate was used mainly for cell growth instead of xylitol production.

In the same way, productivity increased with increasing aeration rate. These results are in agreement with those obtained by Felipe and collaborators (Felipe, Vitolo and Mancilha, 1996). In their work, they compared the agitation at 200 rpm under three different flow rates: 0.4, 0.6, and 0.8 vvm. They found that at 0.4 and 0.6 vvm, the same number of cells was produced. However, twice as much xylitol was produced under the 0.6 vvm condition. In our results, for both aeration conditions, xylose was fully consumed after 60 h of culture. It has been reported (Felipe, Vitolo and Mancilha, 1996; Walther, Hensirisak and Agblevor, 2001) that *Candida* reabsorbs xylitol once there is no more substrate in the medium meaning that xylitol production had to be stopped until no more xylose was present on the media. The xylitol production confirmed that the yeast preferred a smaller sugar ratio (Fig. 2: a), that these operating conditions were adequate for bioconversion.

Effect of lactic acid on C. guilliermondii at different aeration rates with pH regulated at 7

The same two aeration rates were tested in the same culture medium with a glucose:xylose ratio of 15:35 and with 25 g/L of lactic acid added. The presence of lactic acid simulated the end of the first fermentation stage, in which *C. glutamicum* consumed most of the glucose present in the medium.

It was observed that *C. guilliermondii*'s development was limited (it changed from 10^8 to 10^7 cell/mL) in the presence of lactic acid. The yeast was not able to produce xylitol whether the aeration was 0.5 vvm (Fig. 2: a and c) or 0.1 vvm (Fig. 2: c and d). These results suggest that the presence of the organic acid had an inhibitory effect on cell development whatever the aeration rates.

The dissolved oxygen measurements show that, at 0.5 vvm (kLa: 9 h^{-1}), the saturation was maximal after glucose consumption, and even under those conditions, the yeast had difficulty in developing. At 0.1 vvm (kLa: 5 h^{-1}), saturation level reached zero, as kLa was lower, less oxygen was available, obliging the cell to develop as it did at higher aeration. In both cases, two phases were observed in cell development: a first growth stage, corresponding to glucose consumption in the media, during which cells developed from 10^6 cell/mL to 10^7 cell/mL in both cases, and a second, latency phase corresponding to the depletion of glucose and the use of xylose in the culture media.

Felipe and collaborators (Felipe *et al.*, 1995) studied the effect of acetic acid on xylose fermentation to xylitol by *Candida guilliermondii* FTI20037. They found that a maximal concentration of 1 g/L of acetic acid in the culture media favored xylitol yield and productivity. When the concentration increased, the consumption of substrate became slower. This effect has been seen in other *Candida* species. Lauková and collaborators (Lauková, Valík and Görner, 2018) studied the effect of lactic acid concentrations from 0 to 16 g/L on the growth dynamics of *Candida maltose*. They found that the presence of lactic acid retarded the development of *Candida maltose* YP1 in a linear way. This effect was also observed in our experiments with *Candida guilliermondii* when 25 g/L of lactic acid was present at the beginning of the fermentation. This could be the reason why cells did not to develop.

The last condition to test was the effect of pH regulation at different aeration rates in the culture medium when lactic acid was present. The effect of pH on lactic acid dissociation is directly related to toxicity in the microorganisms. Cottier and collaborators (Cottier *et al.*, 2015) compared the effect of weak organic acids (WOA) in *Candida albicans* at different pH values. They found that WOAs had an effect on yeast development that was more fungistatic than fungicidal. Of the organic acids tested (butyric, propionic, acetic, and lactic acid), lactic acid was the one with the weakest fungistatic effect at the pH values tested (between 4 and 6.5). Wang and collaborators (Wang *et al.*, 2013) studied the effect of organic acids on xylitol fermentation by *Candida tropicalis*. They found that the presence of organic acids changed the pH of maximal production. The presence of acids is dramatically affected by pH variations. The combination of pH and the presence of organic acids had inhibitory effects on growth and production. Biomass showed a reduction of 39 %, and xylitol was reduced by 25 %. This could mean that, in our work, the pH regulation at 7 was not adequate for xylitol production.

pH regulation effect in the presence of lactic acid for C. guilliermondii

To verify the influence of pH regulation on *C. guilliermondii* in a medium in the presence of a high concentration of lactic acid, pH was adjusted to 7 at the beginning of the fermentation but not regulated. Two aeration conditions were tested.

Results obtained (Fig. 2: e and f) show that the aeration rate had an influence on *Candida guilliermondii*'s cell growth and xylitol production in these conditions.

Glucose was fully consumed before 20 h at both aeration rates. pH curves had different shapes in the two cases after 20 h of fermentation, the condition with 0.1 vvm was stabilized around 6.2. In contrast, at 0.5 vvm, the pH

quickly attained 5.3, a value approaching the pKa value of the acid (3.8), where the proportion of non-dissociated acid is more significant than that of the dissociated form. The closer the fermentation medium pH is to the pKa value, the more lipophilic the acid is and the easier it is for the acids to damage the cell by permeating it (Halm *et al.*, 2004), thus making less ATP available for cell production (Wang *et al.*, 2013).

At 0.1 vvm, the consumption rate was 0.3 g/L.h but, in the experiment at 0.5 vvm, the xylose consumption rate was 0.11 g/L.h. This change in the sugar consumption rate suggests that, at a higher pH value, the yeast was able to consume more sugar and develop.

Without automatic pH regulation and at 0.1 vvm of aeration, *Candida guilliermondii* produced 0.63 $mol_{xylitol}/mol_{xylose}$ with a productivity of 0.17 g/L.h. This yield is higher than that obtained when *C. guilliermondii* was cultivated in the medium without lactic acid, at 0.1 vvm, and with pH regulated at 7 (0.36 mol/mol). This suggests that the influence of the combined effect of aeration rate and pH regulation had a direct impact on xylose bioconversion to xylitol. The results reported by Wang and collaborators (Wang *et al.*, 2013) for *Candida tropicalis* were a guide to know if, in the presence of organic acids, xylitol production pH was different on *Candida guilliermondii*. In our experiment, we obtained 11 g/L of xylitol in 60 h in the presence of 25 g/L of lactic acid.

Sequential fermentations with the improved conditions for aeration and pH regulation

For the sequential fermentation, after the first 48 h of culture of *C. glutamicum*, *C. guilliermondii* was inoculated and cultivated with the last conditions tested in the presence of lactic acid (glucose:xtylose ratio of 15:35, aeration at 0.1 vvm, no pH regulation). No cell growth or xylitol production was observed in this experiment (Fig. 3), and yet, the conditions had already been tested (ratio 15:35, lactic acid in the medium, 0.1 vvm, and no pH regulation) for xylitol production by *Candida*. It was observed that pH variation also differed from that obtained in Fig. 2(F). In the sequential test, pH passed from 7 to 5.4 in 16 h, while it only decreased from 7 to 6.7 in 64 h in the single culture fermentation. Since no growth was observed for the yeast, it can be suggested that interactions (direct or indirect) between species was the main reason; the lack of xylitol production was probably due to a double effect between cell interaction and pH variation. To prove this, another fermentation was carried out.

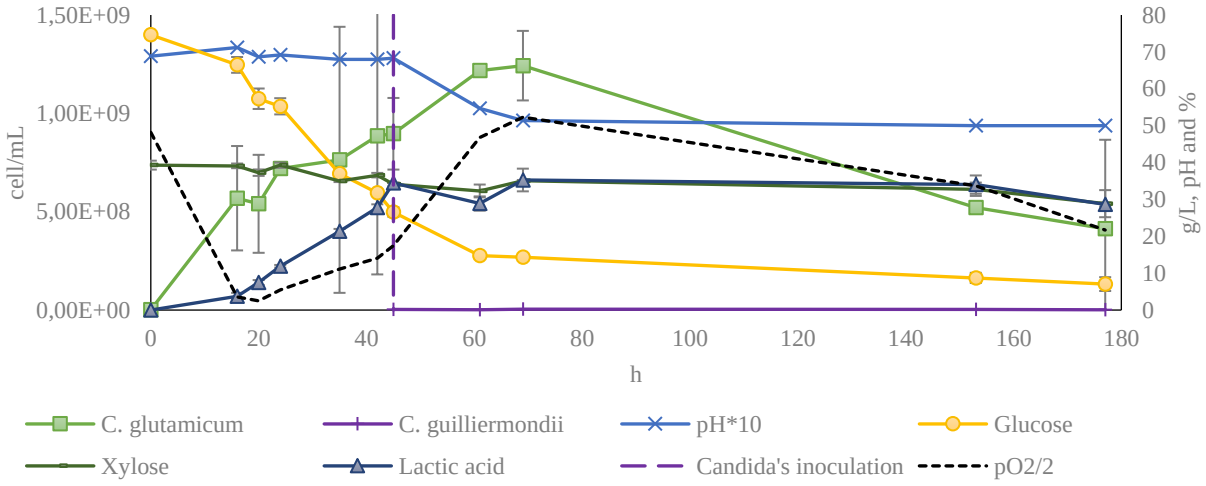


Fig. 3 *Sequential fermentation with 48 h to produce lactic acid with Corynebacterium glutamicum. pH regulated at 7, 0.5 vvm, followed by C. guilliermondii inoculation without pH regulation, and with aeration at 0.1 vvm*

This time, a sequential fermentation was performed with removal of *Corynebacterium glutamicum* when the glucose concentration reached 15 g/L (Fig. 4). The first stage remained the same. The difference lay in the removal of bacterial cells by centrifugation, followed by the filtration of the *C. glutamicum* fermented medium. Once the recycled fermentation medium was obtained, *C. guillermondii* was inoculated and cultivated at 0.1 vvm without automatic pH regulation. Results show that *Corynebacterium* indeed inhibited the yeast production in the sequential fermentation. Without the bacteria in the media, the yeast was able to grow and pH variation was less than in the previous experiment (from 7 to 5.5 in 200 h of fermentation). Even if no xylitol was produced, glucose was fully consumed at 200 h of fermentation. Xylose, however, remained almost unchanged between 100 and 200 h of fermentation.

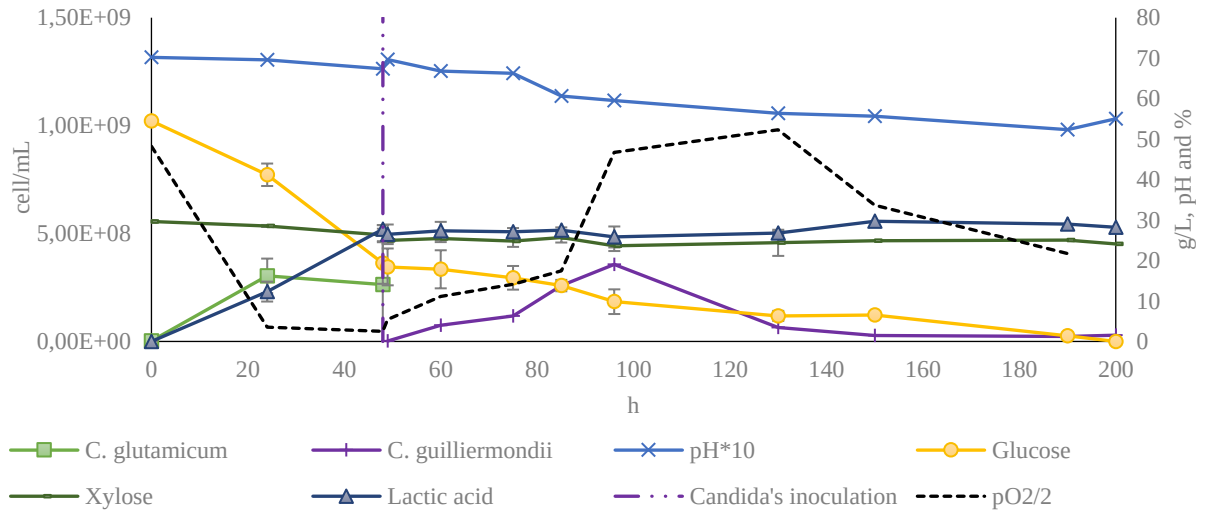


Fig. 4 *Sequential fermentation for 48 h to produce lactic acid with C. glutamicum. pH automatically regulated at 7 and 0.5 vvm of aeration, followed by centrifugation and filtration of C. glutamicum and later by C. guilliermondii inoculation to produce xylitol without pH regulation and with aeration at 0.1 vvm*

The absence of xylitol production may have been due to the co-production of other metabolites by *Corynebacterium glutamicum* in the first stage of the fermentation, which may have inhibited *Candida* production in the second stage. For *C. glutamicum*, lactic acid was the majority product but three other peaks were observed by HPLC with RI detection and a last one was seen with UV detection at 210 nm.

The presence at this wavelength suggests that the species were other organic acids. Production of a majority organic acid and a mix of different products has been previously reported for *coryneform* bacteria (Çalik, Ünlütabak and Özdamar, 2001; Shi *et al.*, 2014b). As yet, the peaks remain unidentified. The following metabolites have, however, been dismissed: aspartic acid, fumaric acid, glycerol, isocitric acid, malic acid, fumaric acid, pyruvate, succinic acid, tartaric acid, and valine.

Strelkov and collaborators (Strelkov, von Elstermann and Schomburg, 2004), utilized gas spectroscopy coupled with a mass spectrogram to determine the metabolome of *C. glutamicum* 13032. They report a list of 121 metabolites in the cell, 77 of which could be detected in our conditions and included organic acids (45), sugar alcohols (2), sugar-phosphates (8), and sugars (10).

Conclusion

Mixed fermentation is not a good strategy for lactic acid and xylitol production by *C. glutamicum* and *C. guilliermondii* because, when they are together, they cannot develop. Only *Corynebacterium* can develop under the conditions of glucose:xylose ratio 65:35, pH regulated at 7, agitation at 200 rpm, and 0.5 vvm. Therefore, sequential fermentation was proposed. In the first step in our conditions, after 48 h of culture, 25 g/L of lactic acid had been produced by *C. glutamicum*. Fermentation conditions were defined for the second step of the fermentation: mixed sugars at a glucose:xylose ratio of 15:35, lactic acid at 25 g/L, no pH regulation, and aeration at 0.1 vvm. In these conditions, *C. guilliermondii* produced 0.63 mol/mol of xylitol with a productivity of 0.17 g/L.h. In sequential fermentation, *C. glutamicum* had to be eliminated from the medium before xylitol production could begin because it inhibited *C. guilliermondii*'s cell growth. Operating conditions have to be optimized to produce the desired metabolites.

Acknowledgments

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Conclusions and perspectives

Biorefineries were implemented to cope with the use of petroleum-based molecules for the obtention of different products. However, several issues keep biorefineries from becoming competitive in the market. The variability of the raw material, in which each lignocellulosic material has different sugar composition, the specificity of the hydrolysis process with one specific microorganism to produce one molecule, in general only one molecule will be obtained by one implemented process and, the low transformation yields result of the mix of sugars in the media, just to mention some.

In this work, we focused on two issues resurfaced over the years to overcome and be competitive in the market. These are: 1) The use of the mix of sugars without going through a sugar separation stage and, 2) The production of multiple value-added molecules to minimize investing costs.

In this work, a model medium was used. This media simulated the sugar composition of the agave bagasse residue from the production of alcoholic beverages. In Mexico, production grows year by year, and the waste is approximately 40% of the total agave used for the production.

The main objective of this work was to propose a process in which the sugars did not have to be separated before the fermentation stage. A couple of microorganisms compatible in fermentation conditions had to be chosen. Two of the main important points of our strategy was the use of non genetically modified strains and the production of value-added molecules different from bioethanol. The choice of non-GMO was to maintain stability over time, and the production of molecules other than bioethanol was chosen because bioethanol prices have to be lowered down to be competitive in the market against other fuels.

Several phases were followed to the pursuit of this work.

First, a literature review was realized to find a couple with similar fermentation conditions that would produce synthon molecules from a mix of glucose and xylose. At the end of this first part, *Corynebacterium glutamicum* ATCC13032 and *Candida guilliermondii* FTI20037 were chosen to produce organic acids and xylitol. One of the principal advantages of the use of *C. glutamicum* was its potential to produce different organic and amino acids. The main compatibility founded in literature between these microorganisms was the temperature and the nature of both as facultative anaerobic microorganisms.

Assays in Erlenmeyer flasks were realized to determine the composition of the model media, followed by verification of cell growth in both microorganisms and the fermentation conditions.

The chosen conditions obtained in this first part were:

- Chosen glucose: xylose ratio 65:35. Salts in the inoculation and fermentation media were chosen with the cross-linked nutrients for each strain.

- For the inoculation media, final composition was: K_2HPO_4 1 g/L; KH_2PO_4 1.49 g/L; $MgSO_4 \cdot 7H_2O$ 3 g/L, $CaCO_3$ 1.6 g/L, $(NH_4)_2(SO_4)$ 3 g/L; $MnSO_4 \cdot H2O$ 0.1 g/L; $FeSO_4 \cdot 7H_2O$ 0.01 g/L; $CaCl_2 \cdot 2H_2O$ 0.1 g/L and Yeast extract 1 g/L.
- Fermentation media was the same with the addition of Biotin 5 μg/L and urea 5 g/L. Supplementary addition of 1 mL of a urea solution (10 % v/w) every 6 hours until a total urea concentration at 12 g/L. pH was regulated manually to between 6 and 9 with NaOH or HCl 4 M,

Under these conditions, *C. glutamicum* produced 1.18 mol/mol of lactic acid, possibly because aeration wasn't enough for glutamic acid production. Furthermore, even if xylitol wasn't produced by *Candida guilliermondii*, ethanol was produced at a yield of 1 mol/mol. This because, the yeast preferred glucose as the first substrate, and aeration conditions weren't enough for xylitol production.

Once the fermentation conditions were tested and chosen in Erlenmeyer flask, preliminary individual single culture were realized in bioreactors. Tests in reactor were realized to verify the metabolite production in pure and mixed cultures.

Lactic acid was chosen as the main metabolite obtained from glucose, therefore urea supplementary addition – proposed for amino acids production – was stopped.

It was found that $CaCO_3$ in the formulation decreased by 30 % lactic acid production by *C. guilliermondii* in the conditions tested. It was also observed that its presence was essential for the production of xylitol by *C. guilliermondii* under the proposed fermentation conditions.

Mixed fermentation was realized, but no cell growth was obtained from either strain. It seemed that the presence of the other species created a competitive environment for the substrate procuration. Sequential fermentation was therefore proposed as an adequate strategy. In the first stage, *C. glutamicum* was used to produce lactic acid from glucose at a concentration of 25 g/L after 48 h of culture.

For the second part of the tests, in which *C. guilliermondii* was used to produce xylitol from xylose in a media with lactic acid, results were presented in the form of an article. Different variables (aeration, lactic acid presence and pH regulation) were tested to determine the best fermentation conditions for xylitol production by *C. guilliermondii* in the second part of the sequential fermentation. At the end, *C. guilliermondii* was able to produce xylitol at 0.63 mol/mol, with a productivity of 0.17 g/L.h in a media with mixed sugars at a ratio glucose:xylose of 15:35, lactic acid at 25 g/L with no pH regulation and aeration at 0.1vvm.

Sequential fermentation assays were realized and it was found that *C. glutamicum* presence in the media affected the yeast cell development. Therefore, a sequential fermentation without the bacteria was realized. Results showed that indeed, the presence of the bacteria affected yeast development, but the presence of lactic acid co-metabolites –yet unidentified- prevented xylitol production by the yeast.

The main contributions of this work were: the definition of a specific culture media for the selected pair of microorganisms. A proposal was started in which the production of xylitol from a lactic acid medium previously produced by *Corynebacterium* is possible. Although different

results were obtained from the tests, the process remained in a first version that is still to be optimized in sequential culture in reactors.

Some issues were left aside in the investigation, which could be tested in the future and verify or refute the validity of the proposed fermentation process. Two main issues were left out of the project due to lack of time: 1) The exhaustive identification of the co-metabolites produced by *Corynebacterium glutamicum*. 2) Aeration studies to optimize production or to test the effects of these metabolites on xylitol production by *Candida guilliermondii*.

For the continuity of this work, several perspectives are presented, mainly categorized into two parts: Fermentation and Biorefinery. Figure VI - 1 presents the different subcategories in the fermentation process: Analytic, aeration, and conditions.

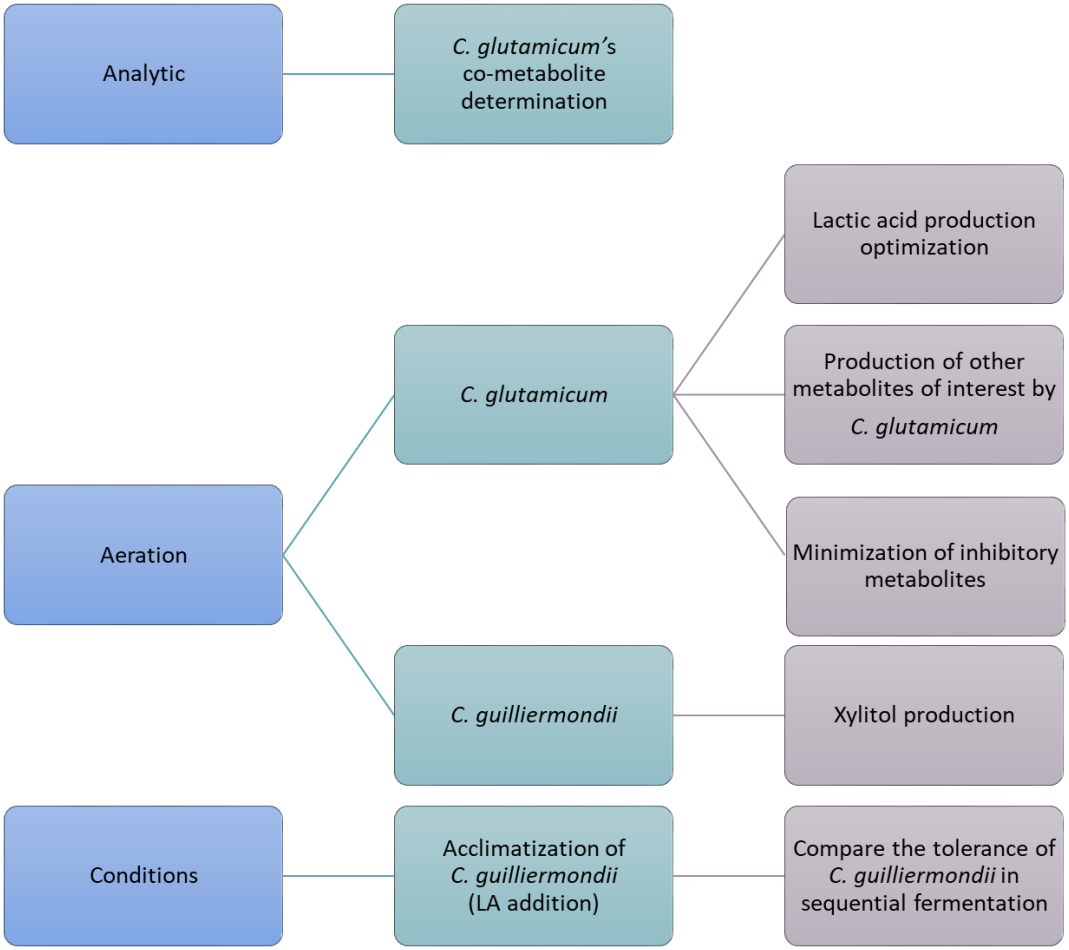


Figure VI - 1 Schema of the fermentation process future derivations of this work.

For the analytic, the determination of *C glutamicum*'s co-metabolites is proposed. The knowledge of their nature. Aeration could be optimized both for *C. glutamicum* and *C. guilliermondii*. For the bacteria, aeration conditions could be optimized to maximize lactic acid production, change the objective metabolite to another, or to minimize the inhibitory metabolites. Once the fermentation conditions are determined for the first fermentation stage then *C. guilliermondii's* aeration will be optimized for xylitol production.

For the fermentation conditions, lactic acid at 25 g/L is proposed to be added in the synthetic inoculation media for *C. guilliermondii's* acclimatization. Then, tolerance to the

organic acid could be compared in sequential fermentation conditions. A test on the macro and micronutrients for the second stage of the fermentation is suggested.

Biorefinery perspectives in the work are classified into two parts: separation techniques and the hydrolysates. The general schema is presented in Figure VI - 2.

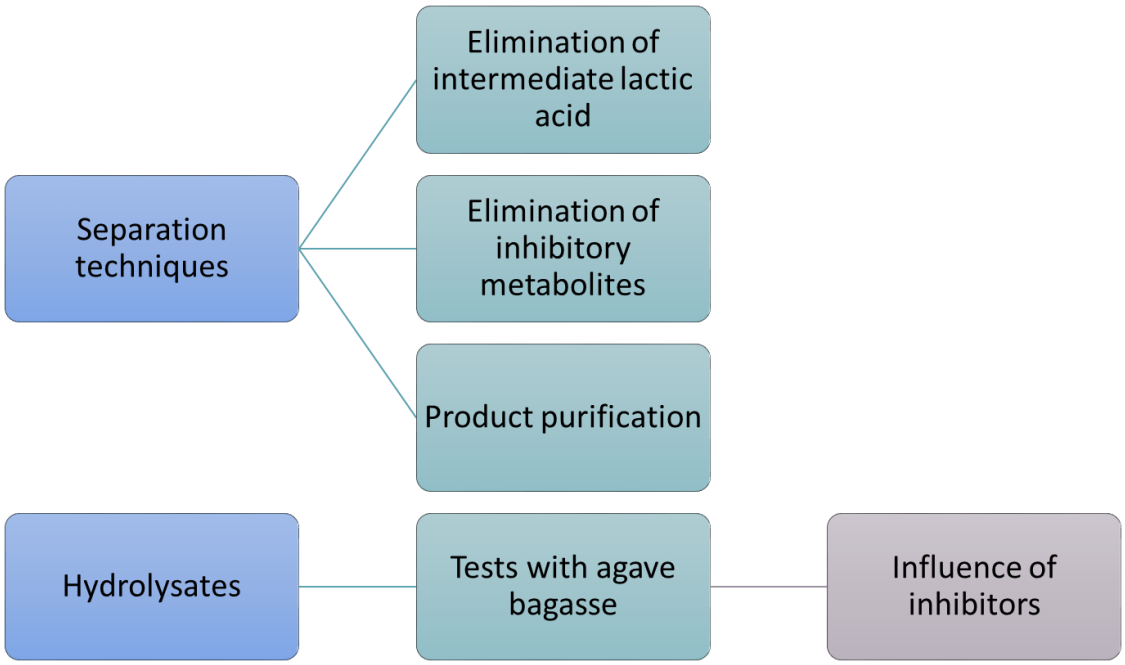


Figure VI - 2 Schema of the biorefinery process future derivations of this work

Separation techniques are proposed to be tested for either for the elimination of lactic acid produced at the end of the first culture or for the elimination of the metabolites inhibiting *C. guillermonidii's* cell growth or xylitol production. Also, for the further recovery and purification of the metabolites produced.

Also, the test on direct agave bagasse hydrolysates is proposed. The sugar and inhibitory compounds composition of the raw material would have an impact in cell growth and metabolite production.

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Appendix 1: Test realized in Erlenmeyer for culture medium optimization

This table summarize the conditions tested for cell growth in both strains in Erlenmeyer culture. Some of the variables were: Nitrogen source, tampon used, and pH regulation were analyzed. In the table are presented: the main objective of the experiment along with observations and conclusions. At the end, the condition with initial urea concentration at 5 g/L, pH regulation in the synthetic inoculation and fermentation media was chosen.

Code	Microorganism	Fermentatio media	Name	Objective	Nitrogen source	Urea addition	Temperature	Agitation	CaCO3	maximal cell concentration	pH regulation	Conclusion	Comments	Carbon source
1710E150	*Corynebacterium + Candida*	*Synthethic*		Determinate the tampon "CaCO3 vs MOPS"	Ammonium sulphate 3 g/L	-					No	CaCO3 was chosen for the price per Kg	The tampon power was roughly the same for both tampons	Glucose + Xylose
1803E200	*Corynebacterium*	*Synthethic*	M	Compare the best nitrogen source	Urea 12.5 g/L + 3 g/L NH4	-	30°C	200 rpm (100 ml in 500ml)	Yes	7.52E+08	No	Urea was a better nitrogen source	CaCO3 is usefull for a smaller latence phase.	Glucose + Xylose
	Corynebacterium	*Synthethic*	N		Ammonium sulphate 28 g/L	-				7.83E+08				
1803E220	*Corynebacterium*	*Patent*	O/P	Replication of the US Patent	Patent	Yes	28°C	220 rpm (100 ml in 500ml)	No	1.10E+09	With urea	Urea addition had to be standarized	The fermentation conditions and the rich inoculation medium	Glucose
1804E220	*Corynebacterium*	*Patent*	Q	Determination of the urea initial concentration	Urea 12.5 g/L + 2 ammonium	-				8.75E+08	HCl - NaOH	Best results are obtained with the urea addition		
	Corynebacterium	*Patent*	R		Urea 5 g/L + 2 ammonium	Yes				1.02E+09				
1806E200	*Corynebacterium*	Synthethic	U	Determine cell growth in synthetic media with supplementary urea addition	Urea 5 g/L + 2 ammonium	Yes	30°C	200 rpm (100 ml in 500ml)	Yes	1.20E+09	HCl - NaOH		Lactic acid production	Glucose + Xylose
	Candida	Synthethic	V							1.00E+09	HCl - NaOH		Ethanol production	
	Candida	Patent	W	Test Candida's growth on the patent media	Urea 5 g/L + 2 ammonium	Yes	28°C	220 rpm (100 ml in 500ml)	No	1.00E+09	HCl - NaOH		Ethanol production	Glucose
1808E200	*Corynebacterium + Candida*	Modified Patent		Determine whats the effect when xylose is added into the inoculation and fermentation media of the Patent	Urea 5 g/L + 2 ammonium	Yes	28°C	220 rpm (100 ml in 500ml)	No		HCl - NaOH	Patent media with xylose is not suitable for cell development	Instead of 24 h in the Inoculation media, 72 h were needed to observe cell developpement	Glucose + Xylose

Appendix 2: Test realized in Reactors for xylitol production optimization

This appendix, summarize the general conditions tested for lactic acid and xylitol production in bioreactor and the results obtained. Experiments presented were mainly for xylitol production with *C. guilliermondii*. Some of the fermentation conditions tested were: aeration, initial glucose concentration, calcium carbonate in the culture media and the presence of lactic acid. Yield, and productivity were the main values used to compare the efficiency of the experimental condition.

				Fermentation conditions						g/L				h	g/Lh	mol/mol
Code	Numero		Reactors	Aeration (vvm)	Glucose (g/L)	Urea 1: oui 0:no	CaCO3 1: oui 0:no	Lactic acid 25g/L 1: oui 0: no	pH	Consummed glucose	Consummed xylose	LA	XOH	Time	Productivity	Yield
1901R0.5	1	Mix	2	0.5	2	1	1	0	7	44	11.9	6.7	0	135	0.050	0.305
	2	Sequential	2	0.5	2	1	1	0	7 --> 5	38.8	4.1	32.9	0	84	0.392	1.696
1902R0.5	3	Sequential	2	0.5	2	0	1	0	7	47.9	5	31.67	0	123	0.257	1.322
	4	Corynebacterium	2	0.5	2	0	1	0	7	30.88	8.96	21.77	0	43.75	0.498	1.410
	5	Candida	1	0.5	2	0	1	0	7	26.7	15.3	0	0	123	0.000	0.000
1902R0.5	6	Corynebacterium	2	0.5	2	0	0	0	7	56.22	5.59	27.6	0	120	0.230	0.982
	7	Candida	1	0.5	2	0	0	0	7	51.3	14.83	0	0	136	0.000	0.000
	8	Candida	2	0.5	1	0	1	0	7	16	32.71	0	23.39	62	0.377	0.710
	9	Candida	2	0.5	1	0	1	1	7	15	2	0	0	136	0.000	0.000
1903R0.5	9	Candida	1	0.5	1	0	1	1	7	14.66	6.58	0	0.196	137	0.0014	0.0294
	10	Candida	1	0.5	0	0	0	0	7	0	7.8	0	0	64	0.000	0.000
1903R0.1	11	Candida	2	0.5	1	0	0	0	7	8	3.47	0	0	64	0.000	0.000
	12	Candida	1	0.1	0	0	0	1	7	0	16.29	0	7.32	64	0.114	0.446
1904R0.1	13	Candida	2	0.1	1	0	0	1	7	13.7	22.22	0	6.32	148	0.043	0.283
1905R0.5	14	Candida	1	0.1	1	0	0	0	7	13.3	11.66	0	0	172	0.000	0.000
1906R0.1	15	Candida	1	0.5	1	0	0	1	7	15	Repaser HPLC	0	0	48	0.000	0.000
	16	Candida	2	0.1	1	0	1	0	7	14.93	31.3	0	11.6	63	0.184	0.368
	17	Candida	2	0.1	1	0	1	1	7	13.13	20.49	0	1.31	140	0.009	0.064
1906E0.5-0.1	17	Candida	1	0.1	1	0	1	1	7	15	17	0	4.4	159	0.028	0.257
	17	Candida	1	0.1	1	0	1	1	7							
1909	18	Candida	2	0.1	1	0	1	1	V	15.22	28.31	0	15.39	150	0.103	0.540
	18	Candida	1	0.1	1	0	1	1	V	15	32.3	0	19	159	0.119	0.584
	18	Candida	1	0.1	1	0	1	1	V	14.27	22.48	0	8.82	137	0.0644	0.3875
	19	Sequential	2	0.5	2	0	1	0	7 --> V	58.78	14.19	28.1	0	69	0.407	0.956
1910- triplicate 17 y 20	20	Candida	2	0.5	1	0	1	1	V	15	8.4	0	0.72	140	0.005	0.085
	20	Candida	1	0.5	1	0	1	1	V	14	12.34	0	1.4	137	0.0102	0.1121

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Design d'un bioprocédé pour la valorisation des résidus d'agave pour la production de molécules plateforme

Le concept de bioraffinerie a été évoqué pour la première fois à la fin des années 80 comme une proposition pour faire face au changement climatique et à la crise pétrolière dans le monde. Cependant, à ce jour, plusieurs problématiques empêchent les bioraffineries de devenir compétitives sur le marché. Dans ce travail, nous nous concentrons sur deux problématiques apparues au fil des années : 1) L'utilisation de l'hydrolysat lignocellulosique sans passer par une étape de séparation des monomères de type C5 et C6 et, 2) la production de diverses molécules à valeur ajoutée afin de minimiser les coûts d'investissement et être compétitif avec les molécules obtenues à partir du pétrole. La matière première sur laquelle on se centre est un milieu modèle de bagasse d'agave. En effet, la production des boissons alcooliques à base d'agave augmente chaque année en générant jusqu'à 40 % des résidus par litre produit. La stratégie présentée lors de ce travail a été la conception d'un procédé pour la production des deux métabolites à valeur ajoutée, chacun issu d'un type de sucre dans le mélange. Pour chaque métabolite, une souche a été sélectionnée en fonction du rendement, de la productivité et de sa capacité à être cultivée dans des conditions de fermentation compatibles avec l'autre souche. Avec ces critères, des souches de *Candida guillermondii* et *Corynebacterium glutamicum* ont été choisies. Trois propositions différentes de processus de fermentation ont été testées : mixte, séquentielle et séquentielle avec élimination du premier microorganisme. *C. glutamicum* a produit de l'acide lactique à un rendement de 1,3 mol/mol après 48 h de culture. Les conditions de fermentation proposées pour la production de xylitol par *C guilliermondii* dans un milieu avec un mélange de sucres et d'acide lactique produit dans la première étape de la fermentation séquentielle sont : 0,1 vvm, pH initial ajusté à 7 sans régulation. Dans ces conditions, le xylitol a été produit avec un rendement de 0,63 mole/mole après 63 h de culture.

Bioprocess design for agave residue valorization to produce chemical building blocks.

The concept of Biorefinery first appeared in the late 1980s as a plan to cope with climate change and the oil crisis in the world. However, several issues keep biorefineries from becoming competitive in the market. In this work, we focused on two issues resurfaced over the years to overcome them and be competitive in the market. These are: 1) The use of the lignocellulosic hydrolysates without subsequent C5 and C6 sugars separation stages and, 2) The production of multiple value-added molecules to minimize investing costs and be competitive against the molecules obtained from oil. The raw material we focus on is a model medium of agave bagasse. In fact, the production of alcoholic agave-based beverages increases each year, generating up to 40% of residues per liter produced. The strategy presented during this work would be the conception of a process for the production of two value-added metabolites, each coming from a type of sugar in the mixture. For each metabolite, one strain has been selected based on yield, productivity and its ability to be cultivated in compatible fermentation conditions with the other strain. With these criteria, strains of *Candida guillermondii* and *Corynebacterium glutamicum* were chosen. Three different propositions of fermentation processes were tested: mixed, sequential, and sequential with the removal of the first microorganism. *C. glutamicum* produced lactic acid at a yield of 1.3mol/mol after 48 h of culture. Proposed fermentation conditions for xylitol production by *C guilliermondii* in a media with a mix of sugars and lactic acid produced in the first stage of the sequential fermentation are: 0.1 vvm, initial pH adjusted at 7 without regulation. Under these conditions xylitol was produced at a yield of 0.63 mol/mol after 63 h of culture.