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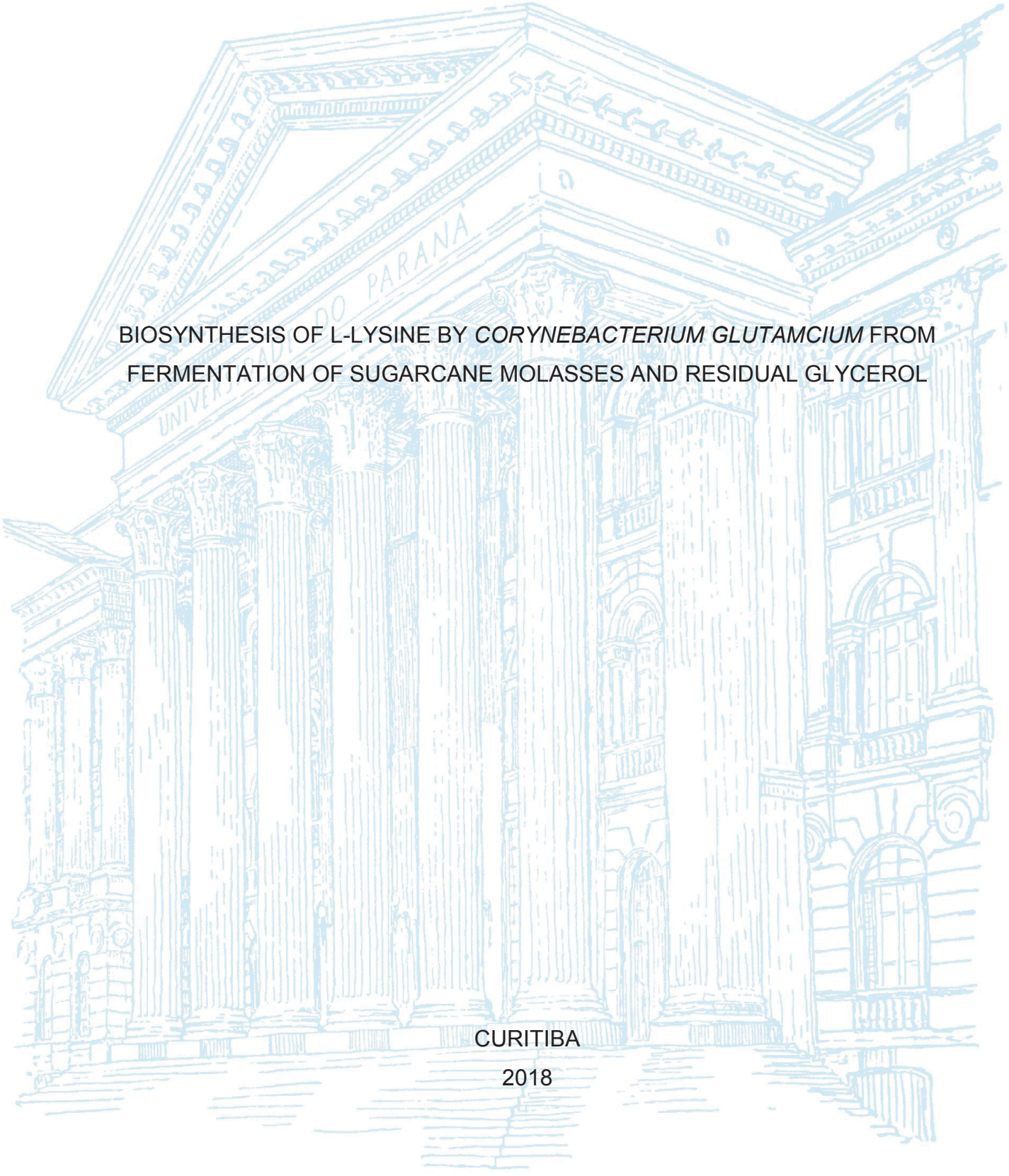
FERNANDA KARINE DO CARMO FÉLIX



BIOSYNTHESIS OF L-LYSINE BY *CORYNEBACTERIUM GLUTAMCIUM* FROM FERMENTATION OF SUGARCANE MOLASSES AND RESIDUAL GLYCEROL

CURITIBA

2018

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Dissertação apresentada ao curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Mestre em Engenharia de Bioprocessos e Biotecnologia.

Orientador: Prof. Dr. Carlos Ricardo Soccol

Coorientador: Prof. Dr. Luiz Alberto Júnior Letti

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da dissertação de Mestrado de **FERNANDA KARINE DO CARMO FELIX** intitulada: **Biosynthesis of L-lysine by *Corynebacterium glutamicum* from fermentation of sugarcane molasses and residual glycerol**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua aprovação no rito de defesa.

A outorga do título de mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 13 de Março de 2018.

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“A vida é uma questão. O amor é a resposta.”

(Pe. Zezinho)

RESUMO

O melaço da cana-de-açúcar e o glicerol são co-produtos industriais interessantes para o uso como fonte de carbono em fermentações microbianas devido, principalmente, ao seu baixo custo e alto valor energético. Essas duas fontes de carbono foram utilizadas como meio de cultura em fermentações utilizando diferentes cepas de *Corynebacterium glutamicum*, selvagens ou mutantes, para produção de L-lisina, com o objetivo de reduzir os custos de produção. Nenhuma das cepas analisadas apresentou crescimento significativo ou produção de L-lisina no meio com glicerol devido à baixa expressão dos genes *glpK* e *glpD*. A hidrólise ácida do melaço foi realizada com o objetivo de liberar açúcares redutores. H_2SO_4 a 0,5% foi a melhor condição de hidrólise com 97% de eficiência. Bons resultados foram apresentados para o crescimento e consumo de açúcares em meio contendo melaço de cana-de-açúcar hidrolisado, com ênfase para os mutantes M5M10 (uma cepa de *C. glutamicum*) com biomassa seca de 9,98 g/L e taxa de consumo de açúcar de 62%. A maior produção de L-lisina foi observada para as cepas mutantes, com ênfase para a M5 com rendimento de 9,84 g/L.

Palavras-chave: Melaço de cana-de-açúcar. Hidrólise ácida. Glicerol. Sacarose. Açúcares redutores. L-lisina.

ABSTRACT

Sugarcane molasses and glycerol are interesting industrial co-products for use as carbon source in microbial fermentations, mainly due their low cost and high-energy value. These two carbon sources were used as culture media in fermentations using different strains of *Corynebacterium glutamicum*, wild or mutant, in order to produce L-lysine and reduce production costs. None of the strains analyzed showed significant growth or L-lysine production in glycerol medium due to low expression of *glpK* and *glpD* genes. The molasses acid hydrolysis was carried out in order to release reducing sugars. H_2SO_4 at 0.5% was the best hydrolysis condition with 97% efficiency. Good results were presented for the growth and consumption of sugars in hydrolyzed sugarcane molasses medium, with emphasis on the M5M10 mutant (a *C. glutamicum* strain) with 9.98 g/L dry biomass and a sugar consumption rate of 62%. The highest L-lysine production was observed for the mutant strains, with emphasis on M5 with a yield of 9.84 g/L.

Keywords: Sugarcane molasses. Acid hydrolysis. Glycerol. Sucrose. Reducing sugars. L-lysine.

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1 INTRODUCTION

Animals have the capacity to synthesize their own proteins through the processes of transcription and gene translation, however, some amino acids (the essential ones) cannot be synthesized by the animals, being necessary their ingestion (KIRCHER; PFEFFERLE, 2001). CHART 1 shows a relationship between essential, non-essential and semi-essential amino acids (which are synthesized by the body from essential amino acids).

CHART 1 – ESSENTIAL AMINO ACIDS IN PIGS AND POULTRY

Essential	Arginine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, Tryptophan, Valine
Semi-essential	Cystine, Cysteine, Tyrosine
Non-essential	Alanine, Asparagine, Aspartic Acid, Glutamic Acid, Glutamine, Glycine, Proline and Serine

SOURCE: Adapted from Dalibard et al. (2014).

The lysine is the first limiting amino acid of several animals, its supplementation in vegetable protein based diets is a very common practice (POWELL; CHOWDHURY; BUREAU, 2015; VAN DEN BORNE et al., 2012). This compost is especially reactive when compared to others containing an epsilon amino group, and it can be obtained by microbial fermentation, besides being present in several sources of plant proteins, however some treatments, such as hydrothermal, reduce its bio-viability in these sources, which are often used for feeding animals (ruminants and non-ruminants).

L-lysine chemical synthesis generally involves costly steps and result in a racemic mixture of L- and D-isomers (in addition to high biological impact residues) (KIRCHER; PFEFFERLE, 2001; POWELL; CHOWDHURY; BUREAU, 2015). As D-lysine, is not biologically catalyzed for the organisms due to a lack of enzymes that catalyze the conversion of D-lysine to the L-isomer, synthetic production of this amino acids is unusual.

Then the biotechnological method is the most suitable for the production of lysine, since it produces only the biologically active form (L-isomer) (POWELL; CHOWDHURY; BUREAU, 2015). Furthermore, the biotechnological method has advantages in terms of cost of production, being able to use cheap carbon sources and processes with low energy requirements.

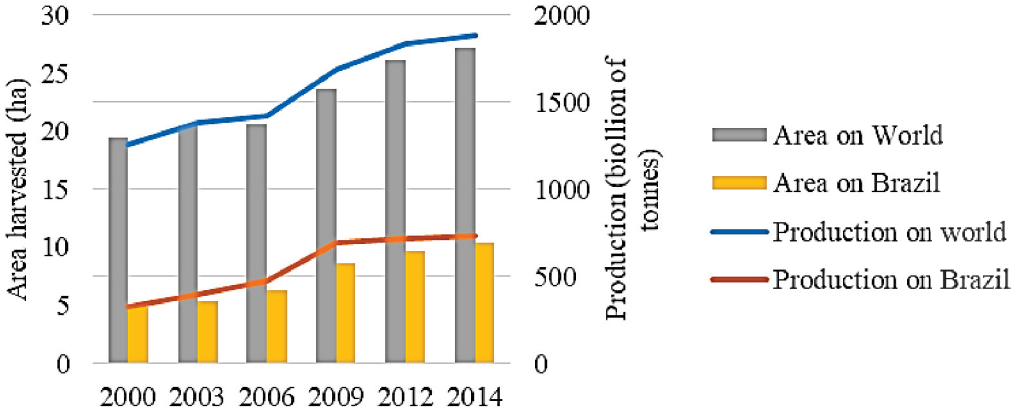
Corynebacterium glutamicum is the microorganism more closely investigated and used as generally-regarded-as-save organism (GRAS) in the food industry for the amino acid production, mainly L-glutamic acid and L-lysine, which in 2012 reached the production of 2.93 and 1.95 million tons respectively (PÉREZ-GARCÍA et al., 2017; WOO; PARK, 2014). It is a facultative Gram positive anaerobic bacterium belonging to the Actinobacteria phylum (the largest and oldest phylum of the Bacteria domain) (HARST et al., 2017; WOO; PARK, 2014); and it was described first as a superior producer of L-glutamate in the 1950s for Kinoshita when it was isolated from Japanese soil (HERMANN, 2003; ZAHOOR; LINDNER; WENDISCH, 2012; LETTI et al., 2016).

C. glutamicum assimilates varied substrates for its growth and production of amino acids, such as monosaccharides (glucose, fructose and ribose), disaccharides (sucrose, mannose, and maltose), alcohols (inositol or ethanol) and organic acids (pyruvic, propionic, acetic and gluconic acids), as well as starch-derived sugars (glucose) and molasses (sucrose and fructose) hydrolysates (SRISIMARAT et al., 2013; ZAHOOR; LINDNER; WENDISCH, 2012).

Glucose is the main fermentable carbon source used by *C. glutamicum* in industrial processes for the production of glutamate and lysine, mainly due to its conversion rate of about 50-55% (ANUSREE; NAMPOOTHIRI, 2015). Its uptake is catalyzed mainly phosphotransferase system (PTS), which phosphorylates the glucose. This pathway is composed by PEP-utilizing enzyme (component EI, encoded by *ptsI*), phosphocarrier histidine protein (HPr, enconcoded by *ptsH*) and glucose-specific EII permease EII^{Glc} (enconded by *ptsG*) (GRUNWALD, 2014; KRAUSE et al. 2010). Glucose can also be absorbed by PTS-independent pathway, in which it is imported by specific transporters and then phosphorylated by energy-consuming glucokinases (ZAHOOR; LINDNER; WENDISH, 2012; GRUNWALD, 2014).

Glucose is mainly obtained through the pre-treatment and enzymatic hydrolysis of sugarcane bagasse (MUSTAPHA, 2010), and although the annual production of sugarcane is relatively high (1.88 billion tonnes wordwide, according to the FAO data for 2014, see FIGURE 1), this crop is mainly intended for the production of ethanol, electricity, alcoholic beverages and refined sugar (which competes with diverse biotechnological applications and with human and animal nutrition) (DIAS et al., 2015; WENDISCH et al., 2016; DOTANIYA et al., 2016).

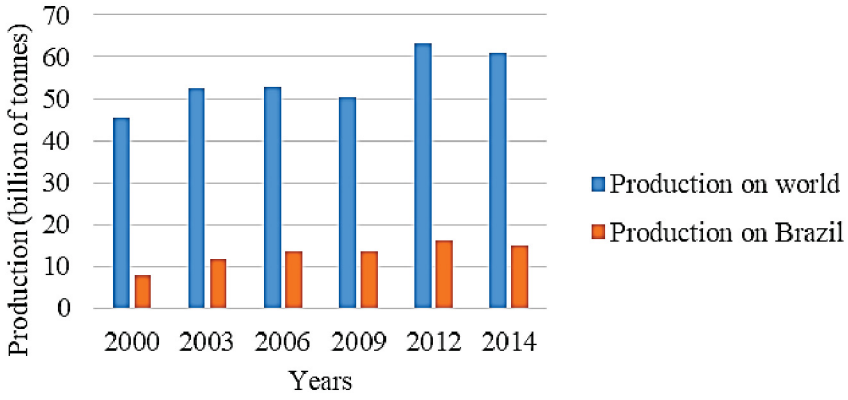
FIGURE 1 – RELATIONSHIP BETWEEN SUGAR CANE PRODUCTION AND AREA HARVESTED



SOURCE: The auteur (2017).

During the sugar production process a number of co-products are generated such as sugarcane press, bagasse, molasses and spentwash; Among these co-products, molasses is biotechnological and economically interesting due to its high fermentable sugars content (DOTANIYA et al., 2016). According to the FAO, in 2014 about 60.96 billion tons of molasses were produced (see FIGURE 2), that is, for every 1 ton of processed sugar cane, 23 L of molasses are produced. This viscous, dark liquid co-product consists mainly of glucose, sucrose and contains about 10-20% fructose (ANUSREE; NAMPOOTHIRI, 2015; DOTANIYA et al., 2016).

FIGURE 2 – RELATIONSHIP BETWEEN SUGAR CANE PRODUCTION AND AREA HARVESTED



SOURCE: The auteur (2017).

Fructose can be absorbed by the cell through three pathways (GRUNWALD, 2014; GEORGI; RITTMANN; WENDISCH, 2005): (1) via phosphoenolpyruvate transferase system PTS_{fru}, in which the fructose will be phosphorylated to fructose-1-phosphate, and then again phosphorylated to fructose-1,6-bisphosphate by means of

1-phosphofructokinase PfkB to thereby enter into glycolysis; (2) via glucose-specific PTS_{glc}, resulting in fructose-6-phosphate entering glycolysis; and (3) via myoinositol permetase IolT1 and IolT2 which will lead to intracellular free fructose.

When fructose-6-phosphate is an intermediate of glycolysis, fructose absorbed by IolT1 or IolT2 cannot be metabolized, since *C. glutamicum* lacks fructokinase activity. In other words, fructose conversion will not be complete (GEORGI; RITTMANN; WENDISCH, 2005).

Sucrose, a diose that consists of a glucose molecule and a fructose molecule linked by glycosidic attachment, can be absorbed efficiently by specific phosphotransferase system (PTS_{suc}) that will phosphorylate sucrose-6-phosphate; which is then hydrolyzed to glucose-6-phosphate and fructose by means of sucrose-6-phosphate hydrolase. The resulting fructose will accumulate intracellularly as it might not be metabolized due to lack of fructokinase activity (GRUNWALD, 2014; GEORGI; RITTMANN; WENDISCH, 2005; LINDNER et al., 2012). The free fructose is then exported out of the cell by an unidentified carrier and re-imported by the phosphoenolpyruvate transferase system PTS_{fru} (ZHANG et al., 2017). Thus, a long period is necessary for the sucrose to be completely metabolized by the cell.

One of today's great efforts of biotechnology is to produce a large amount of amino acids from agro-industrial waste, with the goal of reducing production costs, as the amino acid market is on the rise (POWELL; CHOWDHURY; BUREAU, 2015). Due to the great production of sugarcane molasses and its properties, it has become an important carbon source for amino acids fermentation.

Since the *C. glutamicum* genome was sequenced in 2003, and with the development of omics technologies, various engineered strains were presented, exhibiting high productivity and growth on various substrates non-glycolytic, such as starch, lactose and galactose, xylose, arabinose, cellulose, amino sugars, acetate and acetoin, methylacetate, dicarboxylic acid, methanol and glycerol (SRISIMARAT, et al., 2013; ZAHOOR; LINDNER; WENDISCH, 2012).

Glycerol is another important low cost industrial waste, in fact it is the main co-product in biodiesel production, accounting for about 20-10% of total production (BAUER; HULTEBERG, 2013; SUN et al., 2015). So, the glycerol production increases in proportion to the growth of the biodiesel market, and this market is growing at an accelerated pace, this results in the generation of a large amount of glycerol, which is not fully assimilated by the chemical industry. This surplus glycerol amount makes the

market for this product very volatile, and decreases its price more (ANITHA; KAMARUDIM; KOFLI, 2016).

Two approaches to aggregating value to crude glycerol have been extensively revised: chemical conversion and biological conversion (ANITHA; KAMARUDIM; KOFLI, 2016; ARDI; AROUA; HASHIM, 2015; HEJNA et al., 2016; LUO et al., 2016). The use of glycerol as a carbon source for the fermentation of amino acids is an excellent alternative due to its non-toxicity, high energy density and low price, however, as *C. glutamicum* does not have a significant expression of glycerol kinase (*glpK*, which catalyzes the conversion of glycerol to glycerol-3-phosphate) and glycerol-3-phosphate dehydrogenase (*glpD*, which converts glycerol-3-phosphate into dihydroxyacetonephosphate), it might not uptake the glycerol (MEISWINKEL et al., 2013; WENDISCH et al., 2016).

Many authors have pointed out in genetic engineering and omics technology to increase and accelerate the growth of *C. glutamicum* in glycerol, such as:

- the endogenous overexpression of *glpK* and *glpD* (RITTMANN et al., 2008);
- the exogenous expression in *Escherichia coli* of these two genes and the aquaglyceroporin (*glpF*, which acts as a growth facilitator) (WENDISCH et al., 2016; WOO; PARK, 2014; ZAHOOR; LINDNER; WENDISH, 2012);
- the deletion of *gpp* gene, that encoding glycerol-3-phosphatase, an enzyme responsible for the hydrolysis of glycerol-3-phosphate (glycolysis intermediate) in glycerol, as a response to osmotic cell stress (LINDNER et al., 2012; WENDISCH et al., 2016).

1.1 OBJECTIVES

1.1.1 *General objective*

To evaluate the viability of L-lysine production by several strains of *Corynebacterium glutamicum* (ATCC 21799 and its mutants, ATCC 21543, Pasteur 828, NRRL B4262, NRRL B2784 and NRRL B3330) from glycerol and sugarcane molasses.

1.1.2 *Specific objectives*

- Reactivation and adaptation of several strains of C. glutamicum in glycerol and sugarcane molasses;
- Selection of the best condition for hydrolysis of molasses;
- Analysis of the sugar consumption rate, biomass formation and L-lysine production in different culture media;
- Optimization sugar consumption and L-lysine production.

2 LITERATURE REVIEW

Glycerol is a low cost industrial waste, non-toxic, has high energy density and is an interesting alternative as a carbon source for bacterial fermentations for amino acid production. Beside *Corynebacterium glutamicum* be a superior producer of amino acids, it is unable to perform the glycerol metabolism, so it is necessary manipulations (using DNA recombinant and omics technologies, for example) to became this gram-positive bacterium able to uptake glycerol (and other agro-industrials residues). This work focused on the use of glycerol as a carbon source for amino acids production and its application to animal feed supplementation, given the growing demand for amino acids for this purpose. The amino acid supplementation of animal feed influences on the animal health, milk yield and meat quality, and other aspects. This influence is allied to some factors such as protein source, preparation of the raw material, physiological age and immune system of the animal. Amino acids unbalance in animal feed can lead to several problems not only economic and environmental but also nutritional, and aiming to optimize the feed energy content, many methods were formulated, based on statistical and experimental techniques.

Keywords: Sugarcane molasses. Acid hydrolysis. Glycerol. Sucrose. Reducing sugars. L-lysine.

2.1 INTRODUCTION

2.1.1 *Glycerol*

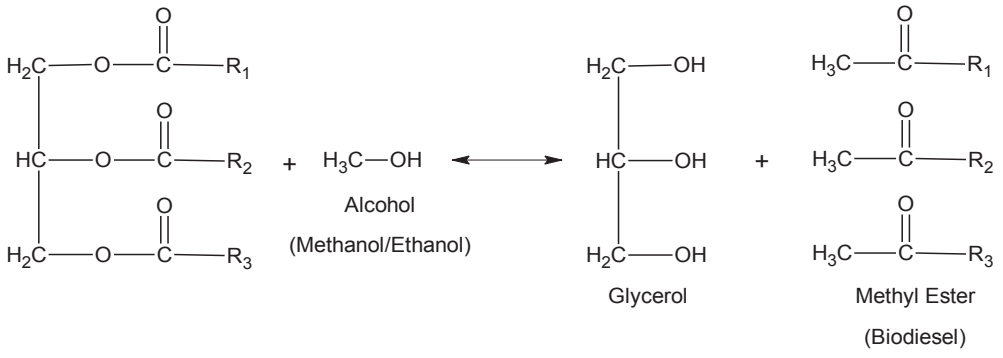
Glycerol can be produced by the saponification of oils and fats; through chemical synthesis from petrochemicals; through fermentation by several microorganisms (*Saccharomyces cerevisiae*, *Candida glycerinogenes*, *Candida magnolia*, *Pichia farinose*, *Bacillus subtilis* and *Dunaliella tertioleta*) by using sugars, starch or cellulose as substrates; and can also be produced within the cell by the glycerol-3-phosphate dephosphorylation (BAUER; HULTEBERG, 2013; COFRÉ et al., 2012). However, the majority of the glycerol produced today is the stoichiometric product outcome of biodiesel industries. In 2011, for example, about 65% of the

glycerol produced worldwide was derived from the plants producing this biofuel (ANITHA; KAMARUDIM; KOFLI, 2016).

Biodiesel is formed by alkyl monoesters produced from the transesterification of vegetable oils and animal fats, which consists of the chemical reaction of these triacylglycerides with an alcohol (methanol or ethanol) in the presence of a catalyst that can be heterogeneous (enzymes) or homogeneous (acids or alkalis) (FIGURE 3) (DUCA et al., 2015). In a world view, the reaction is usually promoted with methanol due to low cost and higher productivity with this alcohol. However, Brazil's biodiesel production is promoted with ethanol mainly due to the ethanol plants stability. Usually the reactions are catalyzed by basic reactives. However, in order to use basic catalysts it is necessary to use anhydrous alcohol to avoid saponification (soap production) (BAUER; HULTEBERG, 2013). For each 3 mols of methyl esters formed from the transesterification reaction, one mol of glycerol is formed, which makes it the main co-product in biodiesel production, accounting for about 20-10% of total production (SUN et al., 2015).

Due to numerous government incentives, the world market for biodiesel is growing at an accelerated pace, with a projection that reaches about 140 billion liters in 2016 (SUN et al., 2015). The increase in biodiesel production will increase glycerol production. The European Union, in 2010, produced 1 million tonnes of glycerol as a transesterification co-product of vegetable oils. The United States of America produced almost 365,000 tonnes of surplus glycerin (BEATRIZ; ARAÚJO; LIMA, 2011). In 2012, a surplus of 220 thousand tons of glycerin were produced in Brazil (regarding the production of 2.4 billion liters of biodiesel) (COFRÉ et al., 2012).

FIGURE 3 – TRANSESTERIFICATION REACTION OF TRIACYLGLYCERIDE



SOURCE: The auteur (2017).

These expressive and alarming numbers represent about 10 times the glycerol amount that chemical industries consume in Brazil, and they tend to increase (POPP et al., 2014). This surplus glycerol amount makes the market for this product very volatile, and decreases its price increasingly more. In the United States in 2007, the price of the pound of crude glycerol fell from US$ 0.25 to US$ 0.05. Several researches have been driven by the need to encourage the reabsorption of glycerol and boost the consumer market (ANITHA; KAMARUDIM; KOFLI, 2016).

75% of the consumer market for glycerol is concentrated in countries in Asia, the United States of America and Western Europe. These countries use glycerol primarily for the manufacture of pharmaceuticals, such as epichlorohydrin (GALADIMA; MURAZA, 2016). Estimates predict an annual increase of 8% in the consumption of this organic molecule for Western European countries and 9% for countries in Africa Estimates predict an annual increase of 8% in the consumption of this organic molecule for Western European countries and 9% for countries in Africa (GALADIMA; MURAZA, 2016; HEJNA et al., 2016).

Two approaches to aggregating value to crude glycerol have been extensively revised: chemical conversion and biological conversion (ANITHA; KAMARUDIM; KOFLI, 2016; ARDI; AROUA; HASHIM, 2015; HEJNA et al., 2016; LUO et al., 2016). **Chemical conversions** employs several routes, such as dehydratation, super-critical water process, hydrogenolysis, selective oxidation, glycerolysis, maleinization, polymerization, on-pot thermochemical process, liquefaction, acetalization, co-pyrolysis, autothermal and stream reforming, etherification, esterification, transesterification and thermochemical process, for the formation of various valuable products, such as acrolein, monoglycerides, maleated glycerides, polyglycerol, hydrogen gas, polyols, solketal, bio-oil and fuel additives (such as, tert-butyl methyl ether and di-tert-butyl glycerol ether) (HEJNA et al., 2016; LUO et al., 2016).

Biological conversions will use glycerol as a carbon source for the microorganisms development (bacteria, fungi and microalgae). These microorganisms can develop through anaerobic digestion or fermentation, and form a varied range of useful products for various industrial sectors (ANITHA; KAMARUDIM; KOFLI, 2016; LUO et al., 2016). Luo and his associates (2016) reviewed some products formed from the microbiological conversion of glycerol, and some of them are shown in CHART 2.

CHART 2 – PRODUCTS FORMED BY THE MICROBIOLOGICAL CONVERSION OF GLYCEROL

Products	Microorganisms
1,3-propanediol	*Klebsiella oxytoca*; *K. pneumoniae; Clostridium butyricum; Lactobacillus diolivorans.*
n-butanol	*Clostridium pasteurianum*
2,3-butanediol	*Klebsiella oxytoca; Klebsiella pneumoniae; Bacillus amyloliquefaciens*
Citric acid	*Yarrowia lipolytica; Clostridium butyricum*
Polyunsaturated fatty acids	*Schzchytrium limacinum*
Lipids	*Cryptococcus curvatus; Chlorella protethecoides*
Poly(hydroxyalkanoates)	*Zobellella danitrificans; Capriavidus necator*
Ethanol	*Enterobacter aerogenes; Citrobacter freundii; Klebsiella oxytoca; Escherichia coli*
Hydrogen	*Enterobacter aerogenes; Clostridium butyricum; Escherichia coli*.
Amino acids	*Propionibacterium acidipropionici; Corynebacterium glutamicum*

SOURCE: Adapted from Luo et al. (2016), Zhang et al. (2015), Meiswinkel et al. (2013) and Anitha, Kamarudim and Kofli (2016).

Crude glycerol has impurities such as soap, alcohols, water, salts and organic matter (ARDI; AROUA; HASHIM, 2015; LUO et al., 2016) that will interfere (positively or negatively) in the conversion reactions, being able to inhibit the fermenting microorganisms growth or even to catalyze some chemical reactions, such as the basic catalysis that is aided by the presence of soaps in the glycerol (LUO et al., 2016; WENDISCH et al., 2016).

However, the vast majority of chemical processes require pure glycerol, and several techniques have been developed in order to promote the glycerol purification and to incorporate this process into the biodiesel production plant without causing a dramatic increase in the product price. Such purification techniques may include neutralization, methanol removal, refining (by vacuum distillation, ion exchange or activated carbon adsorption), and membrane filtration. All these techniques were addressed and explained by Ardi, Aroua and Hashim (2015).

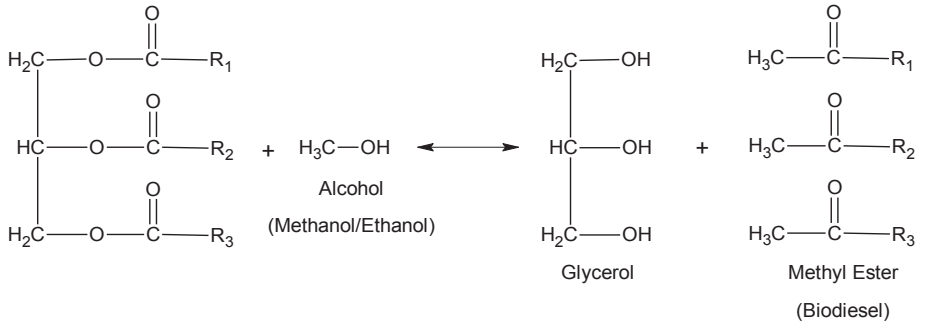
2.1.2 *Amino acids production*

One of today's great efforts of biotechnology is to produce a large amount of amino acids from crude glycerol, with the goal of reducing production costs, as the amino acid market is on the rise. The amino acids cost is strictly related to its obtaining method (POWELL; CHOWDHURY; BUREAU, 2015). There are two routes for the production of several amino acids: the chemical route and the biological route. In

addition, four means of producing amino acids can be highlighted: extraction, synthesis, fermentation and enzymatic catalysis (KIRCHER; PFEFFERLE, 2001).

The amino acids **chemical synthesis** generally involves costly steps that require the handling of hazardous materials, such as ammonia solution (elution of ion exchange columns) and hydrochloric acid (neutralization of the amino acid), resulting in a racemic mixture of L- and D-isomers (in addition to high biological impact residues) (FIGURE 4) (KIRCHER; PFEFFERLE, 2001; POWELL; CHOWDHURY; BUREAU, 2015).

FIGURE 4 – GENERAL STRUCTURE OF AMINO ACIDS



SOURCE: The auteur (2017).

SUBTITLE: Amino acids are characterized by two functional groups: amino group (NH_2) and carboxylic group (COOH). Furthermore, they differ in the R group and their optically active isomers. In (a) we have an L-isomer and in (b) we have a D-isomer.

If an animal is fed a racemic mixture, for example, the D-amino acid should be converted to L-amino acid before being metabolized by the body. This conversion is catalyzed by certain enzymes, which promote deamination and subsequent amination of the amino acid (DALIBARD et al., 2014). This reaction will depend on the enzymatic system of the organism, which varies according to animal species.

Methionine has been synthetically manufactured (chemical route), with a racemate, from acrolein, hydrocyanic acid, mercaptan methyl and ammonia. After the ingestion by the animal, the D-methionine will be enzymatically converted into L-methionine by means of oxidase and transmitase, which allows the use of its racemic mixture (LEUCHTENBERGER; HUTHMACHER; DRAUZ, 2005). However, the other amino acids conversion, such as lysine, is not biologically catalyzed, making synthetic production of these amino acids unusual. In these cases, the biotechnological method is the most suitable for the production of amino acids, since it produces only the biologically active form (L-isomer) (POWELL; CHOWDHURY; BUREAU, 2015), in

addition to using renewable raw material and not generating residues with high pollutant load (KIRCHER; PFEFFERLE, 2001).

Extraction process is also relevant for the production of L-serine, L-proline, L-hydroxyproline and L-tyrosine, however, their large-scale production is not feasible. Latterly, **enzymatic catalysis** has been explored for industrial production because of its economic advantages, and its use can be illustrated by the use of the enzyme membrane reactor for the production of L-methionine and L-valine (LEUCHTENBERGER; HUTHMACHER; DRAUZ, 2005). However, the most employed method for the production of a large amount of amino acids is the microbial **fermentation**, which has launched a large amount of these products on the market (ISHIKAWA et al., 2008).

Microorganisms cultivation for amino acid production can be conducted in batch, fed-batch or repeated fed batch, each of these techniques has its advantages and disadvantages for the biosynthesis of these products and were reviewed in detail by Hermann in 2003. In these processes, cells can be free or immobilized (on agar or alginate gels, for example). Nampoothiri and Pandey (1998) reported conditions for optimization of a L-glutamate production process using immobilized *Brevibacterium* cells, and reached an optimal yield of 13.3 g/L for agar immobilized cells conducted in a repeated fed batch process.

Due to the market expansion and the need to increase the amino acids production to supply it, without interfering in the cost, technologies to improve production both in the fermentation stage and in the production stage were developed (HERMANN, 2003; XU et al., 2013). Viable biotechnological processes for the development of additives for animal feed, should be simple and conducted in a way that reduces production costs (KIRCHER; PFEFFERLE, 2001; MA et al., 2015), in addition to being sustainable and causing minimal environmental impact (KIRCHER; PFEFFERLE, 2001).

As seen in the previous section, glycerol is a low cost industrial waste, non-toxic, has high energy density and is an interesting alternative as a carbon source for bacterial fermentations for amino acid production, however, not all bacteria of industrial interest are able to assimilate this organic compound, and techniques are required to perform the glycerol metabolism by these microorganisms. The next sections seek to summarize some techniques to make viable the use of glycerol for the production of amino acids, a product that is currently in wide and increasing demand.

2.2 STRATEGIES FOR EFFECTIVE PRODUCTION

2.2.1 *Corynebacterium glutamicum: carbon sources*

Kinoshita described C*orynebacterium glutamicum* as a superior producer of L-glutamate in the 1950s when it was isolated from Japanese soil. Until this time, amino acids were produced exclusively by extraction or chemical synthesis (HERMANN, 2003; ZAHOOR; LINDNER; WENDISCH, 2012). This workhorse is a gram-positive bacteria, which has been applied as generally-regarded-as-safe (GRAS) and revolutionized the industry, producing about 2.16 million tonnes of L-glutamate and 1.48 million tonnes of L-lysine annually (ZAHOOR; LINDNER; WENDISCH, 2012).

C. glutamicum assimilates varied substrates, such as monosaccharides (glucose, fructose and ribose), disaccharides (sucrose, mannose, and maltose), alcohols (inositol or ethanol) and organic acids (pyruvic, propionic, acetic and gluconic acids), as well as starch-derived sugars (glucose) and molasses (sucrose and fructose) hydrolyzates (SRISIMARAT et al., 2013; ZAHOOR; LINDNER; WENDISCH, 2012). As mentioned earlier, the search for carbon sources in alternative to the traditional, that does not compete with the food industry, is the current focus of many researchers (KIRAN et al., 2016; ZAHOOR; LINDNER; WENDISCH, 2012).

Since the *C. glutamicum* genome was sequenced in 2003, and with the development of omics technologies, various engineered strains were presented, exhibiting high productivity and growth on various substrates such as starch, lactose and galactose, xylose, arabinose, cellulose, amino sugars, acetate and acetoin, methylacetate, dicarboxylic acid, methanol and glycerol (SRISIMARAT et al., 2013; ZAHOOR; LINDNER; WENDISCH, 2012). CHART 3 displays some of the metabolic engineering studies of this bacterium that allowed access to the aforementioned carbon sources.

CHART 3 – SOME UNCONVENTIONAL CARBON SOURCES AND METHODOLOGIES THAT ALLOW THEIR CONSUMPTION BY *C. glutamicum*

SOURCE	PROCEDURE	REFERENCES
Starch	Expression of the *Streptomyces sp.* (*S. griseus, S. bovis*) α-amilase gene (*amyA*) in *C. glutamicum* for this releases α-amilase in the medium, allowing direct access to the starch materials.	Seibold et al. 2006 Tateno, et al. 2009 Yao, et al. 2009 Tsuge, et al. 2013
Cellulose Cellobiosis	Heterologous expression of *Clostridium cellulovorans CpbA* gene and chimerical endoglucanases of *C. thermocellum* Heterologous expression of endoglucanase (*celE*) and β-glucosidase (*bglA*) of *C. termocellum* (KIM, et al. 2014). Heterologous expression of *X. campestris* endoglucanase and *S. degradans* β-glucosidase.	Hyeon et al. 2011 Sasaki, et al. 2008 Adachi, et al. 2013 Anusree et al. 2016
Lactose Galactose	Heterologous expression for the lacZYA operon of *E. coli.* Heterologous expression for the *galETKM* operon of *Lactococcus lactis subsp. cremoris* MG1363	Brabetz, et al. 1991 Lim et al. 2013
Xylose	Heterologous expression of *E. coli* xylose isomerase (*xylA*) gene. Expression of genes for xylose redutase (*xr*) of NAD(P)H-dependent *Rhodotorula mucilagionosa*, L-arabinose isomerase (*araA*) of *E. coli*, D-psicose 3 epimerase (*dpe*) of *Agrobacterium tumefaciens*, and L-xylulose reductase (*lxr*) of *Mycobacterium smegmati*s.	Kawaguchi et al. 2006 Kiran et al. 2016
Arabionose	Heterologous expression of *E. coli araBAB* operon, which encodes arabinose isomerase (*araA*), ribulokinase (*araB*), and ribulose 5-fosfate 4-epimerase (*araD*).	Schneider, et al. 2011
Methylacetate	Heterologous expression of *Pseudomonas veronii. mekB* gene.	Onaca et al. 2007 Choo et al. 2016
Dicarboxylic acid	Gene expression of ecoding proton- or sodium-dependant transporters *dctA* and *dccT*.	Youn et al. 2008, 2009

SOURCE: Adapted from Wendisch et al. (2016) and Zahoor, Lindner and Wendisch (2012) Luo et al. (2016), Zhang et al. (2015), Meiswinkel et al. (2013) and Anitha, Kamarudim and Kofli (2016).

Meiswinkel et al. (2013) highlighted the need for large-scale processes based on crude glycerol. However, *C. glutamicum* cannot use glycerol naturally; it needs to be manipulated in order to assimilate this carbon source. Generally, this occurs through the heterologous expression of aerobic glycerol utilization *E. coli* genes encoding for glycerol facilitator (*glpF*), glycerol kinase (*glpK*) and glycerol-3-phosphate dehydrogenase (*glpD*) (MEISWINKEL et al., 2013; WENDISCH et al., 2016).

GlpK and *glpD* allow for the assimilation of glycerol and *glpF* accelerates microbial growth (MEISWINKEL et al., 2013; RITTMANN et al., 2008). Another technique to increase the growth rate of *C. glutamicum* in glycerol is the deletion of the

gene glycerol-3-phosphatase (*gpp*) which is responsible for the formation of glycerol as a co-product of *C. glutamicum* (LINDNER et al., 2012).

However, *C. glutamicum* has genes encoding for homologues of *glpK* (Cg3198) and *glpD* (Cg1853), which have 49% and 31% similarity to *glpK* and *glpD* from *E. coli*, respectively. Thus, it is necessary to overexpress these genes (endogenous expression), in order to enable the growth of the strain in glycerol (MEISWINKEL et al., 2013; WENDISCH et al., 2016).

2.2.2 *Reduction of the cost of production: other alternatives*

In addition to the application of more viable sources of carbon and nitrogen (salts, yeast extract, hydrolyzed peptone or maize), the optimization of others fermentation parameters such as pH, aeration, carbon source feed rate or process temperature has been extensively used to reduce production costs and increase the productivity of the industrial plant (HERMANN, 2003; VAN DER BORNE et al., 2012; XU et al., 2013).

As reported, the high relevance of *C. glutamicum* has stimulated the efforts of researchers to analyze and modify their metabolic line in order to obtain, in a targeted manner, better yield and productivity of the desired amino acid. Techniques such as proteomics, fluxomics and metabolomics for the construction of superproductive mutant strains or possessing desirable characteristics to the process have been extensively reported (HERMANN, 2003; LUO et al., 2016; XU et al., 2013). For instance, the optimum temperature for the culture of coryneform bacteria is in the range of 30 to 34°C; however, the use of more thermotolerant strains is desirable as it reduces the cost of cooling the bioreactor (HERMANN, 2003).

In 2009, the need to understand the physiological characteristics of the strains as to the oxygen supply conditions were highlighted by Xu and his associates, who emphasized that dissolved oxygen levels determine the interactions between metabolic reactions and genetic regulation mechanism, as well as co-products formation (such as acetic acid and ethanol), which may inhibit production of the desired amino acid.

Since lysine biosynthesis requires a high redox force, modifications in metabolic flux should be conducted to increase the supply of NADPH. For this purpose, techniques such as the amplification of the pentose phosphate pathway (PPP) at (1)

glucose 6-phosphate dehydrogenase, (2) *6-phosphogluconate dehydrogenase*, (3) *glucose 6-phosphate isomerase* or (4) *fructose-1,6-bisphosphatase* can be employed (KIND; BECKER; WITTMANN, 2013; XU et al., 2013).

Xu et al. (2013) have developed a superproductive strain of *C. glutamicum* through the heterologous expression of *fructose-1,6-bisphosphatase*, which has the important role of regenerating glucose-6-phosphate in the pentose phosphate pathway and, consequently, increasing lysine production. The researchers also evaluated the absorption capacity of sugars from molasses by the microorganism, and noted that the consumption was related to the components (glucose, sucrose and fructose) and to gene expression.

Kind, Becker and Wittmann (2013) describe another interesting example about the metabolomic and fluxonomic analysis of *C. glutamic*. These authors noted that *succylinase* from the lysine biosynthesis pathway accounted for approximately 70% of lysine productivity by *C. glutamicum*, then discontinued the tricarboxylic acid (TCA) cycle at the *succinyl-CoA* synthase level, avoiding the conversion of *succinyl-CoA* *succinate*; the surplus *succinyl-CoA* was integrated into the lysine biosynthetic pathway, which resulted in an increase in lysine productivity of the *C. glutamicum* mutant strain by 60% compared to the parental strain.

In sum, sophisticated experiments and computational tools for biological systems have provided an excellent platform for increasing the yield of the fermentations of the amino acid producing strains. These amino acids will be used in various industrial sectors, such as in the pharmaceutical, food and animal feed industries. This work will focus on the application of amino acids to animal feed supplementation, given the growing demand for amino acids for this purpose.

2.3 ANIMAL FEED

The demographic and economic development observed with evident prominence at the end of the 20th century and the beginning of the 21st was one of the main factors for the increase in the production of cattle, poultry and pigs, due to the increment in the consumption of meat, milk and eggs. In a global setting, the United States of America is a leader in livestock production, however, the Brazilian Assiciation of Meat Exporting Industries (Abiec) said that Brazil, for historical, economic and

geographic reasons, has potential to surpass the USA in beef production in 2020 (DEPEC 2015; SNA 2015).

Some Asian countries (such as China and Japan) are gaining prominence as cattle and buffalo producers (SNA 2015). However, China has also been recognized on the world stage with the creation of pigs and poultry. In 2016, China led the production of swine, followed by the European Union and Brazil. With Germany, Spain and France among the main pig producers in Europe (DEPEC 2015). As for poultry production, the United States, Brazil and China stand out as the first, second and third producers in the world (ABPA 2016).

The producers started to invest in techniques to improve pasture and livestock, driven by the need to increase productivity through rational intensification, stimulated by consumer demand and the need to adjust to the national/international market (DIAS-FILHO, 2016). Techniques such as pasture fertilization, pasture structure and nutritive value control, and food supplementation in the dry season are widely used by cattle ranchers to increase dairy cattle productivity (SIQUEIRA; ZOCCAL, 2016).

Cattle raisers empirically noted the association between milk yield and feed nutritional quality, and this relationship has been studied by several researchers in order to formulate an ideal balanced diet (SWANEPOEL; ROBINSON; ERASMUS, 2016; ZHANG et al., 2012). In addition to affecting milk yield, a nutrient unbalanced diet could bring harm to the health of the herd. Bott et al. (2015) reported that protein overfeeding in horses could compromise respiratory health by increasing the release of ammonia; increase urine calcium excretion, thereby reducing bone mineralization and disrupting the acid-base balance; therefore studies on the ideal concentration of proteins and amino acids in the diet are necessary.

Unbalanced feeding also affects pisciculture, a good example of this negative effect on fish was reported by Hassaan, Soltan and Abdel-Moez (2015), who noticed pathomorphological changes in the distal intestine of the fish due to the antinutritional factors present in soybean meal, which may decrease the nutrients absorptive capacity.

The supplementation of animal feed is allied to two aspects: (1) to increase the energy efficiency of the feed and (2) to reduce the production cost of the feed. The nutritional value and quality of structurally different proteins are governed by their amino acid composition, essential amino acid content, susceptibility to hydrolysis

during ingestion, purity and behavior during and after treatments (for instance, enzymatic hydrolysis and heat treatment) (HAN; CHEE; CHO, 2015).

Thus, for the formulation of an ideal diet one must consider several variables such as the **protein source**. Proteins are the essential components of feeds, it is known that due to its molecular size, it has no nutritive value, because it will not be absorbed by the organism, therefore, it must be hydrolyzed, releasing its monomeric units (amino acids), which will be absorbed by the intestine (amino acids) (DALIBARD et al., 2014).

Animals have the capacity to synthesize their own proteins through the processes of transcription and gene translation, however, some amino acids (the essential ones) cannot be synthesized by the animals, being necessary their ingestion (KIRCHER; PFEFFERLE, 2001). Lysine, methionine, threonine, tryptophan, histidine, leucine, isoleucine, valine and phenylalanine are considered essential amino acids for both humans and various other animals of economic interest (HAN; CHEE; CHO, 2015).

The protein source, in addition to notably influence the price will also influence the content of amino acids, which will dictate their nutritional quality. That is, it will determine the rate of growth, reproduction and production of milk and eggs (DALIBARD et al., 2014). Vegetable-derived protein feeds have a characteristic amino acid spectrum, which differs from the animal-derived protein dietary spectrum, mainly in the concentration of essential amino acids, such as methionine, lysine and threonine (KIRCHER; PFEFFERLE, 2001). According to Hamid et al. (2016) most of the plant proteins, including soybean residues, grass leaves and rice bran, have a low lysine content, with soy being highlighted with the greatest deficiency of the amino acid.

The hydrothermal conditions during the **preparation of the raw material** that will be used in the formulation of the feed will have a strong influence on the residual protein of the feed; aiming at this variable, some studies have turned to the analysis and evaluation, through the use of statistical models, the influence of temperature, acidity and retention time on the constitution of the protein pattern (VAN DER BORNE et al., 2012). As an example, the ethanol production industry uses hydrothermal treatments, acid catalysis or enzymatic hydrolysis for the release of pentoses from the lignocellulosic biomass (wheat bran, corn fibers, sugarcane bagasse, etc.), thus increasing the viability of the pentoses to the fermentation process. This process produces a large amount of protein rich residues, which can be used in animal feed

and replace high commercial value protein sources (*potato protein, animal-derived protein*) (GUPTA; VERMA, 2015; VAN DER BORNE et al., 2012).

However, the hydrothermal pre-treatment can reduce the nutritional quality of the feed through recemination, cross-linking or Maillard reaction, forming compounds of no nutritional value, or even deleterious to the animals (DALIBARD et al., 2014; SHIBAO; BASTOS, 2011). Van der Borne et al. (2012) observed in their experiments that the increase in temperature (120-140°C) accompanied by low acidity increases the digestibility of residual wheat protein by 36%. Temperatures above 140°C, decrease lysine concentration and bioavailability of minerals and may form compounds such as melanoidin, furfural and 5-hydroxymethylfurfural (SHIBAO; BASTOS, 2011).

Another variable to be considered during the formulation of the feed is the **genus of the animal**, since the requirement for amino acids will vary according to species, as confirmed by recent studies on fish (HAMID et al., 2016; HASSAAN; SOLTAN; ABDEL-MOEZ, 2015), equine (MASTELLAR; COLEMAN; URSCHEL, 2016), ovine (MILIS et al., 2005) and swine nutrition (MA et al., 2015; TOLEDO et al., 2014). However, the energy requirement will also vary within the species, according to the **physiological age** of the animal, being necessary the formulation of feeds suitable for each stage, for example, initial phase, weaning, gestation and lactation (GARCIA-LAUNAY et al., 2014; KIRCHER; PFEFFERLE, 2001). Ma et al. (2015) and King et al. (2000) noted that when they work with pigs, a higher intake of amino acids is required for *late finishing gilts* because of the higher growth rate of lean mass and reduced food intake.

According to Zhang et al. (2012), breeding conditions (for example, housing density, lack of environmental control, local hygiene, diseases) affect the animal's immune system, and as the **immune system** is stimulated the needs for amino acids will be modified.

2.3.1 *Protein source*

The aquaculture sector moves US$ 600 billion dollars and US$ 55 billion in annual exports, surpassing the soy, beef and chicken market, however, despite its ascendancy in recent years, aquaculture in Brazil is confronted with several problems, mainly due to the productive chain to attract the consumer (CNA, 2016). It is estimated

that due to changes in dietary habits and population growth, about 37 million tonnes of fish will be required worldwide in 2020 (HAMID et al., 2016).

With the increase in demand, production is increased and, consequently, the requisition for feeds to supply the nutritional needs of these fish. At this point, we are faced with one of the problems for aquaculture: the price of the feed; common ingredients of animal feed such as fishmeal and blood meal contain high levels of essential amino acids (such as lysine), however due to the cost of these ingredients, many breeders have been seeking the addition of plant protein sources as a total or partial substitute for fishmeal (EL-SAIDY; GABER, 2002; HASSAAN; SOLTAN; ABDEL-MOEZ, 2015; POWELL; CHOWDHURY; BUREAU, 2015). Agroindustrial wastes (e.g., flour, soybean meal, soybeans, sorghum, maize, wheat and rice) are highly needed as partial or complete substituents to more expensive protein sources of animal feed (LLATA et al., 2002).

Among these protein sources derived from the residual biomass are the bran and soybean meal that represent very nutritious plant proteins, however they can negatively interfere in the growth of the animals (EL-SAIDY; GABER, 2002; HASSAAN; SOLTAN; ABDEL-MOEZ, 2015). Researches were conducted to infer the reasons for the altered growth performance with the alteration of the feed protein source, and some reasons were weighed: soybean meal may (1) be less energetic than fishmeal, (2) contain inhibitors of protease (trypsin), (3) lack some essential amino acids (EL-SAIDY; GABER, 2002).

Different soybean strains may show considerable variation in total protein content; Cartter and Hopper (1942) evaluated ten soybean varieties and noted that protein values ranged from 36.6 to 53.2%. Kuiken and Llyman (1949) applied microbiological methods to evaluate the composition of ten amino acids in soybean meal and noted that the greatest variation occurred in the methionine content; and that the concentration of lysine could fall precipitously in case of heating under high pressure.

Chee (2005) compared the potential of soybean hulls and wheat bran feeding pigs with growth range close to slaughter, and analyzed the chemical composition, the digestion test and the preference test of these two components. The authors concluded that the energy contained in the hulls (2.070 kcal/kg) was lower than the energy contained in the wheat bran (2.420 kcal/kg), and noted that the hulls contained a high content of fibers that were poorly digested by non-ruminant animals. However, they did

not notice a significant difference in the limiting amino acid level of the two diets, thus concluding that soybean hulls could be inserted into the pigs diet (10 to 12%), without causing nutritional problems when compared to wheat bran.

Seymour, Polan and Hrbein (1990) compared the performance of cows fed with (1) soybean meal and corn silage and (2) soybean meal with corn gluten meal, and noted that, under the same treatment, cows fed with the first diet (which is deficient in some proteins) tended to lose more weight than those fed with soybean meal and corn gluten. The effects of corn gluten meal (which has low degradability by the enzymes present in the rumen), soybean meal (which is highly degradable) and wheat flour has also been studied by Milis et al. (2005), who concluded that gluten-based feeds of corn supplemented with lysine would provide sheep milk with high fat, protein and solid contents and that the feeds corresponding to the soybean-wheat flour mixture would increase milk yield.

As seen, these alternative sources, although being excellent options to sustain the increase in cattle ranching and the consequent demand for animal feed and the need to reduce the cost of production, present some problems that need to be overcome during its formulation (MILIS et al., 2005). Issues such as the occurrence of anti-nutritional factors, low digestibility, palatability issues, and limitations of certain essential amino acids (EL-SAIDY; GABER, 2002; HASSAAN; SOLTAN; ABDEL-MOEZ, 2015).

In terms of palatability, we have as an example the "red drum" fish (*Sciaenops acellatus*) that does not consume diets that do not contain any percentage of fish meal, being necessary the addition of animal co-products in their diet to improve the flavor (EL-SAIDY; GABER, 2002). In the case of amino acid deficiency or presence of anti-nutritional factors, treatments and supplements are necessary in these diets, with the purpose of mitigating the possible nutritional problems that these protein sources could entail. Hassaan, Soltan and Abdel-Moez (2015) cited heat treatment as an alternative to eliminate anti-nutritional factors (thermolabile proteinase inhibitors and agglutinating lectins) and fermentation as a way of increasing the nutrient digestibility and nutritional value of the feed, as well as reducing trypsin inhibitors and increasing the concentration of peptides.

Aimed at controlling the productivity and composition of the final product through nutrition, researchers have sought to lower crude protein levels in the diet, and use non-degradable, fiber-rich protein sources and possibly supplemented with

synthetic amino acids (MILIS et al., 2005). The advantages of amino acid supplementation are routinely reported in the literature for different animals.

Ma and associates (2015) observed that pigs close to the time of slaughter tend to become fatter when fed a diet with low crude protein concentration and lysine supplementation assists in the deposition of proteins, having a positive effect on meat treatment. The amino acids addition, besides exerting influence on the meat quality, also assists in the fat content manipulation and the productivity of milk proteins (MILIS et al., 2005). Amino acids such as L-lysine, DL-methionine, L-threonine, L-tryptophan, L-valine and L-isoleucine are commonly added to animal feeds because of their positive effects on growth performance and productivity (TOLEDO et al., 2014).

2.3.2 *Amino acids supplementation*

There are some questions related to the protein chemical form (intact or in the form of amino acids) and its ingestion and absorption efficiency. While some evidence suggests that animals utilize intact proteins more efficiently than a corresponding mixture of amino acids (SEYMOUR; POLAN; HRBEIN, 1990), There are studies that report exhaustively on the biological/environmental problems of the crude protein use in the herd feeding (MA et al., 2015; MASTELLAR; COLEMAN; URSCHEL, 2016; TOLEDO et al., 2014).

It is noteworthy that the formulation of diets with low crude protein supplemented with amino acids is not only an economic and nutritional strategy, but also brings environmental benefits. As the animal will not use most of the feed components, this excess will be excreted in the urine and feces (HERMANN, 2003). The feed supplementation with an amino acid profile that meets the animal nutritional requirements will reduce the protein content, contributing to the reduction of energy necessary for the deamination of excess amino acids, and consequently the nitrogen and urea synthesis and excretion in the blood and urine (MA et al., 2015; TOLEDO et al., 2014; ZANG et al., 2011). In addition, diets with high concentrations of crude protein or with unbalanced proportions of amino acids increase the water intake by the animals, what consequently, results in an increase in the slurry volume and increase in pollution caused by excessive excretion (TOLEDO et al., 2014).

Amino acid balance is required for animal homeostasis, since diets with excess protein may result in amino acids accumulation such as tryptophan, isoleucine and

valine, which will be excreted in large volumes, increasing the rate of nitrogen released into the environment through urine (TOLEDO et al., 2014; ZANG et al., 2011). Garcia-Launay et al. (2014) conducted a feed supplementation experiment to pigs of different ages, with the aim of reducing the content of protein, the authors tested three kinds of feeds: (1) without addition of amino acids (2) with incorporation of amino acids and reduced protein content and (3) with amino acids and free of protein contents; and noted that the incorporation of amino acids (lysine, threonine, methionine, tryptophan and valine) into low protein diets clearly reduced the impacts of pig rearing on climate change, acidification and eutrophication.

Toledo et al. (2014) stated that the ideal crude protein concentration in animal feed is 15% to avoid recurring environmental problems. With the decrease in body protein content, less energy will be required for protein degradation and nitrogen excretion, thus reducing heat production (ZANG et al., 2011). The decrease in the crude protein concentration will consequently decrease the other amino acids concentration, therefore the economic and environmental advantage of the crude protein reduction in animal feed is confronted with the need for amino acid supplementation in the feed (CHEE, 2005; SWANEPOEL; ROBINSON; ERASMUS, 2016).

2.3.2.1 Lysine

Lysine is an especially reactive amino acid when compared to others containing an epsilon amino group, it can be obtained by microbial fermentation, besides being present in several sources of plant proteins, however some treatments, such as hydrothermal, reduce their bio-viability in these sources, which are often used for feeding animals (ruminants and non-ruminants). As lysine is the first limiting amino acid of several animals, its supplementation in vegetable protein based diets is a very common practice (POWELL; CHOWDHURY; BUREAU, 2015; VAN DER BORNE et al., 2012).

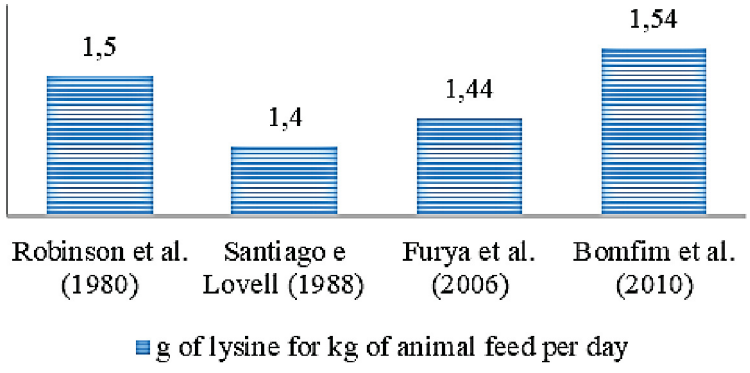
Pigs and fish growth performance depends on dietary lysine intake and the energy content of their diet; inadequate or inexpressive rates of lysine/energy will have a negative effect on growth (HAMID et al., 2016; ZANG et al., 2011). In pisciculture, the main lysine function is the deposition of proteins in the tissues, thus improving the yield of the meat; in addition, this amino acid increases immune responses and aids in

the gastrointestinal development of young catfish (HAMID et al., 2016; NGUYEN; DAVIS, 2016). Among other lysine benefits for aquaculture, Nguyen and Davis (2016) cited palatability, pH change, leaching and rapid absorption, emphasizing that catfish fed with higher lysine levels gain greater weight with lower feed conversion rate.

Researchers reported that the addition of 0.15% crystalline lysine (L-lysine HCl) in the feed of final growth stage pigs (when they require more of this amino acid) is sufficient to accelerate growth and prevent weight loss (LLATA et al., 2002; MOSER et al., 2000; NGUYEN; DAVIS, 2016; RICHERT et al., 1997). Ma et al. (2015) Reported that adjusted levels of dietary lysine could improve the meat characteristics of the animals. According to Kircher and Pfefferle (2001), the diet supplementation of 0.2% of L-lysine HCl causes the nitrogen release rate to fall because the amino acids will be used for the animal protein production and will not be in excess in the organism where it would be broken through an expensive energy process that would release nitrogen into the environment.

Several authors have proposed different ideal concentrations of dietary lysine for fish, as can be observed in FIGURE 5. The non-agreement in the ideal concentrations is due to the different species studied and the habitat influence.

FIGURE 5 – DIFFERENT CONCENTRATIONS OF DIETARY LYSINE FOR VARIOUS FISH SPECIES



SOURCE: The auteur (2017).

The classic form of lysine on the market is the L-lysine HCl, and there may be other forms, such as granulated lysine sulfate and liquid lysine, which generate less solid waste during its production (LEUCHTENBERGER; HUTHMACHER; DRAUZ, 2005). L-lysine HCl is obtained during the post-fermentation process, when the bacterial biomass is separated from the fermented broth; if such separation does not

occur, the resulting lysine will be referred to as L-lysine sulfate. Recently it has been proven that the two forms of lysine have relative ability to supply the nutritional need of the animal (POWELL; CHOWDHURY; BUREAU, 2015). In addition, Nguyen and Davis (2016) have stated that there are no significant differences between the supplementation of rations with L-lysine HCl or intact lysine obtained from highly concentrated corn protein with lysine. Therefore, despite the different sources for obtaining lysine, all have potential to improve the nutritional values of feed.

Lysine production reached about 550,000 tons in 2001 and about 1.5 million tons in 2013. This significant increase in the market is still present and the main producers that supply it are: Ajinomoto (Japan), ADM (USA), Cheil Jedang (South Korea), Global BioChem (China), BASF and Degussa (Germany) (HERMANN, 2003; KIND; BECKER; WITTMANN, 2013; LEUCHTENBERGER; HUTHMACHER; DRAUZ, 2005).

2.3.2.2 Another amino acids

Methionine is known to facilitate the transfer of blood lipids and the supply of methyl groups thereto for the choline and phosphatidylcholine synthesis (which plays an important role in metabolism) (SEYMOUR; POLAN; HRBEIN, 1990). There are reports that for dairy cattle, methionine and lysine are colimitants for the milk proteins synthesis and may aid in milk yield and fat accumulation in the milk of cows and sheep fed soybean meal. However a positive answer was not observed in some studies (MILIS et al., 2005; ROGERS et al., 1987; SEYMOUR; POLAN; HRBEIN, 1990).

The addition of different essential amino acids to the feed may explain differences in milk composition (milk protein and fat content and produced milk amount) (MOSER et al., 2000; RICHERT et al., 1997; SWANEPOEL; ROBINSON; ERASMUS, 2016). Rogers et al. (1989) affirmed that the supplementation of lysine and methionine increased the concentration of milk proteins, but did not interfere in the milk yield.

Valine, leucine and isoleucine are considered important amino acids in the dietary composition of lactating sows, in order to increase farrow weight, with valine being the most critical amino acid for this purpose. Older studies have already reported on the importance of adding these amino acids in the feed to raise the piglets weight, understanding that these branched-chain amino acids, mainly valine, are important

energy sources for the mammary glands, being absorbed by them and secreted as milk proteins (MOSER et al., 2000; RICHERT et al., 1997; TROTTIER et al., 1997).

Both the *succinyl-CoA* pathway and the *acetyl-CoA* pathway can metabolize isoleucine, while the *succinyl-CoA* pathway metabolizes valine. Both isoleucine and valine can be oxidized to become energy sources for the mammary gland (TROTTIER; EASTER, 1995).

Moser et al. (2000) did not notice any effect of leucine on milk yield and composition, however, when the valine content increased from 0.80% to 1.20% in the feed (independent of isoleucine and leucine concentration), observed a 2 kg weight gain in the farrow. An even greater weight gain was observed when the valine concentration increased for a diet supplemented with lysine as well. The authors associated this increase in weight with the increase in milk production, since the composition of this milk was not altered with the amino acid concentration. However, a few years earlier it was reported by Richert et al. (1997) that valine increases the milk fat concentration, having a minimum effect on the concentration of total milk proteins.

The increase in isoleucine content interferes with milk composition, increasing fat and crude protein concentrations (RICHERT et al., 1997). However, the increase in milk fat does not necessarily result in an increase in the animal's body weight. Richert et al. (1997) observed that the farrow weight gain has an independent response to the valine ratio to isoleucine, and that increasing the concentration of these two amino acids in the diet in addition to the recommended levels could increase weight loss during lactation and decrease feed intake and consequently the fat accumulation of the sows.

Another important amino acid in animal feed supplementation is tryptophan, which, like threonine, is commonly described as the second or third amino acid limiting crude protein for corn or soybean diets (LLATA et al., 2002; ZHANG et al., 2012). The ideal concentration of tryptophan in animal diets has not yet been established, but this amino acid is already recognized as playing an important role in the economic production of pigs fed diets based on corn meal and soybean meal containing low crude protein (ZHANG et al., 2012). The tryptophan requirement increases when the immune system is stimulated, because during the immune and inflammatory responses, the indoleamine 2,3-dioxygenase route limits the viability of this amino acid (MOFFETT; NAMBOODIRI, 2003; ZHANG et al., 2012).

Threonine and tryptophan become potentially limiting in the final stages of animal growth (LLATA et al., 2002). Threonine, together with lysine, influences the increase in body weight, increasing protein deposition. The threonine/lysine ratio is strongly influenced by the variation in the growth stage, and increases with the increase of weight and age, and the digestibility of threonine presents great variability in relation to the protein source used (MA et al., 2015; MASTELLAR; COLEMAN; URSCHEL, 2016), therefore it is necessary to know the diet concentrations necessary for an effective performance of the use of these amino acids and to avoid unnecessary expenses. For swines, the threonine/lysine ratio apparently does not influence the meat characteristics (except for the color), being its major function in the animal growth performance (MA et al., 2015; ZANG et al., 2011).

While for swines, threonine is a highly limiting amino acid in the final growth stage of the pig (100-135 kg), methionine is limiting during the growth phase corresponding to 30-70 kg (LLATA et al., 2002), and together with lysine, threonine and tryptophan have the potential to increase growth performance (LLATA et al., 2002; ZHANG et al., 2012). Methionine is an important amino acid not only for swine breeding, it is also used in aquaculture to supplement rations based on soybean meal, however with variable performance. There are study reports in which methionine favored the growth and weight gain of different fish and works in which its performance was irrelevant (MURAI et al., 1986; SHIAU et al., 1978), however it is necessary to consider that the anti-nutritional factors can affect the result (EL-SAIDY; GABER, 2002).

The substitution of expensive animal feed ingredients for low-cost ingredients (plant proteins) has raised the problem of meeting the amino acid needs of the herd, since nutrient digestibility is associated with protein sources and how amino acids are incorporated into the diet (NGUYEN; DAVIS, 2016). For example, the above recommended proportions addition of lysine and the decrease in protein source may cause deficiency in several essential amino acids (LLATA et al., 2002).

Amino acids unbalance in animal feed can lead to several problems not only economic and environmental but also nutritional (SWANEPOEL; ROBINSON; ERASMUS, 2016), and aiming to optimize the feed energy content, many methods were formulated, based on statistical and experimental techniques, including: (1) oxidation of amino acids indicator which evaluates the amino acids necessity in other species (MASTELLAR; COLEMAN; URSCHEL, 2016); (2) *serum amino acid*, which

produces standard references that can be used to evaluate protein quality; (3) *dose-response study*; (4) *broken-line regression model*; (5) *curvilinear-plateau*; (6) *quadratic model*, (7) linear slope-ratio assay to evaluate the effects and bioavailability of amino acids (MA et al., 2015; POWELL; CHOWDHURY; BUREAU, 2015; ZHANG et al., 2012). These methods aim to study the relationship between nutritional quality and animal development (individually or as a population) for a future ideal diet formulation.

2.4 CONSIDERATIONS

This review introduced glycerol as a low cost, non toxic and one of the most promising agro-industrial residues, resulting from biodiesel production, to be used as substrate in biotechnological processes; *Corynebacterium glutamicum* as a superior producer of amino acids, although this bacteria has to be manipulated to be able to metabolize glycerol; and the importance of aminoacids in animal feed supplementation.

The amino acid supplementation of animal feed influences significantly on the animal health, milk yield and meat quality. For example: methionine and lysine are colimitants for the milk proteins synthesis and may aid in milk yield and fat accumulation in the milk of ruminants, but other amino acids (like branched-chain amino acids - valine, leucine and isoleucine) are also important energy sources for the mammary glands. Threonine, lysine and tryptophan become potentially limiting in the final stages of animal growth, increasing body weight and protein deposition. This influence is allied to some factors such as protein source (nutritional value and quality of structurally different proteins), preparation of the raw material (susceptibility to hydrolysis during ingestion, purity and behavior during and after treatments), physiological age and immune system of the animal.

Amino acids unbalance in animal feed can lead to several problems (i.e., economic, environmental and nutritional), and aiming to optimize the feed energy content, many methods have been formulated to evaluate the relation between nutritional quality and animal development (individually or as a population) for a future ideal diet formulation.

3 MATERIAL AND METHODS

3.1 GROWING MEDIA

3.1.1 *Microorganism and reactivation*

The *Corynebacterium glutamicum* strains ATCC 21799 (and its mutants M5, M5M10 and M5M17), ATCC 21543, Pasteur 828, NRRL B4262, NRRL B2784 and NRRL B3330 were used in this work. The mutants of the parental strain ATCC 21799 were kindly donated by Luiz Alberto Junior Letti, the methodology for the mutation, selection and conservation of such strains are described in Letti (2014). All the above strains were reactivated in medium containing nutrient broth (13 g/L) supplemented with glucose (7 g/L) at 30°C under shaking of 120 rpm for 72 h.

3.1.2 *L-lysine fermentation*

After 72 h of reactivation, 12 mL of the fermented medium was deposited in a 15mL Falcon tube, the cells were centrifuged for 15 minutes at 5000 rpm, suspended with saline solution (0.1%). The procedure was repeated twice. Finally, 1.5 mL of the suspension cells were inoculated in fermentation medium (TABLE 1 in the ANNEX 1) and incubated at 30°C under shaking of 120 rpm for 72 h.

For convenience, the fermentation medium in which the carbon sources were sugarcane molasses, hydrolyzed sugarcane molasses and glycerol will be pointed to in this work as M Medium, HM medium and GLY Medium.

Each component of the fermentation medium was autoclaved separately and the vitamins were sterilized by filters with membranes of 22 μm (Milipore).

3.1.2.1 Hydrolysis of sugarcane molasses

An experimental design (TABLE 2 in ANNEX 2) was carried out to obtain the best condition (acid type and concentration) for the hydrolysis of molasses. Samples with 100 g/L of molasses were prepared and submitted to the following conditions: 0.25%, 0.5%, 0.75%, 1%, 2% and 3% of H_2SO_4 and HCl respectively. The hydrolysis was carried out at 121°C for 15 minutes (autoclaving condition). The solutions were

neutralized and diluted 1:100. The DNS method was used (as described in item 4.2.3.1) and plate absorbances were read in the BioTek PowerWave XS plate reader at 540 nm. Afterwards the results were compared with the standard curve (see TABLE 2 and FIGURE 6 in Annex 2) and thus the type and concentration of ideal acids to hydrolyze the molasses used as carbon source of the HM medium were selected.

3.2 ANALYTICAL METHODS

3.2.1 *Evaluation of strains integrity*

3.2.1.1 Fresh smear

In laminar flow chamber, one drop of each culture was transferred with a handle to the slide, covered with a cover slip and observed under the immersion optical microscope.

3.2.1.2 Gram staining

The smear of the desired material was carried out on a slide and the material was fixed with fire. The smear was covered with violet crystal (1st dye) for 1 minute, then the dye was drained and washed with water. The slide was then covered with lugol (sparingly) for 1 minute and washed with running water of low pressure. Cells were decolorized with ketone alcohol for 1-5 seconds and washed with running low pressure water. Subsequently, the smear was covered with Tiehl Neelsen Fuchsin 1:10 (2nd dye) for 30 seconds and again washed with low-pressure tap water. The slides were allowed to dry spontaneously and were then observed under the immersion optical microscope.

3.2.1.3 Drop-plate

The fermented culture medium was diluted with peptone water to the concentrations of 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}, then 30 µL of the dilutions were transferred to a Petri dish containing nutrient agar.

3.2.2 *Biomass quantification*

3.2.2.1 Dry weight

The Falcon tubes were dried in a kiln and the mass was measured (m_e). After that, they received 12 mL of fermented (v_{ms}) and they were centrifuged for 15 minutes at 5000 rpm. Cells were washed twice in distilled water followed by centrifugation. After centrifugation, the supernatant was discarded and the pellet (Falcon + sedimented) was dried for at least 24 h in an oven at 80°C. The mass of the set was measured (m_f). Cell dry weight was calculated by the difference:

$$Dry\ weight\ (g/L) = 1000\ \left(\frac{m_f(g) - m_e(g)}{v_{ms}}\right) \quad (1)$$

3.2.3 *Evaluation of sugar consuption*

The evaluation of sugar consumption by microorganisms was performed using the DNS method, described by Miller (1959) and adapted for smaller volumes and for reading in a 96-well plate.

3.2.3.1 Curve for reducing sugar

First, solutions were prepared with 0, 0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8 and 2 g/L of glucose. 0.1 mL of these solutions were transferred to Eppendorf tubes along with 0.1mL of 3,5-dinitrosalicylic acid (DNS). The reaction was carried out in a water bath (96°C) for 5 minutes and quenched with the addition of 0.5 mL of deionized water in Eppendorf. Absorbance reading was performed on the BioTek PowerWave XS card reader at 540 nm. Afterwards, the graph was plotted with the points and absorbances given (see TABLE 2 and FIGURE 6 in Annex 2).

3.2.4 *Evaluation of L-lysine production*

3.2.4.1 Modified Chinard method

L-lysine quantification was determined by an adaptation of the Chinard protocol (Chinard 1952). For a quantity of 15 samples, reagent A: ninhydrin (0.375 g), phosphoric acid (6 mL) and glacial acetic acid (9 mL) were prepared by reacting for one hour on a heated magnetic stirrer; and reagent B: phosphoric acid (6 mL) and glacial acetic acid (9 mL) were reacted under the same conditions. After dissolving all the ninhydrin in reagent A, 1 mL of the sample, 1 mL of glacial acetic acid and 1 ml of the reagent (A or B) were added to a sample tube. The reaction occurred for one hour in a water bath at 96°C and then quenched by adding 2 mL of glacial acetic acid. Absorbance reading was performed on the BioTek PowerWave XS plate reader at 510 nm. The concentration of lysine in the fermented medium was given by the comparison between the absorbance supplied and the standard curve (see TABLE 3 and FIGURE 7 in Annex 3).

a) Standard curve

Different test tubes were prepared with concentrations of 0, 0.25, 0.5, 0.75, 1, 1.5, 2 and 2.5 g/L of lysine. The adapted Chinard test was performed and was plotting the absorbance by concentration graph (see TABLE 3 and FIGURE 7 in Annex 3).

4 RESULTS AND DISCUSSION

4.1 COMPOSITION OF THE CARBON SOURCE AND HYDROLYSIS

Due to the great production of sugarcane molasses and their properties, in this work, it was chosen to use it as carbon source of the culture medium (M medium) for *C. glutamicum*. Sugarcane molasses used as a culture medium in this work contains 7.9%, 6.98% and 39.9% (w/w) of fructose, glucose and sucrose (see CHART 4), however this composition may vary due to several factors such as pre-treatment, storage form, sugarcane variety and soil cultivation type and also the classification method (GRUNWALD, 2014; DOTANIYA et al., 2016).

CHART 4 – MOLASSES COMPOSITION

Component	Unit	Value
Calcium	mg/Kg	11455
Ashes	%w/v	8.7
Cobalt	mg/Kg	1
Copper	mg/Kg	4.2
Dextran	mg/Kg	6073
Iron	mg/Kg	71
Total phosphor	mg/L	863
Fructose	%w/w	7.9
Glucose	%w/w	6.98
Magnesium	mg/Kg	3991
Manganese	mg/Kg	29.1
Potassium	mg/Kg	25572
Sucrose	%w/w	39.9
Sodium	mg/Kg	119
Sulfite	mg/Kg	2114
Zinc	mg/Kg	6.2

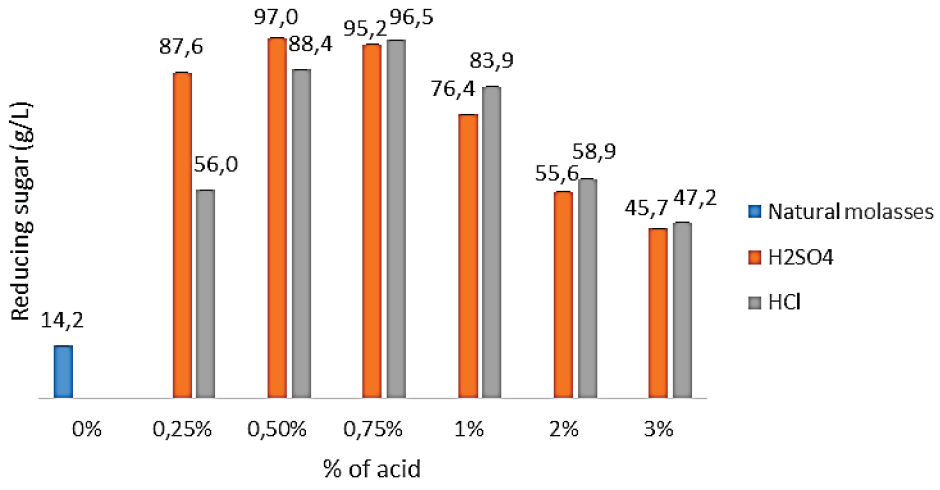
It was decided to hydrolyze the molasses used as a culture medium, which previously contained about 14.2 g/L of reducing sugars, for the liberation of glucose and fructose, accelerating the consumption of sugars due to minimization of the intracellular fructose formation and the need for its efflux to enable metabolization (since *C. glutamicum* lacks fructokinase and the only feasible path of fructose uptake is the PTS_{fru} system). After hydrolysis and pH neutralization the reducing sugars concentrations varied from 47.2-96.5g/L for the use of HCl at concentrations of 0.25-3% and 45.7-97.0g/L for H_2SO_4 at concentrations of 0.25-3% (see TABLE 4 and FIGURE 8 below).

TABLE **Erro! Argumento de opção desconhecido.** – CONCENTRATION OF REDUCING SUGARS AFTER ACID HYDROLYSIS

	Acid concentration	Absorbance (540nm)			Average	Reducing sugar concentration (g/L)
H_2SO_4	0.25%	0.547	0.532	0.553	0.544	87.6
	0.50%	0.589	0.582	0.608	0.593	97.0
	0.75%	0.585	0.576	0.589	0.583	95.2
	1%	0.467	0.489	0.498	0.485	76.4
	2%	0.412	0.363	0.353	0.376	55.6
	3%	0.358	0.343	0.270	0.324	45.7
HCl	0.25%	0.392	0.371	0.372	0.378	56.0
	0.50%	0.559	0.533	0.551	0.548	88.4
	0.75%	0.593	0.587	0.591	0.590	96.5
	1%	0.553	0.539	0.481	0.524	83.9
	2%	0.398	0.391	0.391	0.393	58.9
	3%	0.296	0.374	0.325	0.332	47.2
Natural molasses		0.158	0.161	0.160	0.160	14.2

Source: The auteur (2017).

FIGURE **Erro! Indicador não definido.** – AMOUNT OF REDUCING SUGAR RELEASED AFTER DIFFERENT HYDROLYSIS CONDITIONS



Source: The auteur (2017).

Other alternative to increase the consumption of sucrose is the heterologous expression of the *scrK* gene (encoding fructokinase) from *Clostridium acetobutylicum*, to allow phosphorylation of intracellular fructose without the need for efflux (ZHANG et al., 2017), however, at this stage we do not work with genetic engineering.

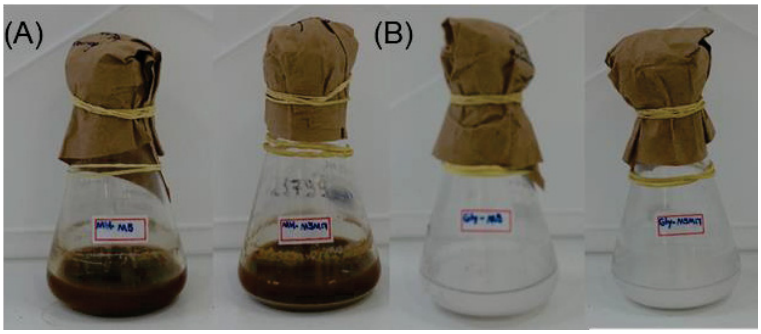
As observed in TABLE 8 (in Annexes) and FIGURE 4, at 0.75% the efficiency of both acids was similar, and below that concentration H_2SO_4 proved to be more effective for the hydrolysis of molasses. The acid hydrolysis condition that releases more reducing sugars from molasses is H_2SO_4 at 0.5%, having an efficiency of 97%, and since sulfuric acid has a relatively cheaper market price than hydrochloric acid, this was the chosen condition for the pretreatment of molasses that will be used as the carbon source of the culture medium (medium HM).

As in a fermentation process the objective is to obtain the highest productivity with the lowest cost, it was also chosen, using the glycerol in parallel, a non-glycolytic and nontoxic compound, and one of the most promising agro-industrial residues, resulting from biodiesel production, as carbon source in the biotechnological process carried out in this work (GLY medium) (ANITHA; KAMARUDIM; KOFLI, 2016; SUN et al., 2015).

4.2 GROWTH IN DIFFERENT CARBON SOURCES

Microorganisms need specific conditions that stimulate their growth and the formation/accumulation of the product of interest. For this purpose, the *C. glutamicum* strains were cultivated for 72h under 120rpm and at 30°C, which are the conditions that allow good growth of most *C. glutamicum* species (BLOMBACH et al., 2011; LETTI et al., 2016; EGGELING; BOTT, 2005), in M, HM and GLY formulated media (see FIGURE 9 bellow and TABLE 1 in Annexes). Biotin and threonine used as micronutrients in these media play an important role in the physiology of cells and in the ability to form L-glutamate and L-lysine (EGGELING; BOTT, 2005).

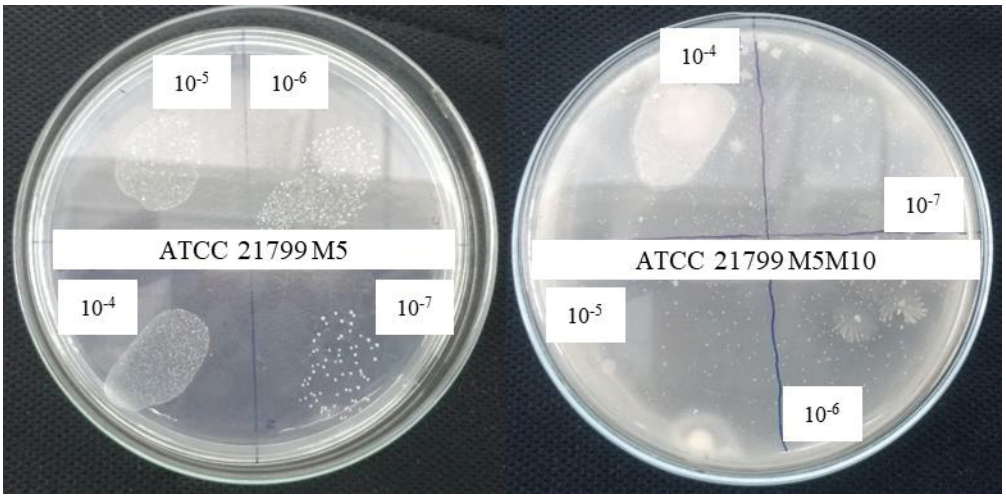
FIGURE **Erro! Argumento de opção desconhecido.** – *Corynebacterium glutamicum* strains ATCC 21799 M5 and ATCC 21799 M5M10 after culturing of 72 h at 30°C and 120rpm in HM medium (in A) and in GLY medium (in B)



Source: The auteur (2017).

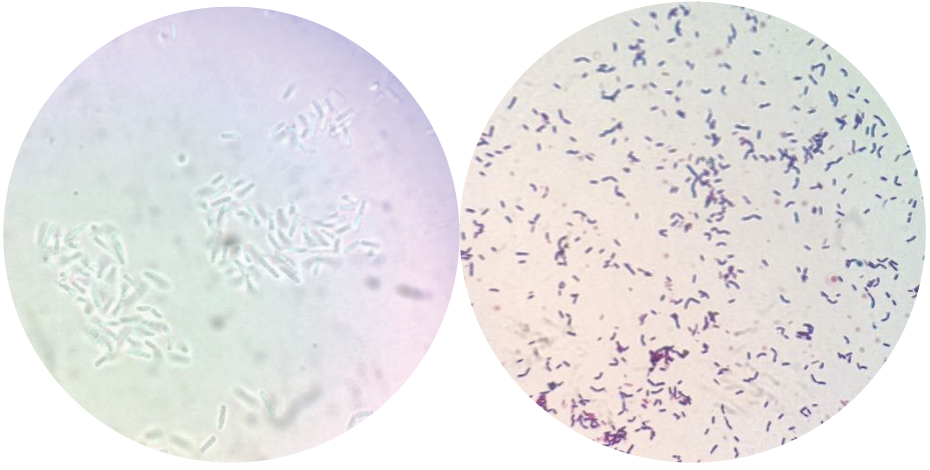
Strains integrity was verified at the beginning and at the end of each fermentation by microscopy (fresh and Gram staining) and Petri dish culture (drop plate). From FIGURE 10 we can observe gram-positive pleomorphic bacilli, which are commonly agglomerated, typical of *C. glutamicum*. And in FIGURE 11, it is verified that cultures (mainly mutants) present several stages of development, and may present as small, smooth, regular-edged colonies or form large, rough, irregular-edged colonies (EGGELING; BOTT, 2005, LETTI, 2014).

FIGURE **Erro! Indicador não definido.** – *Corynebacterium glutamicum* strain ATCC 21799 M5 (in A) and ATCC 21799 M5M10 (in B), with dilutions of 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4}, after 72h of culture at 30°C in agar Nutrient Broth



Source: The auteur (2017).

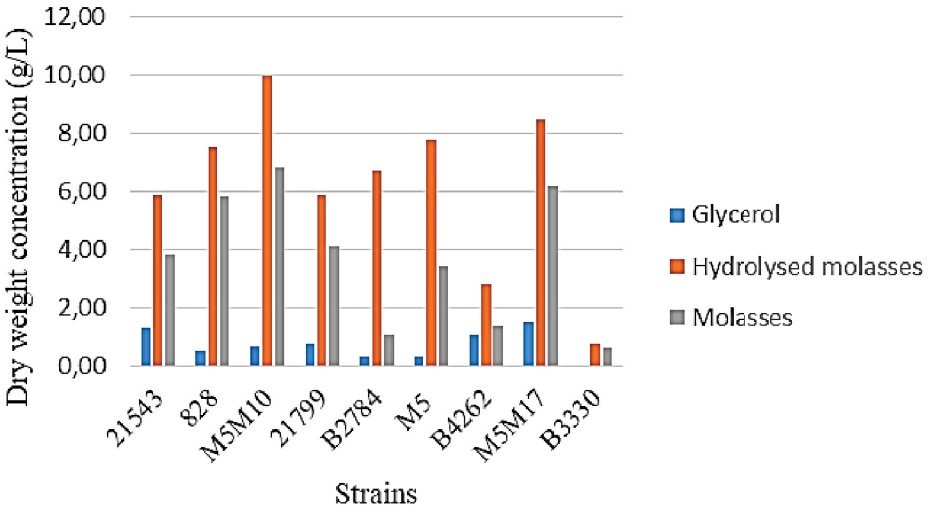
FIGURE **Erro! Indicador não definido.** – Fresh microscopy and gram stain of *Corynebacterium glutamicum* ATCC 21799 M5



Source: The auteur (2017).

During cultivation, *C. glutamicum* strains presented a regular growth, turbidizing some media after 30h of cultivation. After 72h of culture, it was noted the formation of films on the surface of the (LETTI, 2014), these films accumulated on the surface of the bioreactor due to rotational agitation (FIGURE 9). By observing the relationship between the growth of the different strains with the type of carbon source tested (FIGURE 12), it is noticed that the strain NRRL B3330 had a loss of viability, probably due to some negative mutation during storage; the other strains presented significant growth in molasses (in non-hydrolyzed an hydrolyzed), proving that *C. glutamicum* is able to co-utilize different sugars when supplemented with a mixture (in the case of glucose, fructose and sucrose) (GRUNWALD, 2014).

FIGURE **Erro! Indicador não definido.** – GROWTH OF STRAINS IN DIFFERENT CARBON SOURCES



Source: The auteur (2017).

C. glutamicum growth rate of in sucrose is lower than in glucose and fructose mixtures (KRAUSE et al., 2010), making pre-treatment of molasses with acid hydrolysis an excellent alternative to accelerate microbial growth, since it provides higher concentration of reducing sugars in the culture medium. Georgi, Rittmann and Wendisch (2005) reported that an alternative to accelerate *C. glutamicum* growth in non-hydrolyzed molasses would be the overexpression of *fbp* gene, which would lead to almost complete use of sucrose. Another alternative that leads to increased biomass at all fermentation stages is the overexpression of *pfkA* gene that encodes

phosphofructokinase (PFK), the enzyme that catalyzes the conversion of fructose-6-phosphate to fructose-1,6-diphosphate in the glycolytic pathway, however, this approach decreases sucrose consumption and increases the concentration of residual fructose (ZHANG et al., 2017).

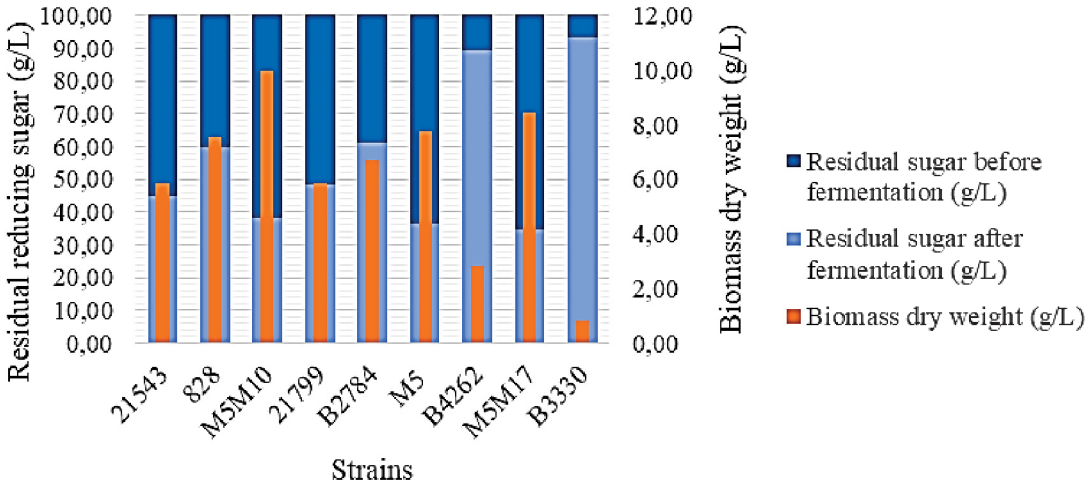
Even *C. glutamicum* being able to encode glycerol kinase (encoded by *glpK* gene) and glycerol-3-phosphate dehydrogenase (encoded by *glpD* gene) (MEISWINKEL et al., 2013; WENDISCH et al., 2016), as can be seen in FIGURE 12, none of the strains analyzed showed significant growth in GLY medium after 72 h of culture, therefore the expression of *glpK* and *glpD* was not large enough to sustain growth. Meiswinkel et al. (2013) also failed to grow spontaneous mutant strains in glycerol due to low expression of these two genes.

Since not all strains, even engineered, adopt glycerol as a good carbon source, an alternative is the use of sub-stoichiometric glucose in the culture medium to stimulate the growth and consumption of glycerol (MEISWINKEL et al., 2013). Cultures with GLY medium supplemented with glucose were performed, although there was no effective growth of cellular biomass (data not shown).

4.3 SUGAR CONSUMPTION AND L-LYSINE PRODUCTION

As bacterial cells oxidize complex carbon sources to obtain the energy required for their metabolism and growth (ALBERTS et al., 2002) it was expected that sugar uptake would be proportional to bacterial growth. Thus, in FIGURE 13 it can be verified that each strain of *C. glutamicum* behaved in a peculiar way in relation to the growth and consumption of sugars, but the mediums with the lowest concentration of remaining sugars also presented the highest concentration of dry biomass (see TABLE 5). The NRRL B4262 and B3330 strains had no effective growth and the residual sugar at the end of fermentation was 89.59 and 93.26 g/L. The strains with the highest cell growth were ATCC 21799 mutants M5M10, M5M17 and M5 that presented sugar consumption of 61.82, 65.26, 63.52 g/L respectively, a consumption 23% higher than that of the parental strain.

FIGURE **Erro! Indicador não definido.** – INITIAL TOTAL SUGAR CONCENTRATION AND THE RESIDUAL SUGAR CONCENTRATION AFTER FERMENTING BY VARIOUS STRAINS OF C. GLUTAMICUM GROWTHING ON HYDROLYZED MOLASSES



Source: The auteur (2017).

TABLE **Erro! Argumento de opção desconhecido.** – CONCENTRATION OF REDUCING SUGARS AFTER ACID HYDROLYSIS

Strain	Absorbance (540nm)			Average	Reducing sugars*
21543	2.292	2.332	2.361	2.328	44.67
828	3.221	3.245	2.898	3.121	59.80
M5M10	2.015	2.009	1.942	1.989	38.18
21799	2.582	2.515	2.488	2.528	48.47
B2784	3.199	3.298	3.095	3.197	61.24
M5	1.986	2.109	1.874	1.900	36.48
B4262	4.674	4.588	4.785	4.682	89.59
M5M17	1.731	1.906	1.788	1.808	34.74
B3330	4.735	4.768	5.118	4.874	93.26

*The reducing sugars present at the culture media after 72h of fermentation were calculated according to equation 2 in Annex 2.

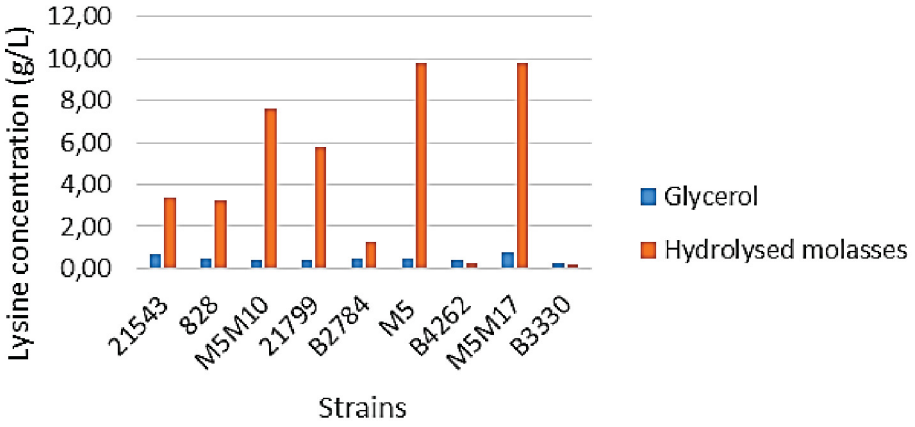
Nelofer et al. (2007), working with Homoserine auxotrophic mutants strains of *C. glutamicum* grown on non-hydrolyzed molasses, obtained a mean sugar consumption of 39.1 g/L. With the intention to increase the consumption of sucrose, Zhang et al. (2017) coexpressed *scrK* and *pfkA* genes and found an increase in cell biomass and a reduction in residual fructose concentration at the end of the culture. In the present work, we opted to break down the glycosidic bonds of sucrose (by acid hydrolysis), giving the cells a higher concentration of glucose and fructose, thus avoiding sucrose absorption and consequent efflux of fructose. From the results

presented it can be confirmed that the consumption of sugar in media with hydrolyzed molasses is higher than in medium with natural molasses.

C. glutamicum amino acids production preferably uses substrates such as glucose, fructose and sucrose consumed through the phosphenolpyruvate-dependent sugar phosphotransferase system (PTS), a process that requires high aeration (ZAHOOR; LINDNER; WENDISH, 2012; ZHANG et al., 2017). *C. glutamicum* has been extensively studied and engineered for the production of mutant superproducers (auxotrophic or regulatory). The techniques employed generally revolve around the modification (1) of the central carbon metabolism, (2) of the terminal routes and (3) of the redox cofactor regeneration system (TOSAKA; ENEI; HIROSE, 1983; XAFENIA; KMEZIK; MAPELLI, 2017).

Several researchers are seeking access to carbon source alternatives such as glycerol and lignocellulosic materials (LINDNER et al., 2012; ZAHOOR; LINDNER; WENDISH, 2012; ZHANG et al., 2017), which are not naturally metabolized by *C. glutamicum*, requiring the formation of mutants with heterologous expression of specific genes that allow absorption of the substrate in question. As can be seen in FIGURE 14, neither the wild strains nor regulatory mutants tested in this work had effective production of lysine in GLY medium, probably because they could not metabolize glycerol (as explained in the previous item).

FIGURE **Erro! Indicador não definido.** – LYSINE PRODUCTION BY VARIOUS STRAINS OF *C. glutamicum* growthing ON GLYCEROL AND HYDROLYZED MOLASSES AS CARBON SOURCES

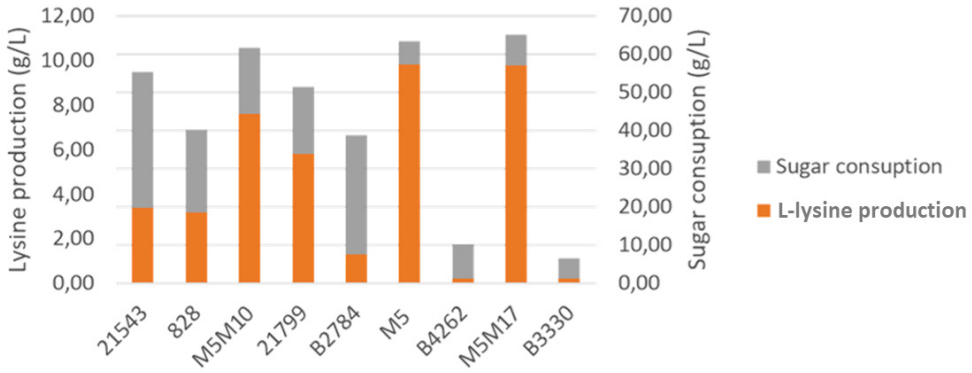


Source: The auteur (2017).

In contrast, for HM medium, L-lysine was produced in all strains analyzed, except for NRRL B4262 and NRRL B3330, which lost viability (FIGURE 14 AND

FIGURE 15). The strains with the highest production of L-lysine were ATCC 21799 (with yield of 5.83 g/L) and their mutants M5M10 (7.60 g/L), M5M17 (9.79 g/L) and M5 (9.84 g/L) (see TABLE 6). Yanase et al. (2016) achieved a similar result (9.36 g/L) for a strain of *C. glutamicum* ATCC 13032 desensitized for inhibition by aspartokinase feedback.

FIGURE **Erro! Indicador não definido.** – RELATIONSHIP BETWEEN SUGAR CONSUMPTION AND L-LYSINE PRODUCTION ON HYDROLYZED MOLASSES CARBON SOURCES



Source: The auteur (2017).

TABLE **Erro! Argumento de opção desconhecido.** – RELATIONSHIP BETWEEN L-LYSINE CONCENTRATION ON HM MEDIA AND ABSORBANCE AT 510 NM, AFTER 72H OF FERMENTATION FOR DIFFERENT STRAINS OF *C. glutamicum*.

Strain	Absorbance (540nm)			Average	L-lysine (g/L)*
21543	0.787	0.496	0.494	0.592	3.39
828	0.635	0.503	0.551	0.563	3.22
M5M10	1.312	1.307	1.264	1.294	7.60
21799	0.996	0.987	1.012	0.998	5.83
B2784	0.255	0.229	0.244	0.243	1.30
M5	1.685	1.679	1.636	1.667	9.84
B4262	0.069	0.060	0.059	0.063	0.22
M5M17	1.670	1.663	1.643	1.659	9.79
B3330	0.043	0.061	0.072	0.059	0.20

*Calculated according to equation 2 in Annex 2.

The mutants evaluated in this work, ATCC 21799 M5, M5M10 and M5M17 were generated after exposure to ultraviolet light and selected by growth resistance in medium containing the thialysine analog. It may be assumed that the mutants possess some key enzyme of L-lysine production with resistance to feedback inhibition, such as aspartokinase. Yanase et al. (2016) also performed mutations induced by chemical

agents (such as N-methyl-N'-nitro-Nitrosoguanidine, NTG), and selected the mutants from the growth resistance on medium containing the lysine analogue S-(2-aminoethyl)-L-cysteine. When analyzing the mutations resulting from this random method, they noticed the predominance of decreased on citrate synthase (CS) activity and phosphoenolpyruvate carboxylase (PEPC) desensibilization for feedback inhibition by aspartic acid.

Subsequently the authors silenced *pyc* genes (which encodes pyruvate kinase) and introduced the D299N mutation in the *ppc* gene (for desensitization to PEPC) and the S252C mutation in the *gltA* gene (for the reduction of the citrate synthase activity), increasing the production in 1.68 times (15.7 g/L) (YANASE et al., 2016). Other strategies that could be adopted for the formation of L-lysine overproducing strains would be the overexpression of key genes in the production route (to quote *lysC*, *dapA*, *asd*, *lysA* and *dapB*) or the expression of genes that favor pathways that increase the generation of NADPH (to quote *zwf*, *gnd*, *fbp*, *icd*, *malE* and *ppnk*) (WU et al., 2015), as for the production of 1 mol of L-lysine, 4 mol of NADPH are required (XAFENIA; KMEZIK; MAPELLI, 2017).

The HM culture medium is rich in glucose and fructose, the use of mixed carbon sources entails a decrease in glucose uptake and lysine production; the low productivity is due to the fact that during the growth in fructose there is a low flow of NADPH (GEORGI; RITTMANN; WENDISCH, 2005; EGGELING; BOTT, 2005). The introduction of fructokinase (expression of the *pfkA* gene) could increase the carbon supply in the pentose phosphate pathway (PPP), resulting in a higher supply of NADPH in media with sucrose (ZHANG et al., 2017). Similar result can be achieved by overexpressing the fructose-1,6-bisphosphatase gene *fbp* (GEORGI; RITTMANN; WENDISCH, 2005).

The lysine productivity found in this work was also similar to that reported by Anusree and Nampoothiri (2015) for *C. glutamicum* DM 1729 cultured in medium containing jackfruit seed hydrolysate (8 g of lysine per liter of medium), hereafter they optimized fermentation parameters to increase lysine production in 1.63 times. In future stages we intend to optimize fermentation parameters using statistical strategies such as Box-Behnken Design under Response Surface.

CONCLUSIONS AND PERSPECTIVES

C. glutamicum presents various stages of development, and it may presents as small, smooth, regular-edged colonies or form large, rough, irregular-edged colonies. This Gram-positive bacterium is able to co-utilize different sugars when supplemented with a mixture of simple sugars, but the sucrose, fructose or mixtures of such sugars with glucose is incomplete. To optimize the use of sugarcane molasses by *C. glutamicum*, a acid hydrolysis of the molasses was performed to release. At 0.75% the efficiency of HCl and H_2SO_4 was similar, and below that concentration H_2SO_4 was more effective for the hydrolysis of molasses. H_2SO_4 at 0.5% was the hydrolysis condition that releases more reducing sugars from molasses. Other strategy to accelerate *C. glutamicum* growth in molasses is the overexpression of *fbp* and *pfkA* gene. None of the strains analyzed showed significant growth or lysine production in GLY medium (or in GLY medium supplemented with glucose) after 72 h of culture due to low expression of *glpK* and *glpD* genes. The strains with the highest cell growth, sugar consumption and L-lysine production, were ATCC 21799 mutants M5M10, M5M17 and M5, grown in HM molasses, that presented a consumption around 23% higher than that of the parental strain. The consumption of sugar in medium with hydrolyzed molasses is higher than in medium with natural molasses because the hydrolyzed molasses have higher concentration of glucose and fructose, thus avoiding sucrose absorption and consequent efflux of fructose from *C. glutamicum* cells. Maybe the higher L-lysine production for mutants is due the presence of some key enzyme of L-lysine production with resistance to feedback inhibition. The low productivity is due to the fact that during the growth in fructose there is a low flow of NADPH.

As perspectives, we intend to (1) test the molasses hydrolysis with phosphoric acid in addition to the H_2SO_4 and HCl previously used; (2) analyze the L-lysine production using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) and compare them with the modified Chinard method; (3) perform sugar and l-lysine production kinetics for strains with higher productivity; (4) generate auxotrophic and regulatory mutants using random mutation techniques and using recombinant DNA and omics technologies; (5) optimize fermentation parameters using statistical strategies; (6) perform anaerobic fermentation with the incorporation of electrodes (cathodes) as energy source, and (7) to scale up the production.

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ANNEX 1 – CULTURE MEDIUM FOR THE FERMENTATION OF L-LYSINE

TABLE 4 – FERMENTATION MEDIUM

Component	**(g/l)**
Carbon source	
Molasses	100
Hydrolyzed molasses	100
Glycerol	100
Nitrogen source	
$(NH_4)_2SO_4$	40
Urea	5
Salts and vitamins	
$MgSO_4.7H_2O$	0,25
K_2HPO_4	0,5
KH_2PO_4	0,5
$CoSO_4.7H_2O$	0,01
$ZnSO_4.7H_2O$	0,001
$FeSO_4.7H_2O$	0,01
$MnSO_4.5H_2O$	0,01
D-biotin	0,01
L-leucine	0,4

Source: The auteur (2017).

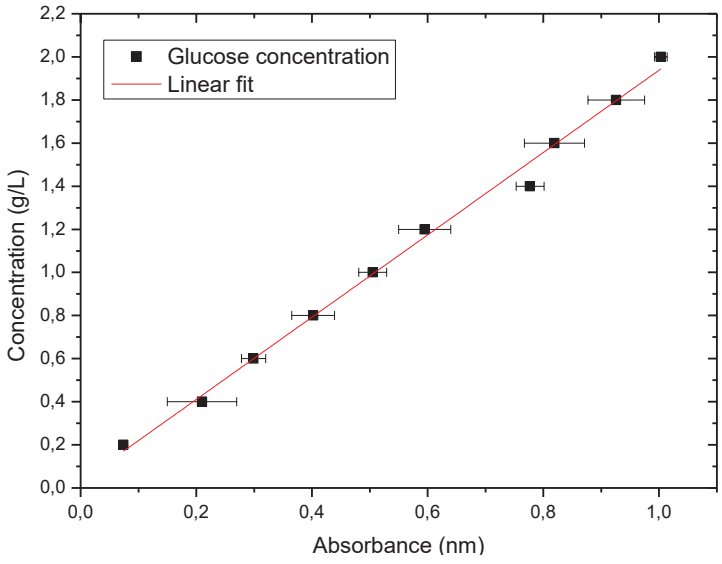
ANNEX 2 – STANDARD CURVE FOR REDUCING SUGARS

TABLE 5 – RELATIONSHIP BETWEEN GLUCOSE CONCENTRATION AND ABSORBANCE AT 540 NM AFTER REACTION WITH DNS

Glucose concentration (g/L)	Absorbance (540nm)			Average	Minus the White:
0.2	0.156	0.161	0.161	0.159	0.074
0.4	0.365	0.264	0.257	0.295	0.210
0.6	0.376	0.408	0.368	0.384	0.299
0.8	0.453	0.527	0.483	0.488	0.402
1.0	0.574	0.579	0.617	0.590	0.505
1.2	0.701	0.711	0.629	0.680	0.595
1.4	0.836	0.866	0.884	0.862	0.777
1.6	0.845	0.924	0.943	0.904	0.819
1.8	1.017	0.960	1.057	1.011	0.926
2.0	1.081	1.101	1.083	1.088	1.003
White	0.086	0.087	0.083	0.085	x

Source: The auteur (2017).

FIGURE 7 – STANDARD CURVE FOR REDUCING SUGARS



Source: The auteur (2017).

Curve standard for reducing sugars equation:

$$y = 1{,}9089x + 0{,}0291 \quad (2)$$

R^2=0,9943

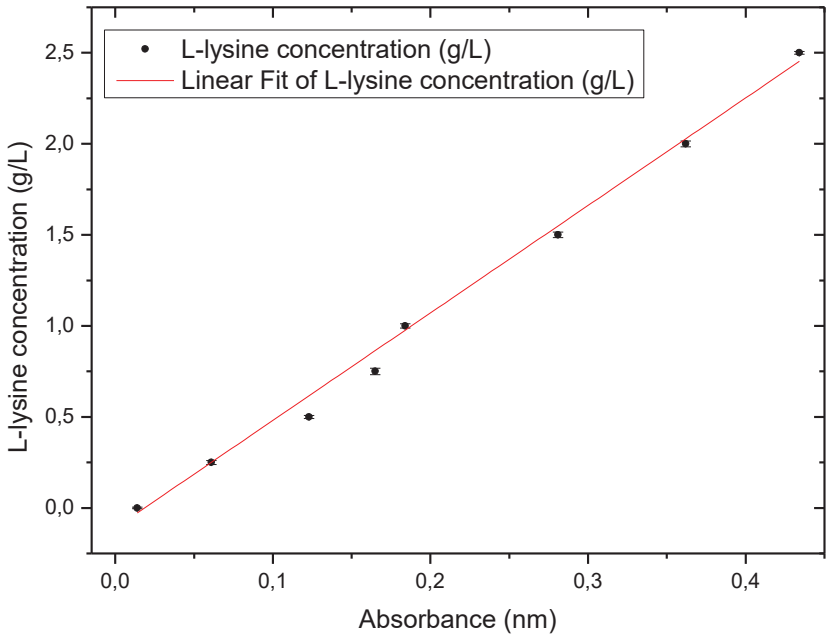
ANNEX 3 – STANDARD CURVE FOR L-LYSINE CONCENTRATION

TABLE 6 – RELATIONSHIP BETWEEN L-LYSINE CONCENTRATION ON BROTH MEDIA AND ABSORBANCE AT 510 NM AFTER CHINARD PROTOCOL

L-lysine concentration (g/L)	**Absorbance (510nm)**			**Average**
0.00	0.011	0.019	0.013	0.014
0.25	0.068	0.049	0.067	0.061
0.50	0.132	0.116	0.122	0.123
0.75	0.171	0.179	0.146	0.165
0.10	0.196	0.173	0.183	0.184
1.50	0.263	0.289	0.291	0.281
2.00	0.361	0.379	0.347	0.362
2.50	0.425	0.437	0.440	0.434

Source: The auteur (2017).

FIGURE **Erro! Indicador não definido.** – STANDARD CURVE FOR L-LYSINE



Source: The auteur (2017).

Curve standard for L-lysine equation:

$$y = 5{,}9942x - 0{,}1541 \quad (3)$$

R^2=0,9940