Université de Provence
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PROPIONIC ACID PRODUCTION BY *Propionibacterium sp.* USING LOW-COST CARBON SOURCES IN SUBMERGED FERMENTATION

Presented in partial fulfillment of the requirements for the Master Degree of Sciences: Mention Microbiology, Plant Biology and Biotechnologies in the University of Provence, University of the Mediterranean Sea and Federal University of Parana.

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ABSTRACT

Propionic acid is widely used as additive in animal feed and also in the manufacturing of cellulose-based plastics, herbicides, and perfumes. Salts of propionic acid are used as preservative in food. Propionic acid is mainly produced by chemical synthesis. Nowadays, propionic acid production by fermentation of low-cost carbon sources has been an interesting alternative. At the present investigation, propionic acid production by eight propionibacteria strains was studied using a basal medium with sugarcane molasses (BMSM), glycerol (BMG) or lactate (BML) in small batch fermentation at 30 and 36°C, in order to select the best one for further experiments in stirred-tank bioreactor. Bacterial growth was carried out under low dissolved oxygen concentration and without pH control in 1L batch assays. Results indicated that *P. zeae* (DSM 20274) was the most adapted propionibacteria to BMG, reaching 8.98g/L of propionic acid at 36ºC and 2.86g/L of dry biomass at 30ºC in small bacth model and the pH controlled fermentation did not improve significantly these productions. In addition, *P. arabinosum* (ATCC 4965) produced more biomass in BMSM than in other media at 30°C (7.55g/L) as well as at 36°C (3.71g/L) and achieved 8.23g/L of propionic acid concentration at 30ºC. When fermentation with pH controlled at 7 was performed using ATCC 4965 in BMSM, its fermentative parameters increased a lot, reaching an improvement of 490% for biomass concentration and 315% for propionic acid concentration. Afterwards, this fermentative broth was tested for antifungal activity and it affected *Aspergillus flavus* and *A. ochraceaus* growth. The best propionic acid productivity was obtained by ATCC 9614 using BML ($0.126g.L^{-1}.h^{-1}$), although the yielding of this metabolite was higher when using glycerol as carbon source by ATCC 4868 (0.962g/g) because there was no detection of acetic acid. Besides, when using the BML or BMSM, acetic acid emerged as an undesirable by-product for further propionic acid purification.

Keywords: *Propionibacterium,* Propionic acid, Batch fermentation, Carbon sources, Sugarcane molasses, Glycerol and Lactate.

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FIELD OF STUDY

Major Field: Bioprocesses Engineering

Specific Field: Anaerobic fermentative processes

Specialty: Propionic acid fermentation

INDEX

1. INTRODUCTION

Propionic acid is known to inhibit cell growth as a result of its antimicrobial activity, thus it is used in poultry and other animal feed to prevent infection from fungus. It is used also as raw material in different industries as intermediate in chemical synthesis; esters of propionic acid are used in the perfume industry and cellulose propionate finds its use as an important thermoplastic in plastic industry. Calcium, sodium and potassium salts of propionic acid have been listed as preservatives which are GRAS (generally recognized as safe) food additives.

Commercial production of propionic acid is mostly done by petrochemical routes, but it could be produced by fermentative processes through groups of bacteria belonging to the genera *Propionibacterium, Veillonella, Selenomonas, Clostridium, Fusobacterium* etc. Although well known, propionic acid production by fermentation has not been economically competitive when compared to the chemical processes; therefore it has not been used commercially. Nevertheless, the cost of oil is increasing and in a short time, biological routes employing renewable feedstocks and industrial wastes might be applied for propionic acid production, leading to a reduction of waste disposals.

Nowadays, propionic acid production is around 440 million pounds with an annual growth rate of 1.8%. The current US market price is $0.51-$0.54 per pound. The high demand of propionic acid for use as a natural preservative in foods and grains has stimulated developments of new fermentation processes to achieve improved propionic acid production from low-cost carbon sources.

Propionibacteria are able to ferment different carbohydrates and certain polyalcohols, producing propionic acid, as major product, and acetic acid. Sugarcane molasses is a renewable carbon source abundant in Brazil which could be used for propionic acid production. Moreover, glycerol has now become a cheap carbon source because of the new processes of biodiesel production from vegetable oils.

However, conventional fermentative processes for propionic acid production undergo from low propionic acid yield, final propionic acid concentration, and propionic acid productivity caused by a strong inhibition of the final products. In order to make propionic acid via microbial production economically attractive, the development of new fermentation processes is required.

Based on these outstanding points, the aim of this work was to evaluate the propionic acid fermentation by *Propionibacterium sp.* using sugarcane molasses and glycerol as carbon sources, comparing with lactate (which is a standard substrate for propionibacteria).

2. OBJECTIVES

2.1. MAIN OBJECTIVE

The main objective of this work was to evaluate the propionic acid fermentation by *Propionibacterium sp.* using sugarcane molasses (which is a renewable source abundant in Brazil) and glycerol (which is a by-product of biodiesel production) as carbon sources, comparing with lactate (which is a standard substrate for propionibacteria).

2.2. SECONDARY OBJECTIVES

- Set fermentative parameters in small scale using 40mL screw-cap flasks;
- Select the best *Propionibacterium sp.* for propionic acid production using 1L screw-cap flasks;
- Study the temperature effect on propionic acid fermentation using 1L screw-cap flasks;
- Study the effect of pH control on propionic acid fermentation using stirred-tank bioreactor;
- Evaluate the capability of the fermentative broth to prevent infection from fungus.

3. LITERATURE REVIEW

3.1. PROPIONIC ACID

The usual name, propionic acid, is from the Greek words *protos* = "first" and *pion* = "fat," because it was the smallest $H(CH_2)_nCOOH$ acid that exhibited the properties of the other fatty acids, such as producing an oily layer when salted out of water and having a soapy potassium salt.

Propionic acid is a naturally-occurring carboxylic acid with chemical formula CH_3CH_2COOH, which is systematically named propanoic acid. In the pure state, it is a colorless, corrosive liquid with a pungent odor (PLAYNE, 1985).

Structural formula:
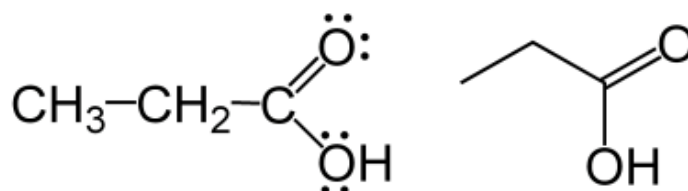
Figure 1 – Structural formula of propionic acid

It is a soluble liquid in water, at any proportion, because its molecules can be attracted by hydrogen bonds. Furthermore, propionic acid reacts with alcohols rendering esters and with basis rendering organic salts (PLAYNE, 1985).

Table 1 – Chemical and Physical Properties of propionic acid

Chemical and Physical Properties	
IUPAC name	Propanoic acid
Other names	Propionic acid and ethanecarboxylic acid
Molecular formula	$C_3H_6O_2$
Molar mass	74.08 g/mol
Appearance	Colourless liquid
CAS number	79-09-4
pKa	4,8
Melting point	-21ºC
Boiling point	141ºC
Density	0,992 g/cm^3
Solubility in water	Miscible
Viscosity	10 mPa.s

Source: http://www.cdc.gov

3.2. ROUTES OF PROPIONIC ACID PRODUCTION

In recent years, concerns about the uncertain supply and the eventual depletion of world petroleum reserves have fostered investigations into alternative ways to produce petrochemicals. One example is propionic acid, which is currently produced commercially by oxidation of liquid-phase propane or propionaldehyde (OZADALI, 1996; PAIK, 1994; PLAYNE, 1985). Although well known, propionic acid production by fermenative processes has not been economically competitive when compared to the chemical processes; therefore it has not been used commercially. Nevertheless, the cost of oil is increasing and

in a short future, biological routes employing renewable feedstocks and low-cost industrial wastes could be applied for propionic acid production and it could become economically competitive, offering some advantages over chemical synthesis (WOSKOW, 1991).

3.2.1. Chemical routes

Propionic acid is obtained mainly by three chemical routes:

- Oxidation of propanol:
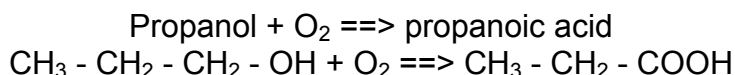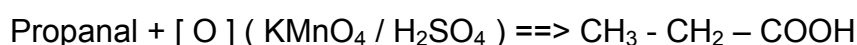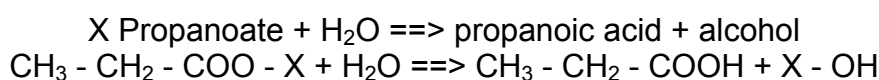
$$Propanol + O_2 ==> propanoic\ acid$$
$$CH_3 - CH_2 - CH_2 - OH + O_2 ==> CH_3 - CH_2 - COOH$$

- Oxidation of propanal:

$$Propanal + [\ O\ ]\ (\ KMnO_4\ /\ H_2SO_4\ ) ==> CH_3 - CH_2 - COOH$$

- Hydrolysis of esters:

$$X\ Propanoate + H_2O ==> propanoic\ acid + alcohol$$
$$CH_3 - CH_2 - COO - X + H_2O ==> CH_3 - CH_2 - COOH + X - OH$$

3.2.2. Biological routes

Several genera of anaerobic bacteria produce propionic acid as a major product, these groups of bacteria belonging to the genera *Propionibacterium, Veillonella, Selenomonas, Clostridium, Fusobacterium* etc (BOYAVAL, 1994; SESHADRI, 1993; PLAYNE, 1985).

Propionibacteria are, in general, Gram-positive, nonmotile, catalase-positive, nonspore-forming, rod-shaped bacteria, facultative anaerobes, capable of utilizing a broad range of carbon sources. A variety of carbon sources such as glucose (HIMMI, 2000; RICKERT, 1998; CHOI, 1994; LEWIS, 1992), lactose (GOSWAMI, 2000; LEWIS, 1992; HSU, 1991), xylose (CARRONDO, 1988), sucrose (QUESADA-CHANTO, 1994), glycerol (BORIES, 2004; HIMMI, 2000; BARBIRATO, 1996) and lactate (GU, 1998; RICKERT, 1998; LEWIS, 1992) could be used by this microorganism as substrate. Their metabolic properties and nutritional requirements have been studied extensively (HETTINGA, 1972a,b,c).

The optimal growth conditions are a pH range of 6 to 7 (SESHADRI, 1993; HSU, 1991; NANBA, 1983) and a temperature range of 30 to 37°C (SESHADRI, 1993). If pH value is below 4.5, there is practically no growth and low organic acid formation (HSU, 1991; PLAYNE, 1985).

Propionibacteria have been studied for the production of Swiss cheeses (BOYAVAL, 1995; HETTINGA, 1972b), vitamin B_{12} (BOYAVAL, 1995; QUESADA-CHANTO, 1998; Playne, 1985), and propionic acid (GU, 1998; CRESPO, 1991; EMDE, 1990; JOHNS, 1951; WOOD, 1936).

Propionic acid is produced mainly by *Propionibacterium*, which was first observed in the cheese manufacture, dates back to the beginning of the nineteenth century with Freudenreich and Orla Jensen, Van Niel, Werkman and Kendall. These authors named some 11 species of *Propionibacterium*.

Propionibacteria such as *P. acidipropionici* (HIMMI, 2000; GOSWAMI, 2000; CHOI, 1994; PAIK, 1994), *P. shermanii* (QUESADA-CHANTO, 1998; NANBA, 1983; LEE, 1974),

P. freudenreichii subsp. *shermanii* (ANASTASIOU, 2006; HIMMI, 2000; MARSHALL, 1995), and *P. freudenreichii* subsp. *freudenreichii* (EMDE, 1990) are commonly used for propionic acid production. However, *P. acidipropionici* has been the most used species for developments of industrial propionic acid production (MARTINEZ-CAMPOS, 2002; WOSKOW, 1991).

Propionate is produced mainly by dicarboxylic acid pathway, but some species produces propionate from lactate by acrylic acid pathway. Propionate is scarcely ever formed as the sole product; its synthesis is usually accompanied by formation of acetate and carbon dioxide, this occurs for stoichiometric reasons (PLAYNE, 1985).

3.2.2.1. Dicarboxylic acid pathway of propionic acid formation

This is the most common pathway for propionic acid formation. Lactate is used preferentially to glucose as a substrate by most propionic acid-producing bacteria. Propionate may be formed from lactate by either the dicarboxylic acid pathway or the acrylic pathway. The route taken depends on the bacterial species. The dicarboxylic acid pathway from glucose and from some carbon sources are shown in *Figure 2*.
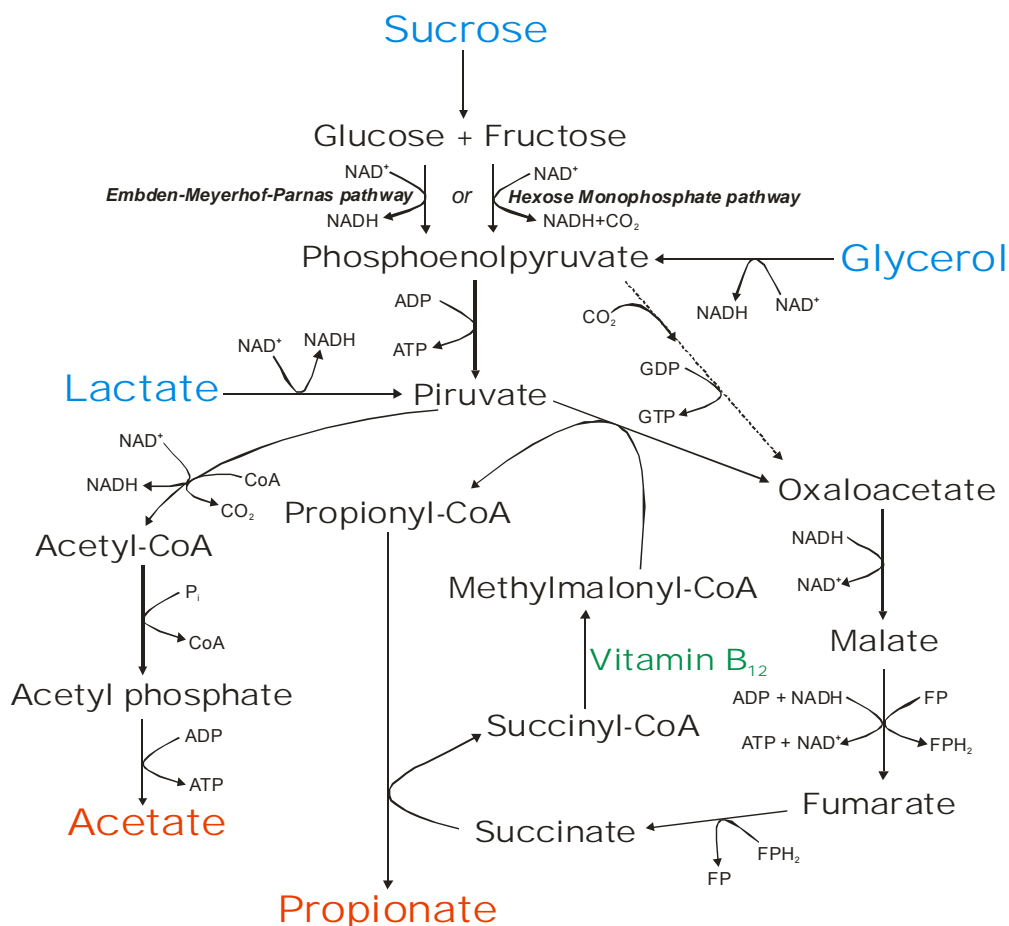
Figure 2 – The dicarboxylic pathway to propionate (Modified from SUWANNAKHAM, 2005)

Product ratios such as the propionic to acetic acid (P/A) ratio are controlled for thermodynamic reasons and for ATP production and entropy generation (HIMII, 2000; LEWIS, 1992). In theory, the P/A molar ratio is 2; nevertheless, it was reported that there is a wide variation for P/A ratio from 2:1 to 10:1 has been observed for glucose as carbon source (MARTINEZ-CAMPOS, 2002; BOYAVAL, 1994; CRESPO, 1991; WOOD, 1936). The P/A ratio changes greatly with growth conditions (MARSHALL, 1995; SESHADRI, 1993; HSU, 1991; NANBA, 1983).

3.3. ANTIMICROBIAL ACTIVITY

Propionic acid is able to inhibit microorganism growth (ANASTASIOU, 2006; MARSHALL, 1995; JOHNS, 1951). The prevailing view is that propionic acid disturbs the pH gradient across the cell membrane, an essential motive force for microorganisms to transport nutrients and metabolites. Due to hydrophobic nature of both the propionic acid and cell membrane, the undissociated acid can diffuse through the bacterial membrane into the cytoplasm, and then dissociate into a proton and propionate anion inside the cell. Thus, an inward accumulation of protons is created. In order to maintain the functional proton gradient across the membrane, extra ATP must be consumed by H^+-ATPase to extrude the proton out, diminishing the available ATP for cell metabolism (GU, 1998; HETTINGA, 1972a). Therefore, the growth of fungus, yeasts, and some bacteria is inhibited.

3.4. MARKET AND USES

Salts of propionic acid are used primarily for animal feed preservation, including hay, silage, and grains (LIND, 2005; HIGGINBOTHAM, 1998), and in human foods, mainly in baked goods and cheeses (LIND, 2005; MARSHALL, 1995; HSU, 1991). Calcium, sodium and potassium salts of propionic acid have been listed as preservatives, which are set by Food and Drug Administration (FDA) in the category Generally Recognized As Safe (GRAS) food additives (http://www.fda.gov).

Propionic acid is used as solvents (alkyl propionate esters) (BOYAVAL, 1995; PLAYNE, 1985) and also as intermediate in the synthesis of:
• Herbicides (BOYAVAL, 1995; LEWIS, 1992);
• Cellulose acetate–propionate plastics (CAP) (QUESADA-CHANTO, 1994; LEWIS, 1992);
• Pharmaceuticals products (mainly for production of drugs against arthritis and Vitamin E) (BOYAVAL, 1995; PLAYNE, 1985)
• Flavors and fragrances (BOYAVAL, 1994; QUESADA-CHANTO, 1994; LEWIS, 1992).
Figure 3 shows the market share for propionic acid.
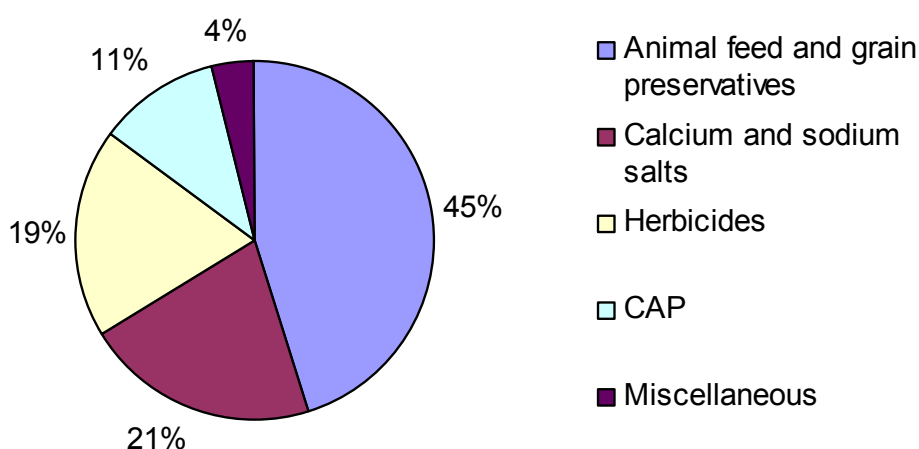
Figure 3 – Market share for propionic acid

3.5. DEMAND AND PRICE

The biggest producers (see *Table 2*) of propionic acid in the world are:
- BASF (Germany);
- BASF (China);
- Chemische Werke Hüls (Germany);
- Distillers Company (United Kingdom);
- USSR (Russia);
- Celanese Chemical Company (USA);
- Eastman Chemical (USA).

Table 2 – Capacity of propionic acid production in USA

Propionic acid producers of USA	Capacity (ton/year)
Celanese, Pampa, Texas	6800
Dow Chemical, Texas City, Texas	122000
Eastman Chemical, Kingsport, Tennesse	50000
Eastman Chemical, Longview, Texas	20000

Source: http://www.the-innovation-group.com

Table 3 – Historical data about propionic acid

Year	Demand in USA (ton)	Price (U$/Kg)
1997	87000	0.91 - 0.97
1998	87500	0.91 - 0.97
1999	88000	0.91 - 0.97
2000	92500	1.02 - 1.08
2001	91500	1.02 - 1.08
2002	92500	1.02 - 1.08
Nowadays	*96000*	*1.13 - 1.19*

Source: http://www.the-innovation-group.com

Nowadays, the consumer's demand for fermentation-produced propionic acid as a natural food preservative is high (BOYAVAL, 1995), therefore fermentation by propionibacteria has a good potential for the production of natural propionic acid to satisfy the market demand (SUWANNAKHAM, 2005).

During 1997 and 2002, the market grew at 1.2 percent per year. Estimate for future is around 1.8 percent per year.

3.6. FERMENTATIVE PROCESSES

3.6.1. Carbon sources

Propionibacteria can grow in a wide range of carbon sources, from carbohydrates (QUESADA-CHANTO, 1994; CARRONDO, 1988; PAPOUTSAKIS, 1985b), organic acids (MARTINEZ-CAMPOS, 2002; LEWIS, 1992; LEE, 1974) to polyalcohols (BORIES, 2004; HIMMI, 2000; BARBIRATO, 1996). The carbon source class as well as the carbon source concentration is able to affect productivities, yields and the cell metabolism (HIMMI, 2000; BOYAVAL, 1995; LEWIS, 1992).

At this moment, there are few reports about propionic acid fermentation using low-cost carbon sources, like sugarcane molasses (which is a renewable source) and glycerol (which is an industrial waste of biodiesel industry).

3.6.1.1. Sugarcane molasses

Sugarcane molasses is a by-product of the manufacture or refining of sucrose from sugarcane. It must contain not less than 46% total sugars. As is often found with many industrial by-products, the chemical composition of molasses shows wide variation. Its composition is influenced by factors such as soil type, ambient temperature, moisture, season of production, variety, production practices at a particular processing plant, and by storage variables. Consequently, considerable variation may be found in nutrient content, flavor, color, viscosity, maturation degree, reducing sugars and total sugar content.

The molasses trade commonly use the term Brix that represents an approximation of total solids content. Brix is a term originally initiated for pure sucrose solutions to indicate the percentage of sucrose in solution on a weight basis. However, in addition to sucrose, molasses contains glucose, fructose, raffinose and numerous non-sugar organic materials that could affect the measure by this method (CURTIN, 1983).

3.6.1.2. Glycerol

Glycerol is a chemical compound also commonly called glycerin or glycerine which is a sugar alcohol, and is sweet-tasting and of low toxicity.

Glycerol is a 10% by-product of biodiesel production (via the transesterification of vegetable oils). This process has led to a excess of crude glycerol in the market, making the epichlorohydrin process no longer economical. Current levels of glycerol production are running at about 350,000 tons per annum in the USA, and 600,000 tons per annum in Europe. This production will increase as it implements EU directive 2003/30/EC which requires replacement of 5.75% of petroleum fuels with biofuel, across all Member States by 2010 (http://en.wikipedia.org, 2008).

3.6.2. Propionic acid fermentation

The interest to improve propionic acid fermentation has been high. New bioprocesses and mutant strains have been developed to improve propionic acid production in terms of its yield, final product concentration, and productivity, but with limited success (RICKERT, 1998; PAIK, 1994; LEWIS, 1992; EMDE, 1990). One significant obstacle in propionic acid fermentation is the strong end-product inhibition caused by propionic acid even at a very low concentration of 10 g/L (GU, 1998; HSU, 1991). A higher final product concentration in the fermentation broth and the absence of by-products would facilitate product separation and recovery, and significantly reduce the production costs (BOYAVAL, 1995; BOYAVAL, 1994).

Many researchers have worked with production of propionic acid through biological processes, using a lot of carbon sources in several fermentation models. However, few researchers developed surveys using industrial wastes or renewable sources, except whey. Some estimates and fermentative processes that were developed are shown in *Table 4* and *5.*

Table 4 – Data about surveys for propionic acid production in small scale

Author	Fermentative process	Microorganism	Propionic acid productivity	Carbon Source
CRESPO, 1991	Continuous	ATCC 25562	0.6 g/(L.h)	Glucose
CRESPO, 1991	Continuous with UF	ATCC 25562	10.3 g/(L.h)	Glucose
LEWIS, 1992	Batch	ATCC 4875	0.22 g/(L.h)	Lactate
SESHADRI, 1993	Continuous	ATCC 25562	0.2 g/(L.h)	Glucose
OZADALI, 1996	Fed-Batch	*P. acidipropionici* P9	0.32 g/(L.h)	Lactate
GU, 1998	Immobilized cells	*P. thoenii* P20	3.9 g/(L.h)	Lactate
RICKERT, 1998	Immobilized cells	*P. thoenii* P20	2.8 g/(L.h)	Glucose
GOSWAMI, 2000	Batch	ATCC 4875	0.23 g/(L.h)	Lactose
GOSWAMI, 2000	Fed-Batch	ATCC 4875	0.31 g/(L.h)	Lactose

Table 5 – Data about propionic acid fermentation

Fermentation Process/ Mode	Substrate	Cell Density (g/L)	Propionic Acid Production			References
			Final Conc. (g/L)	Yield (g/g)	Productivity (g/L/h)	
Cell Recycle (Ultrafiltration)						
continuous	whey permeate	100	25	-	14.3	Boyaval and Corre, 1987
continuous	whey permeate	130	17	-	5	Blanc and Goma, 1989
sequential-batch	whey permeate	50	30-40	-	1.2	Colomban et al., 1993
Cell Immobilization **Fibrous-Bed Bioreactor**						
batch	corn meal hydrolyzate	-	-	0.58	2.12	Huang et al., 2002
recycle-batch	de-lactose whey permeate	34	65	0.5	0.22-0.47 (0.68 with yeast extract)	Yang et al., 1995
Calcium- Alginate Beads						
fed-batch	semidefined medium	9.8×10^9 cells/g beads 0.1 g beads/ mL medium	57	-	0.3 (0.96: continuous mode)	Paik and Glatz, 1994
repeated-batch	glucose	2×10^{11} cells/g beads beads (g)= 40% of broth volume	34	0.45	-	Rickert et al., 1998
Extractive Fermentation (Hollow-fiber membrane) fed-batch	lactose	-	75	0.66	1	Jin and Yang, 1998
Three-Electrode **Amperometric Culture System** batch	glucose	-	-	0.973	-	Emde and Schink, 1990

Source: SUWANNAKHAM, 2005.

Utilization of low-cost raw material for fermentation will allow propionic acid production via biotechnological fermentation to supply propionic acid with low market price. A large amount of fermentative processes, fermentation substrates, integrated fermentation/separation processes have been developed to enhance propionic acid production. Up to now, the highest propionic acid concentration obtained from free-cell semicontinuous fermentation of propionate-tolerant *P. acidipropionici* using glucose was 47g/L with the propionic acid yield of 0.55g/g and the volumetric productivity of $0.37g.L^{-1}.h^{-1}$. A maximum volumetric productivity of $14.3g.L^{-1}.h^{-1}$ was attained by *P. acidipropionici* fermentation in continuous stirred-tank reactor with recycles using an ultrafiltration module. The highest propionic acid yield of 0.973 g/g was achieved in fermentation by *P. freudenreichii* operated with a three-electrode amperometric culture system with fermentation medium containing 0.4mM cobalt sepulchrate (SUWANNAKHAM, 2005).

4. MATERIAL AND METHODS

4.1. MICROORGANISMS AND MEDIA

Strains of propionic acid bacteria used at the research:

- *Propionibacterium arabinosum* (DSM 20273 = ATCC 4965);
- *Propionibacterium jensenii* (DSM 20535 = ATCC 4868);
- *Propionibacterium acidipropionici* (DSM 4900 = ATCC 25562);
- *Propionibacterium pentosaceum* (DSM 20272 = ATCC 4875);
- *Propionibacterium thoenii* (DSM 20276 = ATCC 4874);
- *Propionibacterium freudenreichii* subsp. *shermanii* (DSM 4902 = ATCC 9614);
- *Propionibacterium zeae* (DSM 20274 = CCT 5329);
- *Propionibacterium freudenrichii shermanii* (NRRL 4327 = ATCC 13673).

Propionibacterium sp. were received in the liofilized form and reactivated in Reinforced Clostridial Broth (RCB). These cultures were inoculated in RCB agar plates. Isolated colonies were incubated in deep agar at 30ºC, stored at 4ºC and transferred to new agar monthly. *Propionibacterium sp.* used in this research were grown in a synthetic basal medium making use of lactate, glycerol or sugarcane molasses (supplied by COOPERCANA COMPANY, Brazil) as carbon sources. The composition of conservation medium is shown in table 6; deionised water was used to fill out the medium content. For autoclaving the pH values were adjusted to 6.8 (OZADALY, 1996; QUESADA-CHANTO, 1994).

Table 6 – Composition of conservation medium

Compound	Concentration (g.L^{-1})
Sodium lactate	5.0
Yeast extract	5.0
$(NH_4)_2HPO_4$	2.0
KH_2PO_4	1.0
$MgSO_4.7H_2O$	0.01
$CaCl_2.6H_2O$	0.01
$CoCl_2.6H_2O$	0.01
$FeSO_4.7H_2O$	0.005
$MnSO_4.H_2O$	0.0025
Agar	7.0

Besides this, the strains were stored in Eppendorf tubes filled out with glycerol 50% at -20ºC and -196ºC. All procedures requiring sterility were performed inside a sterile cabine.

The preculture and the inoculum media had the same composition as the conservation medium, except that sodium lactate concentration was increased to 20g.L^{-1}, yeast extract concentration was increased to 10g.L^{-1} and agar was not added.

The fermentation medium, for batch fermentation in 1L screw-cap flasks and 14L bioreactor are shown in table 7. The basal medium and the carbon sources were prepared independently. The pH of these two solutions were adjusted to 6.8-7.0 before autoclaving. After heat sterilization, at 121ºC and 1atm for 20 min, they were mixed aseptically in the fermentation flasks or added aseptically to the bioreactor.

Table 7 – Composition of fermentation medium

Compound	BML conc. (g.L^{-1})	BMG conc. (g.L^{-1})	BMSM conc. (g.L^{-1})
Sodium lactate	40.0	0	0
Glycerol	0	15.0	0
Sugarcane Molasses	0	0	25.0
Yeast extract	10.0	10.0	10.0
$(NH_4)_2HPO_4$	2.0	2.0	2.0
KH_2PO_4	1.0	1.0	1.0
$MgSO_4.7H_2O$	0.01	0.01	0.01
$CaCl_2.6H_2O$	0.01	0.01	0.01
$CoCl_2.6H_2O$	0.01	0.01	0.01
$FeSO_4.7H_2O$	0.005	0.005	0.005
$MnSO_4.H_2O$	0.0025	0.0025	0.0025

4.2. SETTING FERMENTATIVE PARAMETERS IN SMALL SCALE

Propionibacteria can be inhibited by some media elements, like salts and sugars. Thereby, it were carried out trials in order to test the best concentration of sugarcane molasses (1ºBrix, 2ºBrix, 5ºBrix and 10ºBrix), glycerol (10g/L, 15g/L and 20g/L) and sodium lactate (10g/L, 20g/L, 30g/L and 40g/L). Moreover, it was accomplished assays to set fermentative parameters as inoculum rate (1%, 2% and 5%) and fermentation time (48h, 96h and 144h). These trials were conducted in static incubation at 30ºC.

For testing the inoculum rate, one isolated colony (CFU) was transferred from deep agar into a 2mL preculture medium broth in an Eppendorf tube and incubated at 30ºC for 48h. A portion of 0.4mL or 0.8mL or 2.0mL were transferred to a 40mL screw-cap flask containing 40mL of basal medium with 10g/L of sucrose or sodium lactate or glycerol as carbon source. Samples were taken at periodical intervals and they had been analyzed for biomass concentration and frozen. The samples were analyzed by HPLC after finishing the experiments.

For selection of the carbon source concentration and fermentation time, one CFU was transferred from deep agar into a 2mL preculture medium broth in an Eppendorf tube and incubated at 30ºC for 48h. 400µL of this culture were transferred to a 40mL screw-cap flask containing 40mL of basal medium with the carbon source concentration cited above. Samples were taken at periodical intervals until 144h and they were analyzed for biomass and frozen. The samples were analyzed by HPLC after finishing the experiments.

4.3. STUDY THE TEMPERATURE AND CARBON SOURCE EFFECTS ON PROPIONIC ACID FERMENTATION AND SELECTION OF THE BEST STRAIN FOR PROPIONIC ACID PRODUCTION

The selection were carried out with eight strains which were purchased by Biotechnology and Bioprocesses Engineering Division in well-known culture collections (ATCC - USA, DSMZ - Germany and CCT - Brazil). The strains were tested in agreement to the following parameters:

- Acidification;
- Organic acids production;
- Carbon source consumption;
- Biomass growth.

4.3.1 Inoculum preparation

One isolated colony from deep agar plate was transferred to 2mL of preculture medium and incubated at 30°C for 48h. 400µl of this culture were transferred to 40mL screw-cap flask containing 40mL of inoculum medium broth. *Propionibacterium sp.* was grown without agitation for 24–36 h at 30°C in inoculum medium broth (until $A_{660} \approx 0.8$) and was inoculated at 1% (v/v) in the 1L customized flasks containing 1L of fermentation broth.

Figure 4 – 1L screw-cap flask

4.3.2 Batch fermentations

These trials were conducted in 1L screw-cap flasks almost filled to the top using BMSM, BMG and BML, in order to reduce the presence of oxygen. The fermentations were carried out without pH control in static incubation at 30ºC and 36ºC. All assays were performed in duplicate and the obtained results are the average of these independent trials. Samples were taken in the beginning of the fermentation and at periodical intervals during six days.

4.4. STUDY THE EFFECT OF PH CONTROL ON PROPIONIC ACID FERMENTATION

Inoculum were prepared like in section 3.2.3. However, it was used 500ml as inoculum volume in BioFlo 110 (NEW BRUNSWICH SCIENTIFC COMPANY, USA).

The fermentations were performed in a 14L BioFlo 110 bioreactor filled with 5L of BMSM or BMG. After heat sterilization for 30min at 121ºC, the bioreactor was flushed with N_2 gas at 1vvm for 45 minutes and pressurized at 3psig in order to maintain the anaerobiosis. The temperature was controlled at 30ºC, the agitation rate was 100rpm and the pH was maintained at 6.0, 6.5 or 7.0 by the addition of 5N NaOH. Samples were taken in the beginning of the fermentation and at periodical intervals.

Figure 5 – BioFlo 110

4.5. EVALUATION OF PROPIONIC ACID FERMENTATIVE BROTH TO PREVENT INFECTION FROM FUNGUS

The antifungical activity of fermentative broth was determined by the conventional agar dilution method (BAUER, 1966) on Potato Dextrose Agar (PDA), following the recommendations of the National Committee for Clinical Laboratory Standart (NCCLS) (1999). The tests were carried out against *Aspergillus flavus* ATCC1003 and *Aspergillus ochraceaus* ATCC1008. Fungus inoculum was made of approximately 10^5 spores/mL by Neubauer-counting chamber. The plates (150 x 15 mm) were prepared with 50 mL of PDA medium addictioned of inoculun by pour plate. Afterwards, wells with 6mm of diameter were made, where 50 and 25 µL of fermentation broth at three pH values (3.0, 5.0 and 7.0) were placed. Latic acid (50g/L) and sterile water at same pH were used as controls. The plates were incubated at 30°C for 48 hours. The final result was obtained by measurement of the diameter of the halos of inhibition of fungus growth.

4.6. ANALYTICAL METHODS

4.6.1. Biomass determination

Biomass was determined from a standard curve of absorbance versus dry weight. Dry weight were obtained by filtering aliquots of culture broth through prerinsed and preweighted 0.22-µm filters, rinsing with 0.1 M phosphate buffer (pH 7.0), and drying filters in stove at 100ºC for 48h. Regression analysis (least-square method) were performed on the values that were taken from the linear portion of the curve (OZADALI, 1996; BOYAVAL, 1994; QUESADA-CHANTO, 1994; SESHADRI, 1993; CRESPO, 1991).

Optical density of the cells was measured at 660nm in a Shimadzu UV-1601 spectrophotometer. Samples were diluted with distilled water so that OD would be between 0.2-0.8.

4.6.2. pH determination

The pH of the samples were measured in a HANNA HI9321 microprocessor pHmeter, which was calibrated with two standard points before the measures.

4.6.3. Carbon sources and organic acids quantification

Lactate, glycerol, sucrose, succinic acid, propionic acid and acetic acid concentrations were determined by high-performance liquid chromatography (Shimadzu Liquid Chromatograph, model RID-10A refractive index detector), with Aminex® HPX-87H ion exclusion column (Bio-Rad, Hercules, CA) operated at 60ºC, with 5mM H_2SO_4 as the mobile phase at 0.6ml/min flow rate. Samples for analysis were diluted with purified water (milli-Q) according to their concentration, and centrifuged to remove cells, then were filtered through 0.22µm-pore-size filters (Millipore), and stored at -20ºC before analysis. A Shimadzu Chromatopac (model C-R6A) was used to analyze the samples and plot the chromatograms. The product concentration was calculated by comparing the peak areas with external standards (GU, 1998; RICKERT, 1998; OZADALI, 1996; LEWIS, 1992; WOSKOW, 1991). The retention time of these substances are shown in table 8.

Table 8 – Retention time of the HPLC standards

Substance	Retention time (min)
Sucrose	9.3 and 10.1
Succinic acid	12.1
Lactic acid	13.4
Glycerol	14.1
Acetic acid	15.5
Propionic acid	18.2

**Figure 6** – High Performance Liquid Chromatography equipment

5. RESULTS AND DISCUSSION

5.1. DRY BIOMASS VERSUS OPTICAL DENSITY

The standard curves of dry weight *vs.* absorbance was plotted for all the eight propionibacteria growing at the three different carbon sources (sodium lactate, glycerol and sugarcane molasses). It was found that one unit of OD was equivalent around 0,46-0,52g/L of cell dry weight depending the microorganism and the carbon source. *Figures 7, 8* and *9,* are examples of standard curves for the three carbon sources with different propionic acid bacterias.

Table 9 – Data about dry weight of ATCC 4965 *vs.* optical density at 660nm in BML

		BML				Average	
t (h)	Vol (L)	Optical density at 660nm	Membrane (g)	Membrane + biomass (g)	Dry weight (g/L)	Optical density at 660nm	Dry weight (g/L)
9,5	0,15	0,099	0,0881	0,0964	0,055	0,099	0,053
9,5	0,15	0,098	0,0875	0,0952	0,051		
24	0,099	0,357	0,0883	0,1055	0,174	0,358	0,166
24	0,099	0,358	0,0888	0,1044	0,158		
28	0,05	0,478	0,0883	0,1005	0,244	0,478	0,226
28	0,05	0,477	0,0888	0,0992	0,208		
31	0,051	0,598	0,0885	0,1017	0,259	0,593	0,267
31	0,051	0,588	0,089	0,103	0,275		

$$y = 0,4609x$$
$$R^2 = 0,9945$$
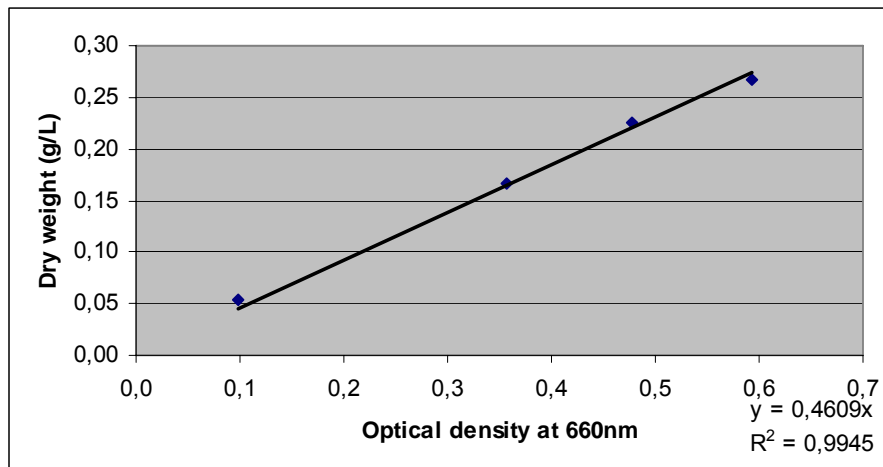
Figure 7 – Relation between dry weight and optical density at 660nm for ATCC 4965 growing in BML

Table 10 – Data about dry weight of ATCC 4875 *vs.* optical density at 660nm in BMG

		BMG				Average	
t (h)	Vol (L)	Optical density at 660nm	Membrane (g)	Membrane + biomass (g)	Dry weight (g/L)	Optical density at 660nm	Dry weight (g/L)
6,5	0,15	0,111	0,0885	0,0971	0,057	0,107	0,050
6,5	0,15	0,102	0,0882	0,0947	0,043		
14,5	0,15	0,345	0,0879	0,1142	0,175	0,294	0,154
14,5	0,15	0,243	0,0873	0,1071	0,132		
17	0,05	0,492	0,0875	0,1005	0,260	0,411	0,217
17	0,05	0,33	0,088	0,0967	0,174		
21	0,05	0,78	0,0871	0,1071	0,400	0,660	0,347
21	0,05	0,54	0,0876	0,1023	0,294		
28	0,048	1,55	0,0887	0,1253	0,763	1,480	0,752
28	0,049	1,41	0,0881	0,1244	0,741		

Figure 8 – Relation between dry weight and optical density at 660nm for ATCC 4875 growing in BMG

Table 11 – Data about dry weight of DSM 20274 *vs.* optical density at 660nm in BMSM

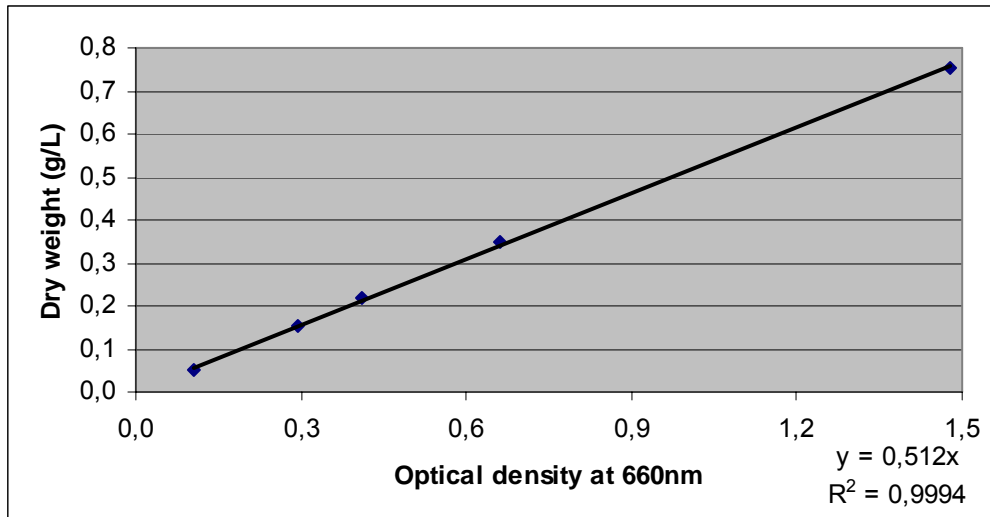| | | **BMSM** | | | | *Average* | |
t (h)	Vol (L)	Optical density at 660nm	Membrane (g)	Membrane + biomass (g)	Dry weight (g/L)	Optical density at 660nm	Dry weight (g/L)
9,25	0,09	0,08	0,0876	0,0918	0,047	0,082	0,046
9,25	0,09	0,084	0,0876	0,0916	0,044		
18	0,09	0,412	0,0876	0,1054	0,198	0,417	0,198
18	0,09	0,422	0,0879	0,1058	0,199		
20	0,05	0,509	0,0868	0,0993	0,250	0,515	0,247
20	0,05	0,521	0,0871	0,0993	0,244		
23,5	0,05	0,706	0,0872	0,105	0,356	0,717	0,376
23,5	0,05	0,728	0,0873	0,1071	0,396		
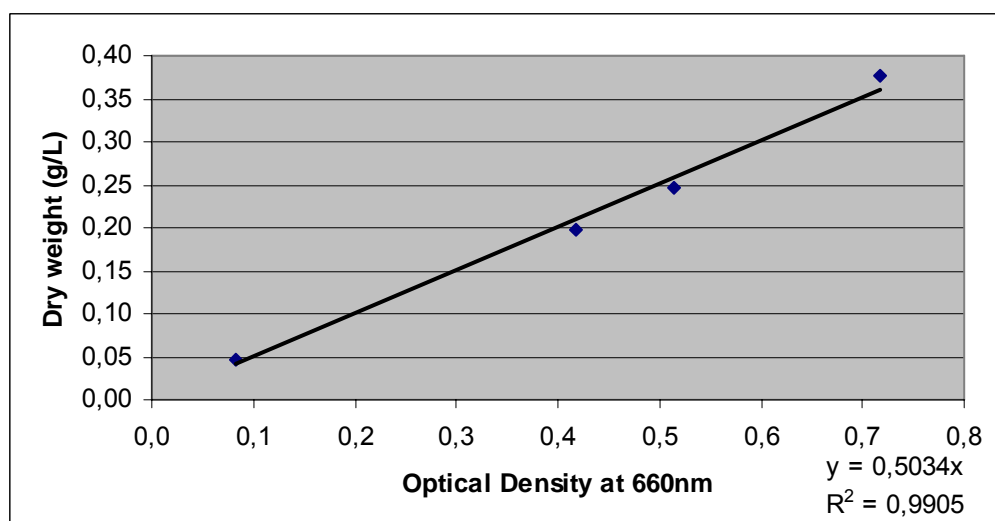
Figure 9 – Relation between dry weight and optical density at 660nm for DSM 20274 growing in BMSM

5.2. SETTING FERMENTATIVE PARAMETERS IN SMALL SCALE

The assays about inoculum rate did not show differences when it was used 1%, 2% or 5%(v/v). Thus, it was chosen 1%(v/v) as inoculum rate for facilitating the procedures of the next experiments.

The carbon source concentration of sugarcane molasses, glycerol and sodium lactate was not able to inhibit propionibacteria growth for all the levels tested. The inhibition was a result of organic acid production and final pH (SUWANNAKHAM, 2005; HSU, 1991; WOSKOW, 1991; NANBA, 1983).

Nevertheless, when it was used BMSM, the microorganisms got to consume all the carbon source just for 1ºBRIX (it is almost equivalent to 10g/L of sucrose) after 96h, so it was used 2ºBRIX as sugarcane molasses concentration for the next experiments. Using BMG 10g/L, *Propionibacterium sp.* almost depleted the entire carbon source after 144h, then it was selected 15g/L of glycerol for the next assays. In the other hand, all the strains tested was able to deplet 40g/L of sodium lactate until 96h, except ATCC 4874. Higher levels of sodium lactate (50g/L and 60g/L) was studied but both concentration caused growth inhibition in some propionibacterias, then it was chosen 40g/L as sodium lactate concentration for the next experiments with BML (data are not shown).

The fermentation time was chosen considering the assays about carbon source concentration and it was selected 144h as fermentation time for the next steps.

5.3. STUDY THE TEMPERATURE AND CARBON SOURCE EFFECTS ON PROPIONIC ACID FERMENTATION AND SELECTION OF THE BEST STRAIN FOR PROPIONIC ACID PRODUCTION IN SMALL SCALE

In order to study the temperature and carbon source effects on propionic acid fermentation, batch fermentation experiments were carried out in 1L screw-cap flasks filled with BML, BMG and BMSM at two temperatures. The results of these trials are summarized in the *Tables 9* and *10* as well the fermentative parameters, such as acidification (ΔpH), average ratio between propionic (P) and acetic acid (A), cell productivity (P_X), propionic acid productivity (P_P), biomass yield from substrate ($Y_{X/S}$) and propionic acid yield from substrate ($Y_{P/S}$), and from biomass ($Y_{P/X}$).

Table 12 – Effects of carbon source and temperature on propionic acid fermentation using 1L screw-cap flasks

Strain	Carbon Source	Temp (°C)	$pH_{initial}$	ΔpH	Final concentration (g.L^{-1})			Average Ratio P:A
					X	P	A	
ATCC 4965 after 133h	Lactate	30±0.1	6.87	0.48±0.03	3.31±0.11	15.06±0.56	5.59±0.22	2.7:1
		36±0.1	6.87	0.46±0.02	1.29±0.12	13.32±0.89	3.58±0.29	3.7:1
	Glycerol	30±0.1	6.98	2.50±0.10	2.42±0.13	6.77±0.11	ND*	15.0:1
		36±0.1	6.98	2.32±0.08	1.81±0.08	4.87±0.36	ND*	15.0:1
	Sucrose	30±0.1	6.77	2.58±0.03	7.55±0.07	8.23±0.12	2.25±0.02	3.7:1
		36±0.1	6.77	2.40±0.09	3.71±0.01	6.92±1.17	1.65±0.09	4.2:1
ATCC 4868 after 140h	Lactate	30±0.1	6.83	0.43±0.01	2.50±0.06	16.31±0.23	5.68±0.04	2.9:1
		36±0.1	6.83	0.41±0.01	1.96±0.05	16.63±0.14	5.76±0.01	2.9:1
	Glycerol	30±0.1	6.87	2.30±0.10	0.98±0.04	6.16±0.13	ND*	15.0:1
		36±0.1	6.87	2.21±0.07	0.78±0.08	4.77±0.66	ND*	15.0:1
	Sucrose	30±0.1	6.70	1.71±0.03	1.75±0.12	2.83±0.24	0.94±0.05	3.0:1
		36±0.1	6.70	1.79±0.01	1.21±0.07	3.04±0.01	1.07±0.02	2.8:1
ATCC 25562 after 146h	Lactate	30±0.1	6.43	0.22±0.01	1.77±0.04	15.80±0.17	4.54±0.45	3.5:1
		36±0.1	6.43	0.18±0.01	1.33±0.07	15.82±0.25	4.98±0.06	3.2:1
	Glycerol	30±0.1	6.56	2.04±0.08	1.12±0.01	5.93±0.12	ND*	15.0:1
		36±0.1	6.56	1.83±0.10	0.53±0.11	3.08±0.11	ND*	15.0:1
	Sucrose	30±0.1	6.49	2.22±0.01	1.91±0.01	7.12±0.03	1.72±0.02	4.1:1
		36±0.1	6.49	2.06±0.03	1.35±0.10	5.06±0.39	1.23±0.01	4.1:1
ATCC 4875 after 144h	Lactate	30±0.1	6.67	0.32±0.01	2.09±0.07	16.73±0.01	5.85±0.01	2.9:1
		36±0.1	6.67	0.38±0.02	1.33±0.03	17.24±0.01	5.89±0.07	2.9:1
	Glycerol	30±0.1	6.76	2.19±0.11	1.68±0.04	7.16±0.48	ND*	15.0:1
		36±0.1	6.75	2.24±0.06	1.21±0.07	6.31±0.85	ND*	15.0:1
	Sucrose	30±0.1	6.68	2.21±0.01	0.94±0.01	5.23±0.19	1.89±0.03	2.8:1
		36±0.1	6.69	2.22±0.04	0.98±0.11	5.59±0.41	1.74±0.17	3.2:1
ATCC 4874 after 132h	Lactate	30±0.1	6.51	0.29±0.02	1.37±0.02	10.92±1.10	3.87±0.35	2.8:1
		36±0.1	6.50	0.29±0.01	1.35±0.15	10.41±1.92	3.70±0.72	2.8:1
	Glycerol	30±0.1	6.64	2.21±0.06	1.99±0.07	7.56±0.06	ND*	15.0:1
		36±0.1	6.63	2.22±0.01	1.72±0.01	7.17±0.13	ND*	15.0:1
	Sucrose	30±0.1	6.57	2.22±0.01	1.83±0.01	6.05±0.01	2.26±0.01	2.7:1
		36±0.1	6.57	2.08±0.04	1.49±0.20	5.18±0.07	1.83±0.23	2.8:1
ATCC 9614 after 139h	Lactate	30±0.1	6.47	0.25±0.01	1.79±0.04	17.15±0.45	6.45±0.37	2.7:1
		36±0.1	6.48	0.26±0.01	1.59±0.02	17.46±0.43	6.14±0.95	2.8:1
	Glycerol	30±0.1	6.65	2.12±0.05	2.33±0.05	5.08±0.51	ND*	15.0:1
		36±0.1	6.66	2.18±0.01	2.26±0.02	5.24±0.08	ND*	15.0:1
	Sucrose	30±0.1	6.53	2.20±0.01	3.81±0.06	7.84±0.06	1.97±0.01	4.0:1
		36±0.1	6.53	2.01±0.01	3.13±0.11	5.35±0.17	1.66±0.03	3.2:1
DSM 20274 after 140h	Lactate	30±0.1	6.47	0.27±0.01	1.81±0.09	10.75±0.23	3.20±0.02	3.4:1
		36±0.1	6.46	0.28±0.01	1.25±0.04	8.86±0.33	2.44±0.08	3.6:1
	Glycerol	30±0.1	6.60	2.26±0.12	2.86±0.12	8.91±0.42	ND*	15.0:1
		36±0.1	6.61	2.36±0.01	2.35±0.03	8.98±0.08	ND*	15.0:1
	Sucrose	30±0.1	6.56	2.34±0.01	1.57±0.01	6.83±0.24	2.43±0.12	2.8:1
		36±0.1	6.54	2.32±0.01	1.52±0.02	6.76±0.12	2.03±0.56	3.3:1
ATCC 13673 after 139h	Lactate	30±0.1	6.50	0.31±0.01	2.57±0.01	17.16±0.10	6.36±0.03	2.7:1
		36±0.1	6.50	0.31±0.01	2.41±0.04	17.18±0.50	6.33±0.20	2.7:1
	Glycerol	30±0.1	6.61	1.84±0.07	1.36±0.08	4.73±0.51	ND*	15.0:1
		36±0.1	6.61	1.88±0.02	1.18±0.02	4.26±0.07	ND*	15.0:1
	Sucrose	30±0.1	6.55	2.30±0.01	2.84±0.05	7.04±0.31	2.41±0.08	2.9:1
		36±0.1	6.54	2.32±0.01	2.91±0.14	6.93±0.02	2.37±0.03	2.9:1

*ND = None detected in HPLC analysis.

Table 13 – Effects of carbon source and temperature on propionic acid fermentation using 1L screw-cap flasks related to productivities and yields

Strain	Carbon Source	Temp (ºC)	Productivity (g.L^{-1}.h^{-1})		Yield (g.g^{-1})		
			P_X	P_P	$Y_{X/S}$	$Y_{P/S}$	$Y_{P/X}$
ATCC 4965 after 133h	Lactate	30±0.1	0.025±0.001	0.113±0.004	0.097±0.004	0.442±0.020	4.57±0.01
		36±0.1	0.010±0.001	0.100±0.007	0.042±0.001	0.436±0.002	10.44±0.34
	Glycerol	30±0.1	0.018±0.001	0.051±0.001	0.259±0.004	0.724±0.015	2.81±0.09
		36±0.1	0.014±0.001	0.037±0.003	0.261±0.002	0.703±0.024	2.72±0.07
	Sucrose	30±0.1	0.057±0.001	0.062±0.001	0.417±0.001	0.455±0.002	1.09±0.01
		36±0.1	0.028±0.001	0.052±0.009	0.297±0.003	0.555±0.089	1.89±0.32
ATCC 4868 after 140h	Lactate	30±0.1	0.018±0.001	0.117±0.002	0.071±0.002	0.468±0.007	6.56±0.05
		36±0.1	0.014±0.001	0.119±0.001	0.056±0.001	0.477±0.004	8.53±0.30
	Glycerol	30±0.1	0.007±0.001	0.044±0.001	0.139±0.001	0.886±0.057	6.40±0.43
		36±0.1	0.006±0.001	0.034±0.005	0.154±0.013	0.962±0.106	6.23±0.17
	Sucrose	30±0.1	0.012±0.001	0.020±0.002	0.329±0.028	0.537±0.038	1.63±0.02
		36±0.1	0.009±0.001	0.022±0.001	0.277±0.024	0.702±0.099	2.53±0.13
ATCC 25562 after 146h	Lactate	30±0.1	0.012±0.001	0.108±0.001	0.046±0.001	0.414±0.004	9.05±0.31
		36±0.1	0.009±0.001	0.108±0.002	0.034±0.002	0.415±0.006	12.10±0.81
	Glycerol	30±0.1	0.008±0.001	0.041±0.001	0.159±0.015	0.858±0.069	5.42±0.08
		36±0.1	0.004±0.001	0.021±0.001	0.092±0.007	0.574±0.055	6.27±1.10
	Sucrose	30±0.1	0.013±0.001	0.049±0.001	0.155±0.001	0.582±0.001	3.76±0.03
		36±0.1	0.009±0.001	0.035±0.003	0.173±0.004	0.659±0.013	3.81±0.01
ATCC 4875 after 144h	Lactate	30±0.1	0.015±0.001	0.116±0.001	0.058±0.002	0.465±0.001	8.04±0.29
		36±0.1	0.009±0.001	0.120±0.001	0.037±0.001	0.479±0.001	13.06±0.29
	Glycerol	30±0.1	0.012±0.001	0.050±0.003	0.197±0.004	0.844±0.053	4.29±0.19
		36±0.1	0.008±0.001	0.044±0.006	0.163±0.013	0.856±0.003	5.26±0.39
	Sucrose	30±0.1	0.007±0.001	0.036±0.001	0.122±0.009	0.682±0.018	5.61±0.26
		36±0.1	0.007±0.001	0.039±0.003	0.128±0.007	0.744±0.013	5.82±0.21
ATCC 4874 after 132h	Lactate	30±0.1	0.010±0.001	0.083±0.008	0.053±0.001	0.425±0.047	8.03±0.93
		36±0.1	0.010±0.001	0.079±0.015	0.051±0.001	0.395±0.041	7.68±0.60
	Glycerol	30±0.1	0.015±0.001	0.057±0.001	0.196±0.008	0.747±0.060	3.82±0.16
		36±0.1	0.013±0.001	0.054±0.001	0.183±0.009	0.763±0.054	4.18±0.09
	Sucrose	30±0.1	0.014±0.001	0.046±0.001	0.161±0.004	0.533±0.007	3.32±0.04
		36±0.1	0.011±0.002	0.039±0.001	0.150±0.031	0.518±0.032	3.52±0.52
ATCC 9614 after 139h	Lactate	30±0.1	0.013±0.001	0.123±0.003	0.048±0.001	0.462±0.012	8.82±0.31
		36±0.1	0.011±0.001	0.126±0.003	0.043±0.001	0.470±0.012	8.96±1.56
	Glycerol	30±0.1	0.017±0.001	0.037±0.004	0.325±0.008	0.713±0.070	2.19±0.27
		36±0.1	0.016±0.001	0.038±0.001	0.318±0.017	0.742±0.044	2.33±0.02
	Sucrose	30±0.1	0.027±0.001	0.056±0.001	0.295±0.005	0.607±0.005	2.06±0.02
		36±0.1	0.022±0.001	0.038±0.001	0.357±0.011	0.611±0.020	1.71±0.01
DSM 20274 after 140h	Lactate	30±0.1	0.013±0.001	0.077±0.002	0.047±0.002	0.282±0.010	5.97±0.43
		36±0.1	0.009±0.001	0.063±0.002	0.036±0.001	0.259±0.011	7.10±0.03
	Glycerol	30±0.1	0.020±0.001	0.064±0.003	0.252±0.006	0.788±0.021	3.12±0.01
		36±0.1	0.017±0.001	0.064±0.001	0.214±0.001	0.819±0.023	3.83±0.08
	Sucrose	30±0.1	0.011±0.001	0.049±0.002	0.138±0.005	0.604±0.049	4.37±0.18
		36±0.1	0.011±0.001	0.048±0.001	0.152±0.009	0.678±0.020	4.48±0.14
ATCC 13673 after 139h	Lactate	30±0.1	0.018±0.001	0.123±0.001	0.071±0.001	0.478±0.003	6.70±0.06
		36±0.1	0.017±0.001	0.124±0.004	0.067±0.001	0.479±0.014	7.13±0.32
	Glycerol	30±0.1	0.010±0.001	0.034±0.004	0.223±0.001	0.774±0.038	3.48±0.17
		36±0.1	0.009±0.001	0.031±0.001	0.217±0.022	0.783±0.080	3.61±0.01
	Sucrose	30±0.1	0.020±0.001	0.051±0.002	0.224±0.003	0.556±0.022	2.48±0.07
		36±0.1	0.021±0.001	0.050±0.001	0.227±0.001	0.543±0.025	2.39±0.10

5.3.1. Batch fermentation using sodium lactate as carbon source

Propionic acid and dry biomass production using sodium lactate as carbon source at two temperatures are shown in *Figure 10.*
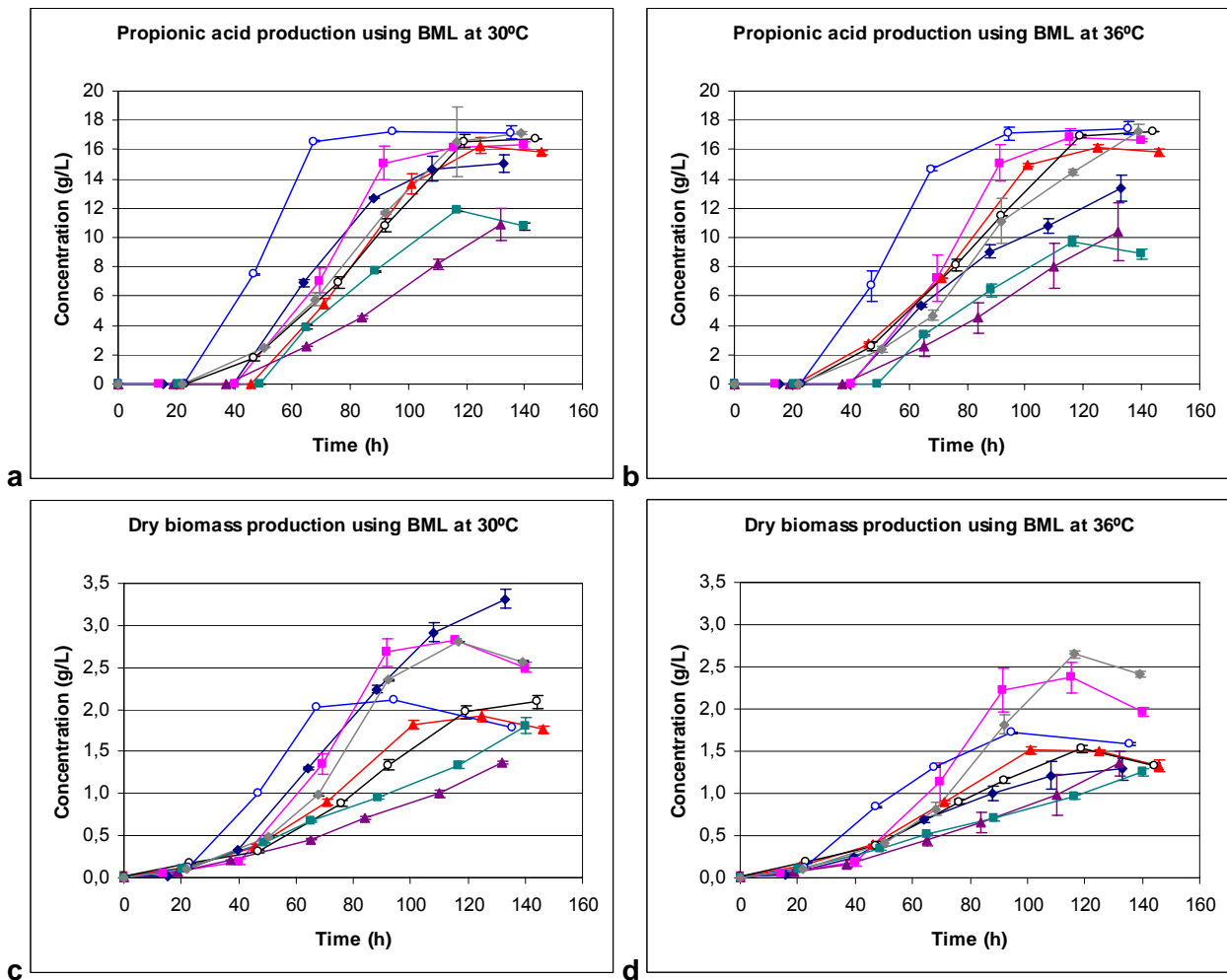
Figure 10 - Propionic acid fermentation using lactate. Symbols: (◆) ATCC 4965, (■) ATCC 4868, (▲) ATCC 25562, (○) ATCC 4875, (▲) ATCC 4874, (○) ATCC 9614, (■) DSM 20274 and (◆) ATCC 13673.

Table 12 shows that propionic acid formation using lactate as carbon source does not depend on temperature levels because the propionic acid concentration are very similar at 30ºC and 36ºC for all the propionibacteria, although ATCC 4965, 4875 and 9614 showed small differences about the production of this metabolite at the two temperatures.

Besides, the data in *Table 12* are not conclusive about the best strain for propionic acid production, since four *Propionibacterium sp.* (ATCC 4868, 4875, 9614 and 13673) had a good propionic acid formation in lactate. However, *Figure 10* **a** and **b** show an interesting propionic acid profile. All the three strains reached almost the same propionic acid concentration, propionic acid productivity and propionic acid yield from substrate in the end of fermentation, but ATCC 9614 was faster than the others at 30ºC and 36ºC, producing more propionic acid in a short time, mainly during 20-60h, even so ATCC 4875 displayed the highest efficiency for propionic acid production per gram of cell at 36ºC ($Y_{P/X}$ = 13.06).

On the other hand, the bacterial growth in sodium lactate was more influenced by the temperature than the organic acid formation, that is all propionibacteria grew better at 30ºC than 36ºC, but ATCC 4874 was indifferent about this parameter. ATCC 4965 at 30ºC

achieved the highest dry biomass concentration (3.31g/L), biomass productivity (0.025g.L^{-1}.h^{-1}) and cell yield from substrate (0.097g/g) of all other bacterias.

However, *P. freudenreichii* subsp. *shermanii* (ATCC 9614) was able to grow faster in the beginning of the fermentaion (20-60h) at 30°C and 36°C than the others microorganims (*Figure 10* **c** and **d)**, as well as produced more propionic acid in this interval, so after 60h ATCC 9614 had been depleted all the carbon source and produced around 17g/L of propionic acid which resulted in a higher inhibition of growth and a lower cell concentration. Using sodium lactate as carbon source, pH did not change so much, that is, the final pH of fermentations was around 6.2-6.4 (*Table 12*), thus the mainly effect of inhibition was only related to the propionic acid concentration.

5.3.2. Batch fermentation using glycerol as carbon source

Propionic acid and dry biomass formation using glycerol as carbon source at two temperatures are shown in *Figure 11*.
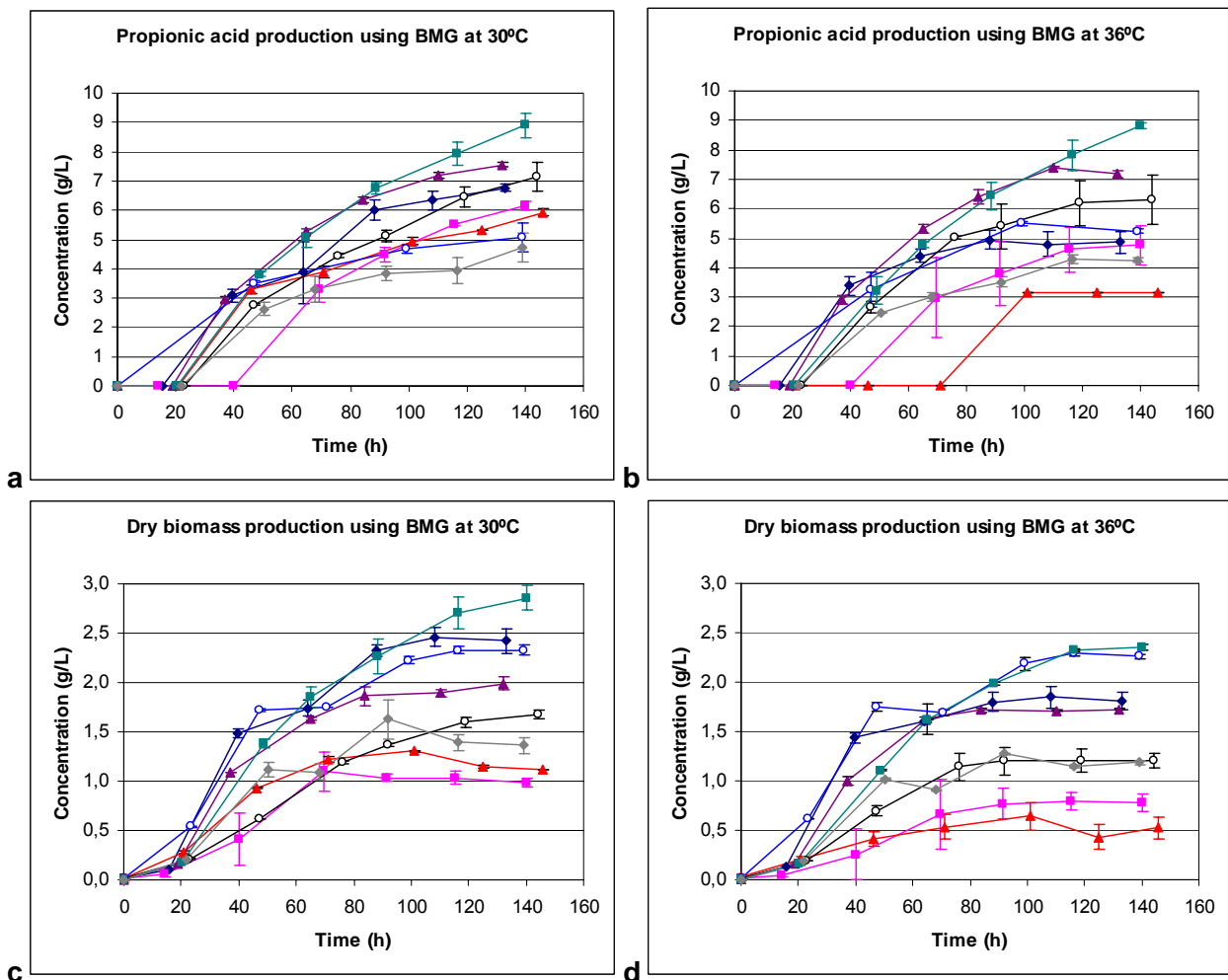
Figure 11 - Propionic acid fermentation using glycerol. Symbols: (◆) ATCC 4965, (■) ATCC 4868, (▲) ATCC 25562, (○) ATCC 4875, (▲) ATCC 4874, (○) ATCC 9614, (■) DSM 20274 and (◆) ATCC 13673.

Table 12 shows that propionic acid formation using glycerol as carbon source are probably related to the temperature levels, once *Propionibacterium* ATCC4965, 4868 and 25562 showed differences between the propionic acid production at 30°C and 36°C, but the other strains did not display so significantly evidences, therefore the temperature level

could influence more some species than other ones. The highest concentration of propionic acid (8.98g/L) in BMG was obtained by DSM 20274, as well the highest propionic acid productivity ($0.064g.L^{-1}.h^{-1}$).

Moreover, the bacterial growth in BMG was influenced by the temperature because all strains grew better at 30ºC than 36ºC, except ATCC 9614 was indifferent about this parameter.

It becomes possible to realize from *Figure 11* **c** and **d** that *P. arabinosum* (ATCC 4965) and *P. freudenreichii* subsp. *shermanii* (ATCC 9614) got to grow in BMG faster than the other ones at 30ºC and 36ºC, during 20-40h, and also ATCC 9614 got the best cell yield from substrate of all (0.325g/g at 30ºC and 0.318g/g at 36ºC), but considering the fermentative parameters in the end of fermentation, DSM 20274, ATCC 4965 and ATCC 9614 obtained similar results, 0.020, 0.018 and $0.017g.L^{-1}.h^{-1}$ (*Table 13*) of dry biomass productivity, respectively. Besides, *P. zeae* (DSM 20274) reached the highest cell concentration (2.86g/L) and propionic acid concentration (8.98g/L) of the other strains in BMG, due to this, DSM 20274 was chosen for the next experiments using BMG in BioFlo110.

Nevertheless, the assays in 1L screw-cap flasks using BMG as carbon sources did not allow a clear conclusion about the best temperature level for propionic acid fermentation in BMG, thus another tests had been done in BioFlo110.

5.3.3. Batch fermentation using sugarcane molasses as carbon source

Propionic acid and dry biomass formation making use of sugarcane molasses as carbon source at two temperatures are shown in *Figure 12*.
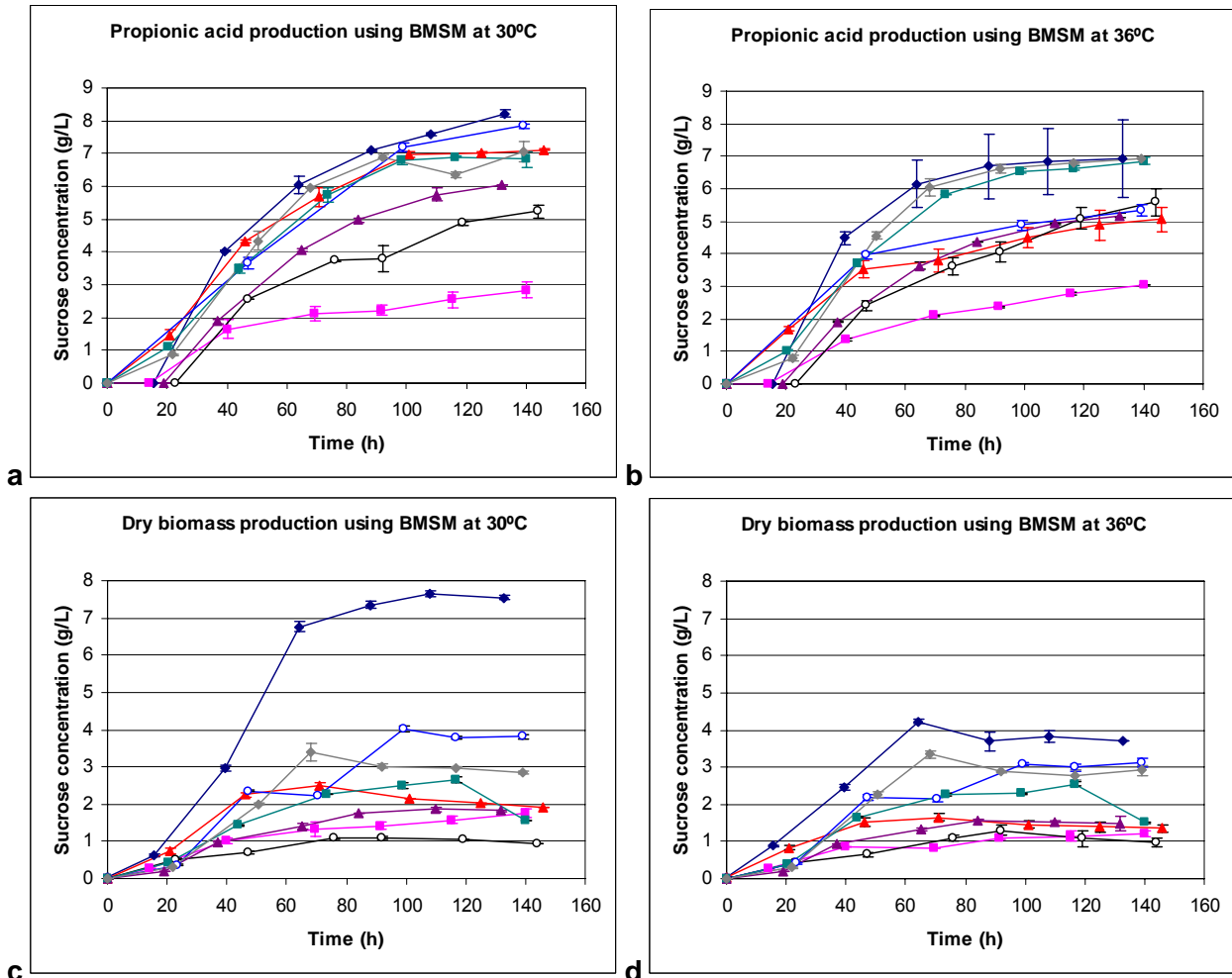
Figure 12 - Propionic acid fermentation using sugarcane molasses. Symbols: (◆) ATCC 4965, (■) ATCC 4868, (▲) ATCC 25562, (○) ATCC 4875, (▲) ATCC 4874, (○) ATCC 9614, (■) DSM 20274 and (◆) ATCC 13673.

It can be noticed that *P. arabinosum* (ATCC 4965) in BMSM showed the highest capability for propionic acid production during all the fermentation time of all propionibacterias, at 30ºC as well as 36ºC (*Fig. 12* **a** and **b**), this behavior can be realized by its propionic acid productivity, 0.062 and 0.052g.L^{-1}.h^{-1} (*Table 13)*, at 30ºC and 36ºC, respectively. In addition, this strain *P. arabinosum* had the best average ratio (4.2:1) between propionic and acetic acid in BMSM, what means around 81% of propionic acid purity.

Cell growth in BMSM was influenced by the temperature levels, that is, almost all microorganisms grew better at 30ºC than 36ºC and this effect was more pronounced with ATCC 4965, which obtained the highest cell concentration (7.55g/L), cell productivity (0.052g.L^{-1}.h^{-1}) and cell yield (0.496g/g) of all trials, growing at 30ºC (see *Tables 9* and *10*).

P. arabinosum (ATCC 4965) was selected for the assays in BioFlo110 using BMSM at 30ºC, as a result of undergoing the higher propionic acid concentration and reached the

higher dry biomass concentration, once propionic acid production is related partially or totally to the bacterial growth (SUWANNAKHAM, 2005; CHOI, 1994).

5.3.4. Comparing parameters: Use of different carbon sources at two temperatures on propionic acid fermentation

As already shown in *Figs. 10, 11* and *12,* propionic acid fermentations in BML, BMG, and BMSM at different temperature levels display different kinetic parameters. The differences were also evident in comparing acidification (ΔpH), propionic-to-acetic acid ratio (P/A), and cell (X) and product yields (*Tables 9* and *10*).

As shown in *Tables 9* and *10,* cell yield depends on temperature and the carbon source used. The cell yield from sugarcane molasses was about 3 - 4 times higher than that from lactate; nevertheless, propionic acid formation was similar for almost all *Propionibacterium,* growing in BML. Cell growth rate as well as biomass final concentration, biomass productivity, and biomass yield was better when using BMSM than BML or BMG, mainly at 30°C.

The propionic acid production had small differences according to the temperature level, but in all of the carbon sources tested for almost all strains, it can be noticed that 30°C temperature presented better condition to produce propionic acid and dry propionibacteria biomass than 36°C, mainly for *P. arabinosum* (ATCC 4965).

The best carbon source for *Propionibacterium,* comparing the propionic acid final concentration and the propionic acid productivity, was BML. However, with glycerol, there was no acetic acid formation, and the propionic acid yield was maximum, as estimative P/A ratio based on the sensibility of HPLC detector was 15:1, that means a minimal of 93% of propionic acid purity.

When it was used sodium lactate as carbon source, which is a neutralized organic acid, for production of other organic acids by propionibacteria, there was a small variation in hydrogen ion (H^+) net concentration; hence sodium lactate showed the best results for organic acid formation because cells did not undergo inhibition by pH when compared to the other carbon sources, where the final pH is around 5, thus little growth occurs as well propionic acid formation (HSU, 1991; PLAYNE, 1985; NANBA, 1983).

5.4. STUDY THE EFFECTS OF pH CONTROL ON PROPIONIC ACID FERMENTATION IN STIRRED-TANK BIOREACTOR

The effects of pH control on propionic acid fermentation was studied in the mainly carbon sources for developing propionic acid fermentation in Brazil, glycerol and sugarcane molasses. The assays were performed in BioFlo110 (NEW BRUNSWICH SCIENTIFC COMPANY, US) using 5L of working volume in anoxic environment by sparging N_2 gas. The results are presented below.

## 5.4.1. Effects of pH control on propionic acid fermentation with BMG

As discussed in *Section 4.3.2*, the experiments using BMG in 1L screw-cap flasks were not concluded about the best temperature for propionic acid fermentation by *P. zeae* (DSM 20274) with BMG. Thereby, it was conducted two experiments in stirred-tank bioreactor with controlled pH at 30ºC and 36ºC (*Fig. 13*).
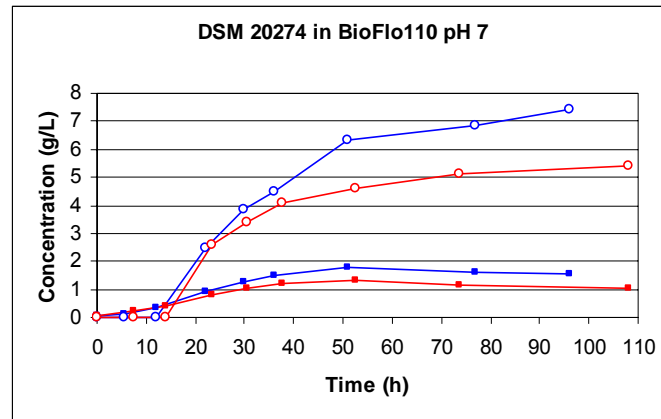
Figure 13- Propionic acid fermentation by *P. zeae* (DSM 20274) with BMG in BioFlo110. Symbols: (■) Dry biomass at 30ºC, (■) Dry biomass at 36ºC, (○) Propionic acid at 30ºC and (○) Propionic acid at 36ºC.

Starting from *Fig. 13*, it is obvious that 30ºC temperature had a clear advantage about propionic acid formation and *P. zeae* growth. Thus, the effects of pH control were studied using this temperature at pH 6, 6.5 and 7, as mentioned by HSU and YANG (1991), and also by SESHADRI and MUKHOPADHYAY (1993) as the best pH range for organic acid formation and cell growth by propionibacterias.
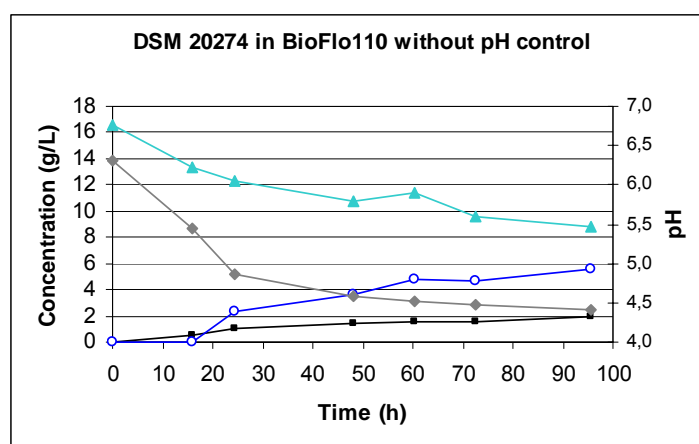
The profiles of propionic acid fermentation at different pH are shown in *Fig. 14*.
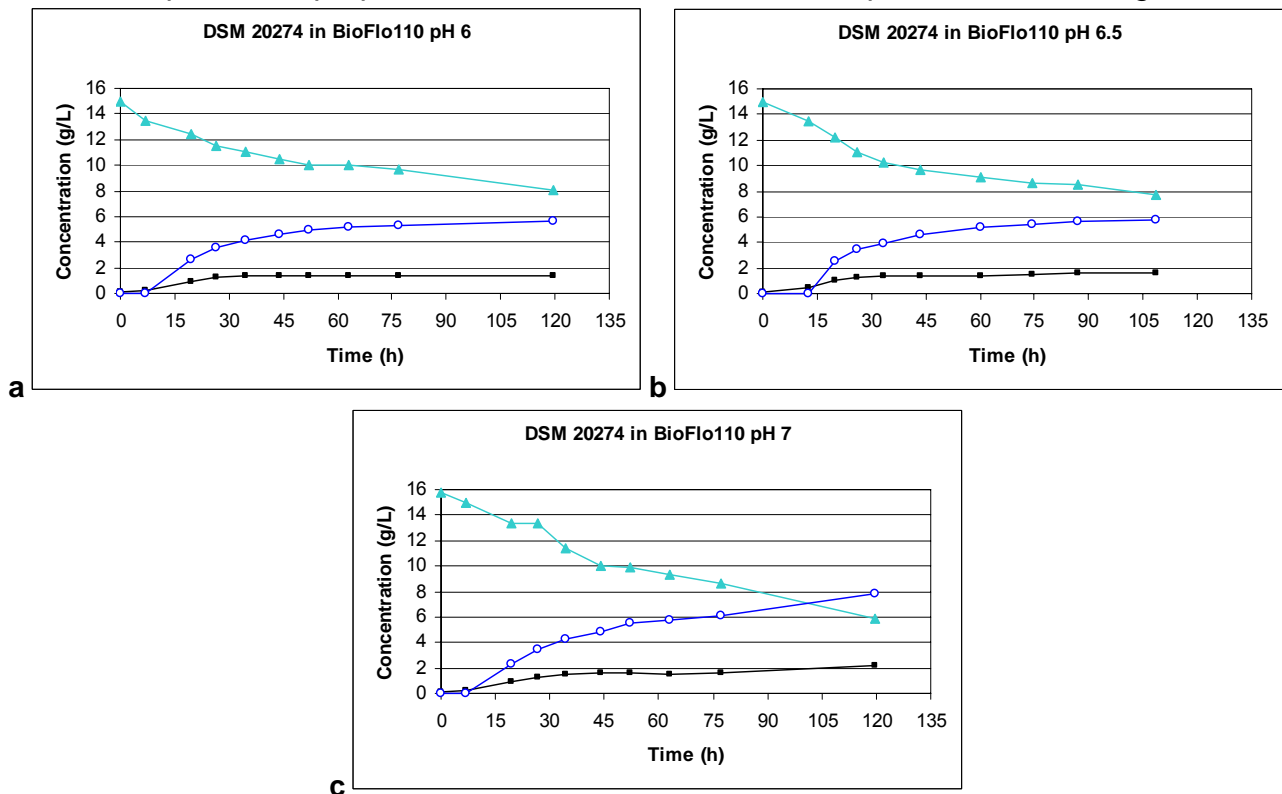
Figure 14 - Propionic acid fermentation by *P. zeae* (DSM 20274) using glycerol as carbon source in BioFlo110. **a** Controlled pH at 6; **b** Controlled pH at 6.5; **c** Controlled pH at 7. Symbols: (■) Biomass – Dry weight; (▲) Glycerol and (○) Propionic acid.

Fermentations using BMG with controlled pH showed that *P. zeae* grew just until 40h and then almost stopped, propionic acid concentration was around 4.5g/L at this time. This pattern was noticed in the propionic acid formation at pH 6 and 6.5 (*Fig. 14* **a** and **b**), there were a good propionic acid production until 45h, reaching a plateau around 5.7g/L. However, in BMG fermentation at pH 7, there was propionic acid formation after 45h but in a slow way.

It was surprising that none of the fermentations with pH control, making use of BMG, was better than those in 1L screw-cap flasks, in this way, it was carried out a fermentation at 30ºC without pH control in BioFlo110 (*Fig. 15*).

Figure 15 - Propionic acid fermentation by *P. zeae* (DSM 20274) using glycerol as carbon source in BioFlo110 without pH control. Symbols: (■) Biomass – Dry weight; (▲) Glycerol, (○) Propionic acid and (◆) pH.

P. zeae (DSM 20274) was not capable to produce propionic acid and dry biomass as good as in small scale (see *Table 12*). This behavior are not clear until this moment, but one possible explanation could be the environmental condition, once in 1L screw-cap flasks there was a low oxygen concentration in the media, different of the stirred-tank bioreactor, because N_2 gas was sparged in the BMG broth in order to simulate an anoxic environment. The *Table 14* shows some fermentative parameters of propionic acid fermentation in stirred-tank bioreactor.

Table 14 – pH effects on propionic acid fermentation of DSM20274 using BMG in BioFlo110.

|	Without pH control	pH 6	pH 6.5	pH 7
Dry biomass final concentration (g/L)	1.92	1.41	1.59	2.15
Propionic acid final concentration (g/L)	5.53	5.69	5.78	7.84
Acetic acid final concentration (g/L)	ND*	ND*	ND*	ND*
Succinic acid final concentration (g/L)	ND*	ND*	ND*	ND*
Dry biomass yield from substrate - $Y_{X/S}$ (g/g)	0.24	0.19	0.21	0.21
Propionic acid yield from substrate - $Y_{P/S}$ (g/g)	0.71	0.82	0.80	0.80
Propionic acid yield from dry biomass - $Y_{P/X}$ (g/g)	2.88	4.03	3.63	3.65

* None detected

Even so propionic acid fermentation in BMG with controlled pH as not good as in small scale, the pH control improved the propionic acid fermentation when comparing pH 6 or 6.5 to pH 7, it means an enhancing around 38% on propionic acid production. When pH was controlled, the cell underwent less inhibition and became more productive ($Y_{P/X}$ increased), it can be realized because of the reduction in $Y_{X/S}$ and improvement in $Y_{P/S}$.

As there was not by-product formation, the propionic acid yields were interesting ($Y_{P/S}$ = 0.8g/g) as also seen in *Table 12*. In this way, fermentative processes making use of glycerol as carbon source deserve further development.

5.4.2. Effects of pH control on propionic acid fermentation with BMSM

Using pH control on propionic acid fermentation with BMSM (which contain 25g/L of sugarcane molasses) all the carbon source was depleted by *P. arabinosum* (ATCC 4965) before 24h of fermentation, then the carbon source concentration was twice increased and all the 50g/L of sugarcane molasses was consume before 48h. Thereby, sugarcane molasses concentration was doubled again and the fermentative profiles with BMSM (x4) are shown in *Figure 16*.
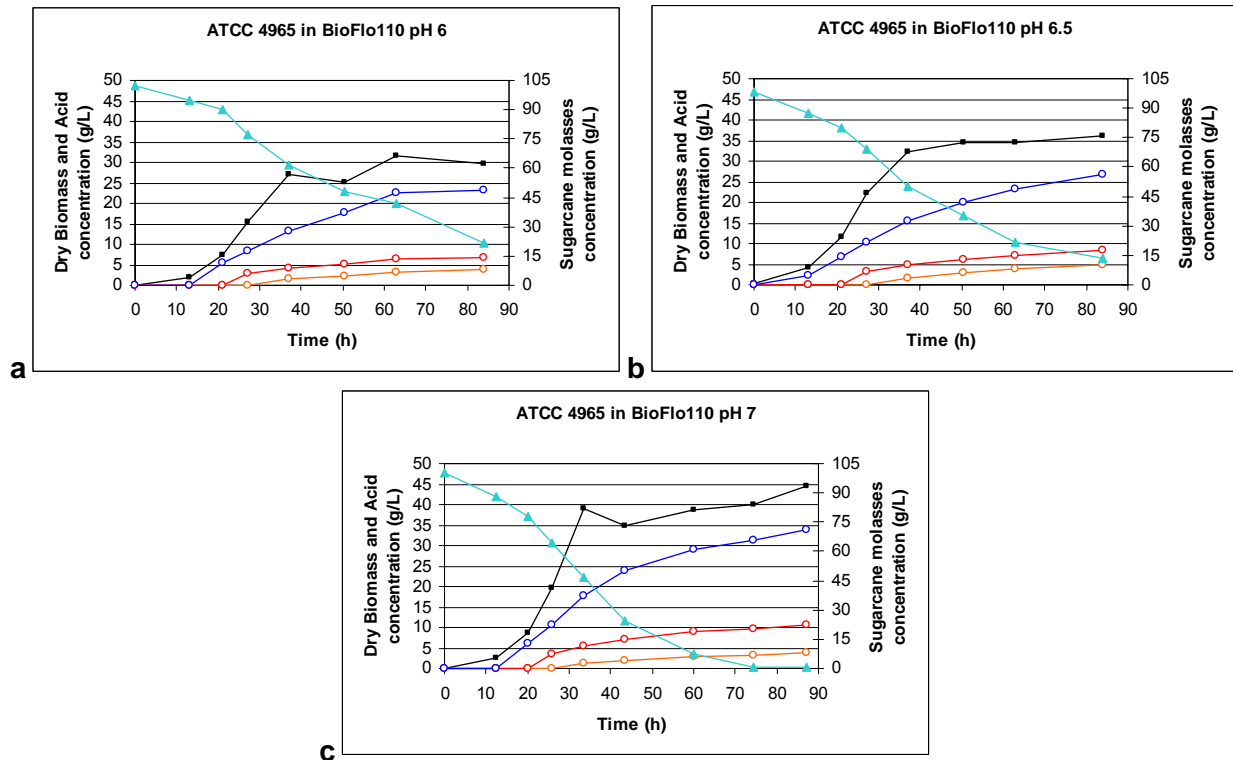
Figure 16 - Propionic acid fermentation by *P. arabinosum* (ATCC 4965) using sugarcane molasses as carbon source in BioFlo110. **a** Controlled pH at 6; **b** Controlled pH at 6.5; **c** Controlled pH at 7. Symbols: (■) Biomass – Dry weight; (▲) Sugarcane molasses, (○) Propionic acid, (○) Acetic acid and (○) Succinic acid.

In spite of *P. arabinosum* fermentation at pH 7 (*Fig. 16***c**) had consumed all the carbon source before 75h, the other fermentations at pH 6 and 6.5 (*Fig. 16* **a** and **b**) did not get this capability and it was detected 21,7g/L and 13,4g/L of residual sucrose in the end of fermentation at pH 6 and 6.5, respectively.

For the first time, succinic acid was measured in this work; it was a result of the wide amount of available carbon source and the controlled pH.

Propionic acid concentration achieved the higher value of all assays (33.82g/L) as well the dry biomass (44.55g/L) in the fermentation at pH 7 (see *Table 15*).

Table 15 – Performance comparisons in the controlled fermentation at pH 6, 6.5 and 7 using BMSM

|	pH 6	pH 6.5	pH 7
Dry biomass final concentration (g/L)	29.82	36.02	44.55
Propionic acid final concentration (g/L)	23.31	26.92	34.19
Acetic acid final concentration (g/L)	6.92	8.43	10.86
Succinic acid final concentration (g/L)	3.82	4.90	4.64
Dry biomass yield from substrate - $Y_{X/S}$ (g/g)	0.37	0.42	0.40
Propionic acid yield from substrate - $Y_{P/S}$ (g/g)	0.29	0.32	0.31
Propionic acid yield from dry biomass - $Y_{P/X}$ (g/g)	0.78	0.75	0.78

Comparing the results in *Table 12* with the controlled fermentation at pH 6, it was an improvement around 295% in dry cell concentration, 183% in propionic acid concentration and so on for acetic acid concentration. In addition, an increment of 0.5 in pH value enhanced dry biomass concentration about 21% and other 15% in propionic acid formation. By the end, the controlled fermentation at pH 7 was the best one as seen in *Table 15* and *Fig 16***c**.

Besides, cell yield from substrate was not so different compared with those ones in *Table 12* (0.3-0.4g/g), but the propionic acid yield from substrate, and from biomass, were lower than those in small scale, because of fermentation with pH control had an amount of the substrate transformed in succinic acid.

Therefore, the fermentative broth with BMSM (x4) at pH 7 was used to evaluate the capability of these products to prevent infections from fungus.

5.5. EVALUATION OF PROPIONIC ACID FERMENTATIVE BROTH TO PREVENT INFECTION FROM FUNGUS

The final fermentative broth with BMSM (x4) at pH 7 in Bioflo110 (see *section 4.4.2*) was used for an inhibition test of fungus growth. The fermentative broth was centrifuged and the supernatant had the pH adjusted to 3, 5 and 7. After filter-sterilization, samples of 25μL and 50μL were tested against *Aspergillus flavus* and *A. ochraceus*, which are common microorganisms that infect grains, cereals and other human foods. The antifungical activity of propionic acid fermentative broth is shown in *Fig. 17* and *Table 16*.
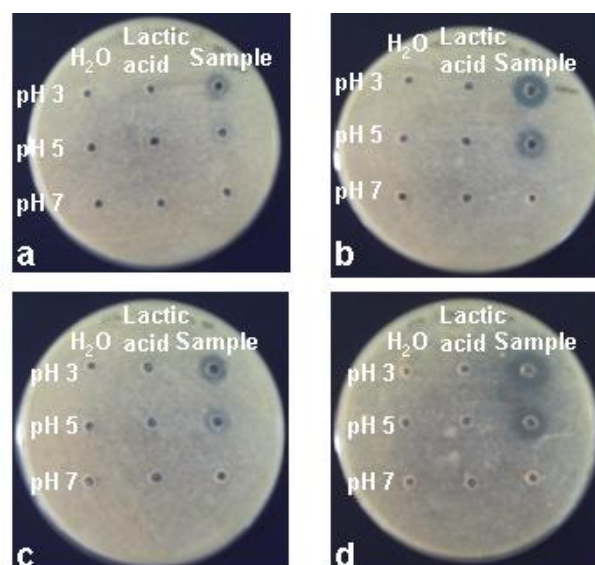
Figure 17 – Tests with propionic acid fermentative broth. **a** *Aspergillus flavus* ATCC 1003 with 25μL of sample; **b** *Aspergillus flavus* ATCC 1003 with 50μL of sample; **c** *Aspergillus ochraceus* ATCC 1008 with 25μL of sample; **d** *Aspergillus ochraceus* ATCC 1008 with 50μL of sample.

Table 16 – Evaluation of fungus inhibition

	Diameter of halos of inhibition (mm)			
	Aspergillus flavus		*Aspergillus ochraceaus*	
	25µL	50µL	25µL	50µL
pH 3	13	20	16	25
pH 5	0	15	12	16
pH 7	0	0	0	0

It was used a lactic acid solution made of 50g/L in order to compare the inhibion powder with propionic acid fermentative broth, because lactic acid is widely applied as food preservative. However, all assays with lactic acid did not show any inhibition against these microorganisms, on the other hand, both fungi was inhibited by the samples and *A. ochraceaus* was more sensible than *A. flavus*, mainly at pH 3 (*Table 16*).

Propionic acid fermentative broth confirmed the strong inhibitory effect of propionic acid, mainly at pH 3, where almost all propionic acid is in the undissociated acid form (LIND, 2005). Therefore, it could be applied for food or feed preservation without any concentration or purification processes.

6. CONCLUSION

The obtained results about propionic acid fermentation in this work are consistent with the following conclusions:

• Inoculum rate did not show differences on propionic acid fermentation;

• 25g/L of BMSM, 40g/L of BML and 20g/L of BMG was not able to inhibit propionibacteria growth;

• The best carbon source for *Propionibacterium*, comparing the propionic acid final concentration and the propionic acid productivity, was BML;

• The best carbon source for *Propionibacterium*, comparing the propionic acid yield from substrate, was BMG;

• The best carbon source for *Propionibacterium*, comparing the biomass final concentration, biomass productivity, and biomass yield was BMSM;

• Cell growth rate as well as biomass final concentration, biomass productivity, and biomass yield was better at 30ºC than 36ºC;

• Using lactate as carbon source ATCC 4965 was faster than the other propionic acid bacterias at 30ºC and 36ºC, producing more propionic and dry biomass in a shortest time of all microorganisms tested;

• *P. zeae* (DSM 20274) was the most adapted propionibacteria to BMG, reaching 8.98g/L of propionic acid and 2.86g/L of dry biomass in 1L screw-cap flasks and fermentations with pH control did not improve significantly these productions;

• *P. arabinosum* (ATCC 4965), or better *P. acidipropionici*, showed the best results when BMSM was used in small scale. Moreover, when it was carried out the fermentation using BMSM (x4) with controlled pH at 7, this strain was able to produce 34.19g/L of propionic acid, 10.86g/L of acetic acid, 44.55g/L of dry biomass and for the first time a small amount of succinic acid was measured (4.64g/L) as well as in the other fermentations with controlled pH at 6 and 6.5;

• By the end, the final broth fermented by ATCC 4965 in BioFlo110 at pH 7 was capable to prevent *Aspergillus ochraceaus* and *A. flavus* growth, showing a feasible application for the future.

INFORMATION SHEET

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Title: Propionic acid production by *Propionibacterium sp.* using low-cost carbon sources in submerged fermentation

Field of study:

    Major field: Bioprocesses engineering

    Specific field: Anaerobic fermentative processes

    Specialty: Propionic acid fermentation

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