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PRODUCTION OF BIO-ETHANOL FROM SOYBEAN MOLASSE BY *Saccharomyces cerevisae*

Dissertação apresentada como requisito parcial à obtenção do título de Mestre em Processos Biotecnológicos, do Programa de Pós-Graduação em Processos Biotecnológicos, Área de Concentração Agroindústria, da Universidade Federal do Paraná

Orientador: Prof. Dr. Carlos Ricardo Soccol
Co-orientador: Prof. Dr. Jean-Luc Tholozan

CURITIBA

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Setor de Tecnologia

UFPR

RELATÓRIO DE DEFESA DE DISSERTAÇÃO DE MESTRADO

Aos vinte e seis dias do mês de agosto de 2005, na Sala de Vídeo Conferência do CESEC, no Centro Politécnico da Universidade Federal do Paraná, Jardim das Américas, foi instalada pelo Prof Dr Carlos Ricardo Soccol, Coordenador do Curso de Pós-Graduação em Processos Biotecnológicos, a banca examinadora para a Terceira Dissertação de Mestrado, área de concentração: Agroindústria. Estiveram presentes no Ato, além do Coordenador do Curso de Pós–Graduação, professores, alunos e visitantes.

A Banca Examinadora, atendendo determinação do colegiado do Curso de Pós-Graduação em Processos Biotecnológicos, ficou constituída pelos Professores Doutores Jean-Luc Tholozan (Université de Provence - França), Jean Lorquin (Université de Provence – França), Vanete Thomaz Soccol (UFPR), e Carlos Ricardo Soccol (UFPR – orientador da dissertação).

Às 10:00 horas, a banca iniciou os trabalhos, convidando a candidata **Paula Fernandes de Siqueira** a fazer a apresentação da Dissertação intitulada: "Production of Bio-Ethanol from Soybean Molasse by *Saccharomyces cerevisae*". Encerrada a apresentação, iniciou-se a fase de argüição pelos membros participantes.

Tendo em vista a monografia e a argüição, a banca composta pelos professores Dr Jean-Luc Tholozan, Dr Jean Lorquin, Drª Luciana Porto de Souza Vandenberghe, e Dr Carlos Ricardo Soccol, declarou o candidato _____ (de acordo com a determinação dos Artigos 32/33/34/35 da Resolução 13/96 de 23.07.96).

Curitiba, 26 de Agosto de 2005.

Prof Dr Jean-Luc Tholozan

Prof Dr Jean Lorquin

Prof Dr Carlos Ricardo Soccol

Profª Drª Luciana P S Vandenberghe

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Agradeço ao Presidente da Imcopa pela confiança depositada na execução deste e de vários outros projetos.

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ABSTRACT

The non-renewable energy resources can not be replaced as fast as they are consumed, and eventually, they will run out. Therefore, alternative energy sources such as ethanol are being considered. Some biological processes have rendered possible routes for producing ethanol in large volumes. The main objective of this work was to define an economically viable bioprocess to produce bio-ethanol from soybean molasses at laboratory, pilot and industrial scale.

In order to increase the reducing sugars concentration of the molasses, acid hydrolysis was tested, but it did not improve the ethanol yield of the fermentation. The enzymatic hydrolysis of the molasses and the acid hydrolysis of the vinasse (waste product) in severe conditions provided an increase of 20% and 17% in ethanol yield, respectively. However, until now, these processes are not considered economic.

The bacterial strains of *Zymomonas mobilis* assayed did not show a good yield in ethanol production from soybean molasses, once these bacteria are more selective to the types of metabolizable sugars. The commercial yeasts Levasaf and Fermol presented the best yields among the four assayed strains; however, Levasaf was chosen considering the lower cost. Among the six wild strains assayed, one showed an interesting result, which will be the subject of further studies.

In the fermentative process, the medium was maintained at 30ºBrix and the previous separation of the molasse's proteins showed no good efficiency in the conditions assayed. The supplementation of the medium with nutrients and pH adjustment were not necessary.

The recycle of biomass was tested and there were expressive contamination problems and losses in cell viability. Therefore, the growth of bacteria needs to be controlled in order to assure a satisfactory ethanol yield. The antibiotic tested in our experiments (Kamoran HJ) and the reduction of the medium pH were not effective in inhibiting bacterial growth. The main contaminants are probably lactic acid bacteria, which are acid tolerant. Consequently, a more effective antibiotic will be necessary in order to control contamination and maintain a good ethanol yield. On the other hand, in pilot scale, the acid treatment of the inoculum provided a better ethanol yield in fermentation.

The major problems identified after scaling-up the process were foam formation and contamination with bacteria. The first problem was solved by carrying out the process as fed-batch and using antifoam and dispersant agents. The second problem was solved by the previous treatment of soybean molasses (removal of solids by centrifugation or filtration), in order to facilitate the separation of the yeast biomass at the end of the fermentation and the acid treatment. These solid particles can act as "support" for contaminants. The yield in ethanol production was maintained after scale-up.

The main by-product generated by the alcoholic fermentation is the waste produced after distillation and ethanol recovery. This residue (called *vinasse*) has a high concentration of organic compounds, essentially non-reducing sugars, and it is being tested as substrate for production of two other products of high commercial value: xanthan gum and lactic acid.

Keywords: Soybean molasses; bio-ethanol; pilot scale; industrial scale; *Saccharomyces cerevisiae*.

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LIST OF ABBREVIATIONS

CAS – Chemical Abstracts Service

CFU – Colony-Forming Units

D-LdH – D-lactate dehydrogenase

FAT – Fundação André Tosello

FDA – Food and Drug Administration

G-C – Guanine-cytosine

GPT – Glutamate-pyruvate transaminase

GRAS – Generally Recognized as Safe

HPLC – High Performance Liquid Chromatography

LAB – Lactic Acid Bacteria

L-LdH – L-lactate dehydrogenase

m.f.b. – moisture free basis

MRS – Man-Rogosa-Sharp

NAD – Nicotinamide-adenine dinucleotide

N. f. m. – Non-fermented medium

NRRL – Northern Regional Research Laboratory

OD – Optical density

P.A. – Pro analysis

UFPR – Universidade Federal do Paraná

USP – United States Pharmacopeia

v/v – volume/volume

w/v – weight/volume

w/w – weight/weight

LIST OF SYMBOLS

% – Per cent

g – Gram

L – Liter

pH – Hydrogen potential

h – Hour

US$ - United States Dollar

m^3 – Cubic meter

β – Beta

α – Alpha

kg – Kilogram

℃ – Celsius degree

μm – Micrometer

mm – Millimeter

g – Gravity acceleration

rpm – Rotations per minute

min - Minute

$CaCO_3$ – Calcium carbonate (chemical formula)

$(NH_4)_2HPO_4$ – Diammonium hydrogen phosphate (chemical formula)

$(NH_4)_2SO_4$ – Ammonium sulfate (chemical formula)

KOH – Potassium hydroxide (chemical formula)

N – Normal

$Ca(OH)_2$ – Calcium hydroxide (chemical formula)

H_2SO_4 – Sulfuric acid (chemical formula)

mM – Millimolar

mL – Milliliter

NaOH – Sodium hydroxide (chemical formula)

nm - Nanometer

cm – Centimeter

Δ – Delta

r_X – Biomass production rate

r_S – Substrate consumption rate

r_P – Product formation rate

$Y_{X/S}$ – Biomass yield from substrate

$Y_{P/S}$ – Product yield from substrate

X – Biomass

mmHg – Mercury millimeter

J – Joule

kJ – Kilojoule

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1 INTRODUCTION

Natural energy resources such as fossil fuel, petroleum and coal have been consumed at high rates over the last few years. These non-renewable resources can not be replaced as fast as they are consumed, and eventually, they will run out. Therefore, alternative energy sources such as ethanol, methane, and hydrogen are being considered. Some biological processes have rendered possible routes for producing ethanol and methane in large volumes. A worldwide interest in the utilization of bio-ethanol as an energy source has stimulated studies on the cost and efficiency of industrial processes for ethanol production (SOCCOL *et al.*, 2005; CYSEWSKI, 1978). Intense research has been carried out for obtaining efficient fermentative organisms, low-cost fermentation substrates, and optimum environmental conditions for fermentation to occur.

Nowadays, the United States produces 14 billion liters of alcohol per year from maize, and Brazil produces 16 billion liters per year from sugarcane, through a simple and cheap process. The worldwide production of ethanol in 2005 reached 45 billion liters, which means 11% more than the amount produced in 2004.

The main countries responsible for this increase were Brazil and United States. Regarding to alcohol demand, Japan is one of the most interested countries in Brazilian alcohol. However, the Japanese government wants to assure that Brazil will be able to supply the necessity of the country, which is estimated in 1.8 billion liters per year. Considering the internal market, preliminary data indicate that the center-south region of Brazil, in the beginning of the harvest, has commercialized about 1 billion liters of alcohol (111 million of liters for exportation and the remaining for the internal market).

The Brazilian production of soybean is estimated in 52.2 millions of tons (2005/2006), which represent around 30% of the global production.

IMCOPA is a company created in 1967 in Ponta Grossa, Paraná state, Brazil, with an initial transformation capacity of 20 tons of soybean per day. Today, that capacity supplies 2 million tons/year of the following products:

Soy Bran = 1,440,000 tons / year
Soybean Oil = 380,000 tons / year
Soy Lecithin = 19,000 tons / year
Soy Molasses = 36,000 tons / year (by-product)

The protein concentrate is a soy bran with 72% of protein (dry matter), obtained by the extraction of sugars with ethanol 60% from soy bran. The final products are soybean meal with 72% of protein (dry matter) and a by-product nominated soybean molasses (3.000 tons/month).

1.1 OBJECTIVES

1.1.1 Main objective

The main objective of this work was to define an economically viable bioprocess to produce bio-ethanol from soybean molasses at laboratory, pilot and industrial scale.

1.1.2 Secondary objectives

- Optimization of the conditions of acid and enzymatic hydrolysis of soybean molasse
- Comparison between acid and enzymatic hydrolysis of soybean molasses
- Screening of *Saccharomyces cerevisae* and *Zymomonas mobilis* strains to produce bio-ethanol
- Optimization of fermentative process conditions: conversion of the hydrolyzed sugars into ethanol
- Scale-up bioprocess to pilot and industrial plants

2 LITERATURE REVIEW

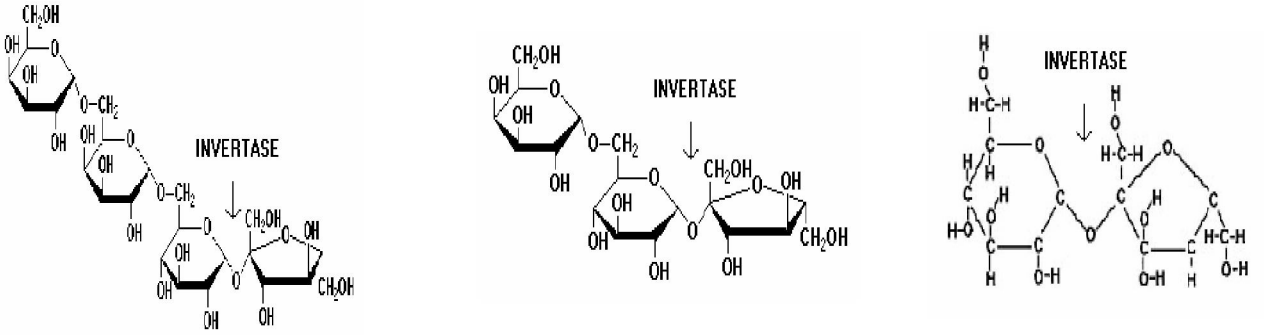
2.1 Soybean molasses

Molasse is a co-product with high concentration of sugars (57% dry weight), nitrogen and other macro and micronutrients, and is currently sold at a low price (US$ 3.00 per ton).

Only sucrose and glucose, among total amount of sugars, are completely converted into ethanol, whereas, 47% of the total sugars in the soy molasse are not fermented by *Saccharomyces cerevisae* or *Zymomonas mobilis.* From stachyose and raffinose, only the terminal fructose unit and terminal glucose unit are consumed, respectively.

Residual sugars are oligomers linked by an alpha-1,6 binding. Since microorganisms do not produce the enzyme alpha-1,6-galactosidase, it is necessary to break these bindings.

The chemical structures of the main sugars in soybean molasses are shown below.



Stachyose **Raffinose** **Sucrose**

Figure 1 - **Structural formula of the predominant sugars in soybean molasses.**

2.2 FLOWCHART: PROTEIN CONCENTRATE OF SOYBEAN

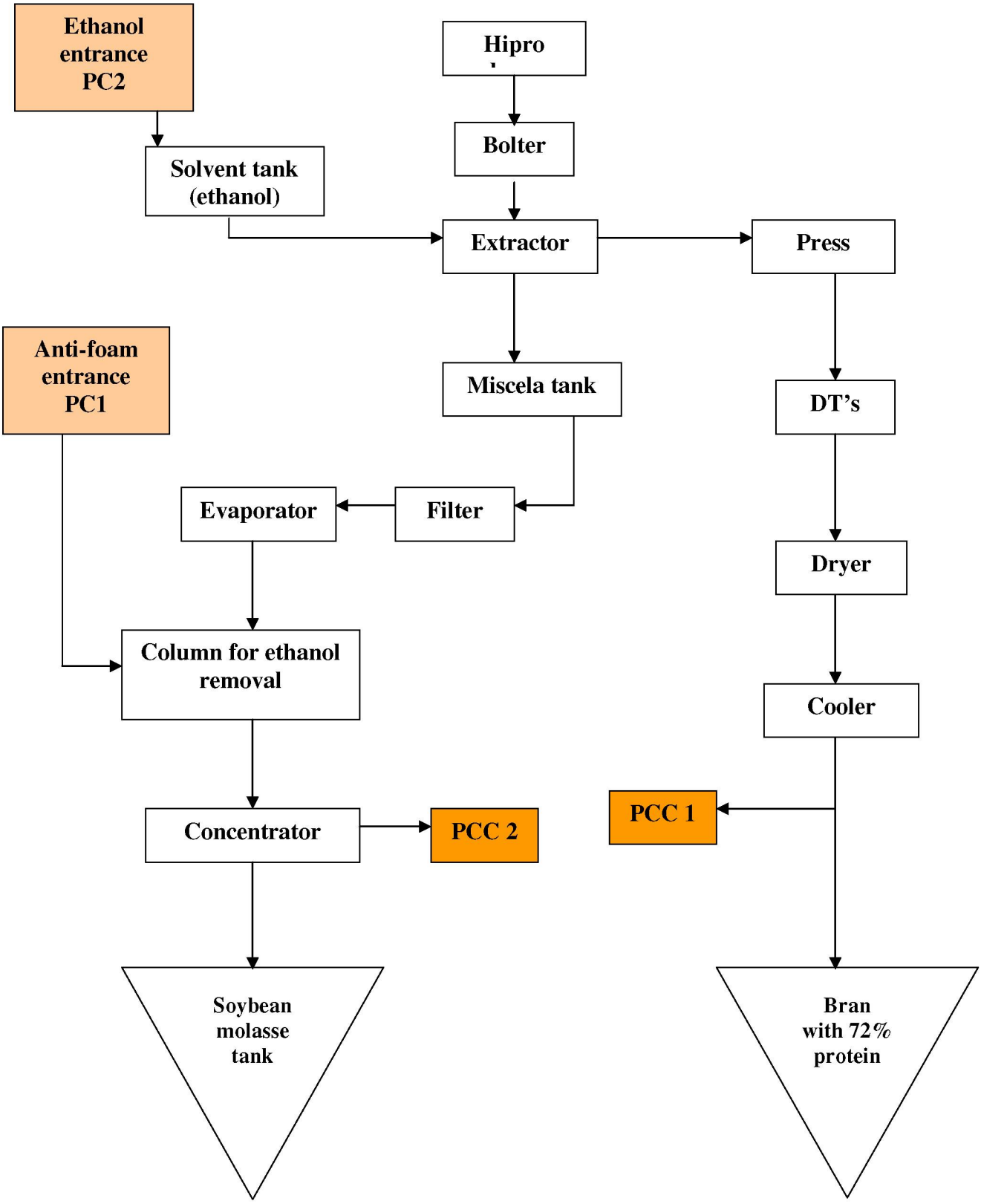


Figure 2 - **Process for production of protein concentrate of Imcopa.**

Imcopa´s project for utilization of bio-ethanol is shown in the following flowchart.

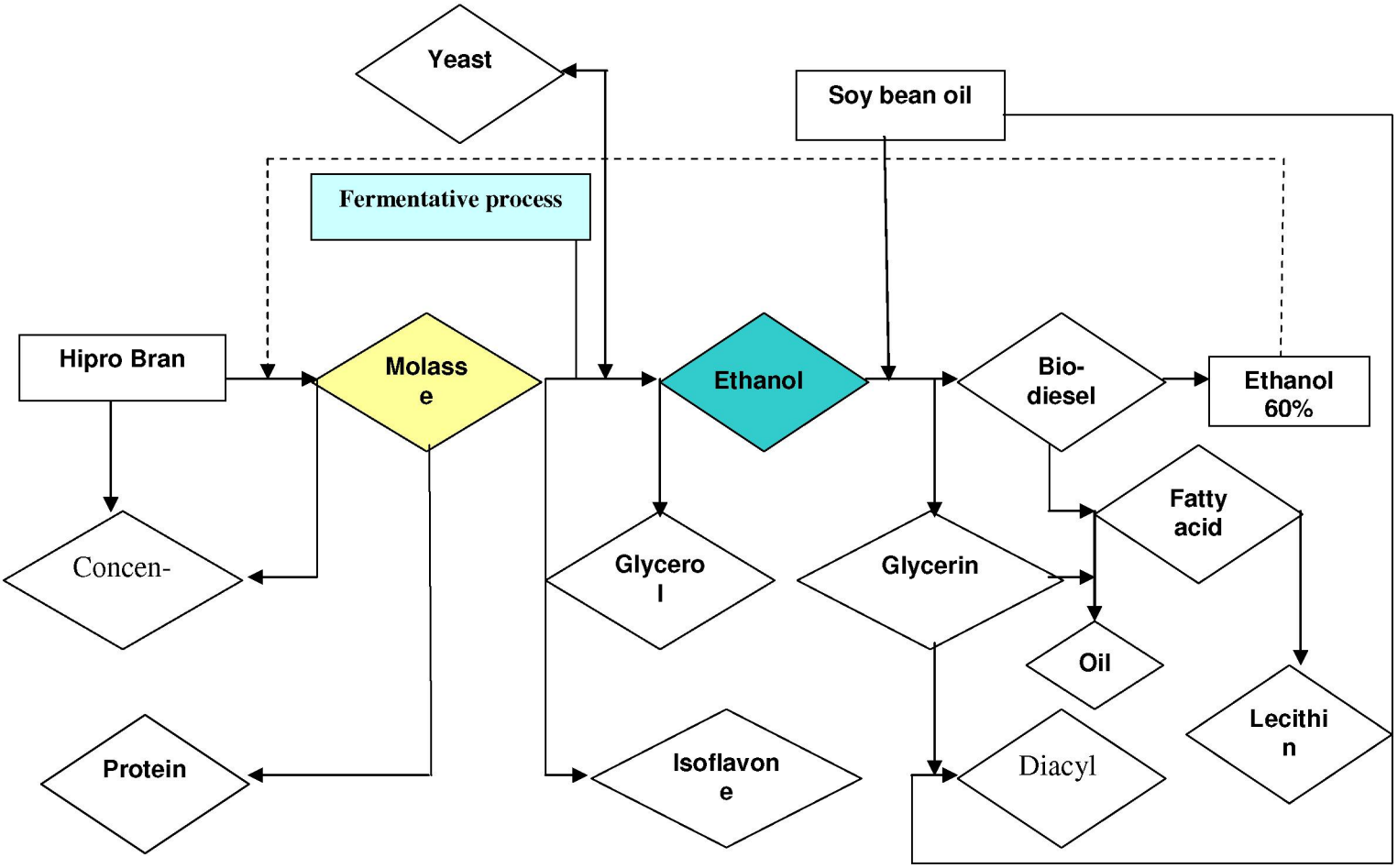


Figure 3 - **Project flowchart for different processes and products of Imcopa.**

2.3 FLOWCHART: GLOBAL MASS BALANCE FROM SOYBEAN TO MOLASSE

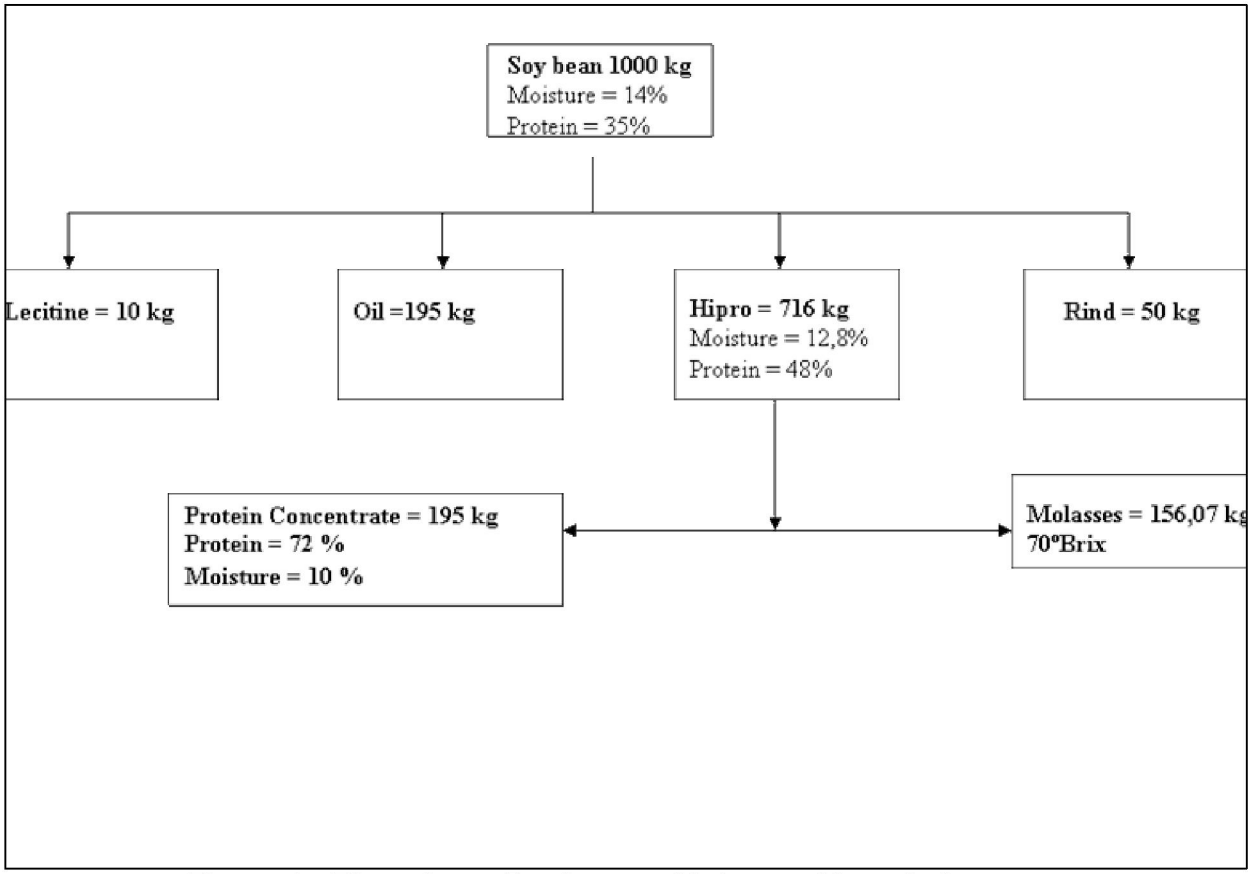


Figure 4 - **Flowchart: Soybean – Molasse Mass balance.**

3 MATERIALS AND METHODS

3.1 SCREENING OF MICROORGANISMS

- *Saccharomyces cerevisae* (commercial name Levasaf)
- *Saccharomyces cerevisae* (commercial name Fermol)
- *Saccharomyces cerevisae* (commercial name Fermol-JP1)
- *Saccharomyces cerevisae* (commercial name Mauri)
- *Saccharomyces cerevisae* sp. (six wild strains from Bioprocess Engineering and Biotechnology Division of UFPR, LPB 1-6)
- *Zymomonas mobilis* (André Tosello Foundation culture collection – São Paulo / Brazil)
- *Zymomonas mobilis* NRRL 806
- *Zymomonas mobilis* NRRL 4286
- *Zymomonas mobilis* NRRL 4491

3.2 ACIDS

The following acids were tested in this work:

- Hydrochloric acid (HCl)
- Sulphuric acid (H_2SO_4)
- Phosphoric acid (H_3PO_4)
- Nitric acid (HNO_3)

3.3 ENZYME

The enzyme chosen for enzymatic hydrolysis was an alpha-1,6-galactosidase named AEO 301 (Prozyn), which breaks the alpha-1,6 bindings from stachyose and raffinose, releasing glucose, fructose and galactose. This enzyme has an optimum activity at pH and temperature of 5.0 and 55°C, respectively.

3.4 SOYBEAN MOLASSE 20-30°BRIX

The composition of the soybean molasse is shown below:

Table 1 – Average composition of soybean molasses.

Component	% dry weight
Total Carbohydrates	57.3
Glucose	0.243
Fructose	0.127
Galactose	0.254
Sucrose	28.4
Lactose	-
Raffinose	9.68
Stachyose	18.6
Proteins	9.44
Lipids	21.2
Fibers	5.7
Ash	6.36

The molasses were received from the company in the concentrated form (75-80ºBrix); for that reason, it was stored at room temperature, being diluted with distilled water to a concentration of 20-30ºBrix for fermentation tests.

3.5 QUANTIFICATION OF BIOMASS

The number of cells per mL was determined either with a Neubauer counting chamber or CFU counting (plate count), and the cell concentration was determined by dry weight analysis. Yeast cells were grown in potato dextrose agar medium (PDA) and *Zymomonas* cells were grown in MRS medium. The viability of yeast cells was determined by methylene-blue staining.

3.6 DETERMINATION OF REDUCING SUGARS

The amount of reducing sugars was determined by the Somogyi-Nelson method.

3.7 CHARACTERIZATION OF FERMENTABLE AND NON FERMENTABLE SUGARS

The fermentable and non fermentable sugars were quantified by HPLC, using a Shodex KS-801 column at flow rate of 1.0 mL/min H_2O milli-Q and temperature of 50°C. The samples were diluted (10 fold) and filtered with cellulose-acetate filters (0.22 □m).

3.8 DETERMINATION OF ETHANOL AND GLYCEROL IN FERMENTED BROTH

Ethanol and glycerol concentrations were measured by HPLC, using the Shodex KS-801 column, in the same conditions mentioned above.

3.9 DETERMINATION OF ACIDITY

The determination of acidity in the broth was made by potentiometric titrimetric analysis, using 20 mL of fermented wine which was diluted in 50 mL of distilled water. This solution was neutralized with NaOH 0.01 N until pH 8.5 (controlled with the potentiometer). The calculus of acidity was made according to the following equation:

Acidity = Volume NaOH used (mL) x 0,245 x correction factor*

* The correction factor is determined by standardization of the NaOH solution with potassium bi-phthalate.

4. PRACTICAL RESULTS AND DISCUSSION

4.1 HYDROLYSIS OF THE MOLASSE ("UP STREAM")

4.1.1 Acid hydrolysis of the molasse

Optimization of hydrolysis conditions (first screening):

The following variables were tested:

Acid concentration – 0.1, 0.5 and 1.0 N
Temperature – 80, 100 and 120ºC
Time – 10, 20 and 30 minutes

Table 2 – Optimized conditions for acid hydrolysis.

Acid	Concentration (N)	Temperature (°C)	Time (min)	Increase in RS* concentration
HCl	1.0	120	30	10.1 x
H_2SO_4	0.5	80	10	12.5 x
H_3PO_4	0.5	120	10	10.6 x
HNO_3	0.5	120	10	14.2 x

*RS = Reducing sugars

The acid hydrolysis using nitric acid showed the best efficiency. However, it spoiled the nutrients of culture medium, and consequently the cells were unable to grow on this hydrolyzed medium.

Characterization of the optimal point to H_2SO_4 (from the conditions optimized above):

The following variables were investigated:

Acid concentration – 0.3, 0.4 and 0.5 N
Temperature – 70, 80 and 90ºC
Time – 5, 10 and 15 minutes

Table 3 – Optimized conditions for acid hydrolysis (H_2SO_4)

Acid	Concentration (N)	Temperature (°C)	Time (min)	Increase in RS* concentration
H_2SO_4	0.5	90	10	21 x

*RS – Reducing sugars

Data were analyzed using the analysis of variance test (ANOVA). Concentration and temperature variables showed to be significantly different at the confidence-level of 95%. Regarding to time, there was no significant difference between 10 and 15 min.

The optimized acid hydrolysis provided a major increase in reducing sugars concentration (21 fold). However, there was no increase in ethanol yield. According

to literature data, high concentrations of sulphuric acid can possibly damage the structure of sugars, so they can not be metabolized by the yeasts and converted into ethanol. Consequently, the hydrolysis was conducted at higher values of pH and temperature in order to maintain the efficiency of the process.

Hydrolysis at a higher pH:

Table 4 – Optimized conditions for acid hydrolysis with higher values of pH.

Acid	pH of the SBM* hydrolysis	Temperature (°C)	Time (min)	Increase in RS# concentration
H_2SO_4	3,5	120	20	20 x

*SBM = soybean molasses, #RS = Reducing sugars

According to the results obtained, it was possible to maintain a good efficiency of hydrolysis at a higher value of pH.

4.1.2 Enzymatic hydrolysis of the molasse

Optimization of hydrolysis conditions using the enzyme Prozyn AEO 301:

In this work the following variables were assayed:

Enzyme concentration – 50; 100; 150; 200, 300, 400… to 900 g of enzyme per ton of soybean molasse at 15°Brix
Concentration of substrate – 10; 15 and 20ºBrix

Table 5 – Results of enzymatic hydrolysis (pH 5.0, 55°C).

Enzyme concentration (g/ton molasse 15°Brix)	Soybean molasses (°Brix)	Time (h)	Increase in RS* concentration
100	10	24	5.69 x
500	20	24	10.7 x
400	10	24	15.9 x

*RS – Reducing sugars

The enzymatic hydrolysis of soybean molasses at 10ºBrix showed the best yield in terms of reducing sugar concentration improvement. Nevertheless, the highest dilution factor was not ideal for economic ethanol fermentation. Thus, the chosen conditions were: 20ºBrix, 500 g of enzyme per ton molasses.

Table 6 – Kinetics of enzymatic hydrolysis (500 g/ton, 20ºBrix, pH 5.0, 55ºC).

Component (g/L)	0h	3h	6h	9h	18h	24h	27h	30h
Stachyose	5.5	0	0	8.2	6.8	0	0	0
Raffinose	46.1	26.9	21.5	15.4	5.6	0	0	0
Sucrose	19.7	10.8	10.3	10.4	1.0	4.0	3.8	0
Disaccharide	11.4	9.9	11.6	14.9	17.2	15.2	14.8	15.3
Glucose	0	5.7	9.5	14.9	22.0	20.9	21.9	21.9
Galactose+ Fructose	4.6	17.5	17.2	30.2	38.1	47.4	42.6	47.1
Total sugars	87.3	70.8	70.1	94.0	90.7	87.5	83.1	84.3
Efficiency (%)	-	41.6	47.5	51.9	63.8	75.9	76.5	75.7

It was observed that the minimum time required was around 24 hours of incubation.

Testing other enzymes: AMG (Novozymes – 55ºC) and Termamyl (Novozymes – 95ºC)

Table 7 – Results of the tests with other enzymes: AMG and Termamyl (500 mL/L, pH 5.5, 24h).

Component (Sugars) (g/L)	Molasse 30ºBrix	AMG	Termamyl
Stachyose	81.0	79.3	89.5
Raffinose	46.5	74.1	56.0
Sucrose	121.8	78.7	137.5
Disaccharide	18.4	18.5	25.5
Glucose	-	16.3	4.6
Galactose+ Fructose	16.2	18.5	25.9
Total sugars	283.9	285.4	339,0
Increase RS*	-	2.15 x	1.88 x

*RS – Reducing sugars

The additional enzymes tested (Termamyl, AMG) did not show a good efficiency in releasing reducing sugars.

4.1.3 Acid hydrolysis of the vinasse with non fermented sugars

Optimization of the hydrolysis conditions (severe hydrolysis to break all the remaining non-reducing sugars):

The following conditions were tested:

Catalyst – HCl and H_2SO_4
Concentration – 0.5 N; 1,0 N
Temperature – 80; 100ºC
Time – 10, 20, 30… to 90 min

Table 8 – Optimized conditions of vinasse acid hydrolysis

Acid	Concentration (N)	Temperature (°C)	Time (min)	Conversion in RS*
HCl	1	100	60	91%
H_2SO_4	1	100	60	50%

*RS – Reducing sugars

Table 9 – Global balance of hydrolysis focusing the increase in ethanol yield from molasses and vinasse.

Type of hydrolysis	Increase in ethanol yield
Acid hydrolysis of the molasses	0
Acid hydrolysis of the molasses with low acid concentration (higher value of pH)	0
Enzymatic hydrolysis of the molasses	20%
Acid hydrolysis of the vinasse	17%

The acid hydrolysis of the molasse did not provide an increase in ethanol yield, because the broken bindings (□-1,2) were the same hydrolysed by the enzyme invertase, which is produced by the yeast. Only enzymatic hydrolysis and severe acid hydrolysis were capable of breaking the □-1,6 bindings, releasing monossacharide units that were not available before (see Conclusions on page 45).

4.2 SELECTION OF STRAINS

4.2.1 *Zymomonas mobilis* – four strains

A - NRRL 4286
B - B 806
C - B 4491
D - FAT (André Tosello Foundation culture collection)

Fermentation conditions of screening: Soybean molasses at 15ºBrix, temperature of 30ºC, fermentations in shaker at 120 rpm (4h, half-filled flask) and static (18h, full flask). Inoculum was previously adapted in soybean molasses.

Table 10 – Biomass production of *Zymomonas mobilis* in soybean molasses.

Sample (strain)	Initial biomass (CFU/mL)	Final biomass (CFU/mL)
A	$2{,}78.10^{7}$	9.10^{7}
B	7.10^{7}	1.10^{8}
C	3.10^{7}	4.10^{7}
D	$2{,}1.10^{8}$	$1.7.10^{12}$

Table 11 – Ethanol production using different strains of *Zymomonas mobilis*.

Sample (strain)	Total sugars (g/L)	Glycerol (g/L)	Ethanol (g/L)	Yield over total sugars (%)	Yield kg abs. et./ton (%)*
A.0h	129.6	2.7	2,6	-	-
A.18h	109.1	2.4	2,5	0	0
B.0h	99.5	2.3	3,7	-	-
B.18h	89.0	4.3	11.1	14.6	8.55
C.0h	115.0	2.7	1.6	-	-
C.18h	114.1	2.7	2.6	1.7	2.00
D.0h	60.6	2.1	8.5	-	-
D.18h	40.6	2.3	18.7	32.9	14.4

*Yield in kg of absolute ethanol (100%) per ton of dry soybean molasses

The *Zymomonas mobilis* strains tested in this work did not reach satisfactory yields in ethanol from soybean molasses; consequently, their use was discarded.

4.2.2 *Saccharomyces* sp. – six wild strains from the culture collection of the Bioprocess Engineering and Biotechnology Division / UFPR

Fermentation conditions: Molasses at 30ºBrix, temperature of 30ºC, in shaker at 100 rpm, 24h. Inoculum previously adapted in molasses.

Table 12 – Ethanol production from different *Saccharomyces* sp. (wild strains).

Sample	Total sugars (g/L)	Glycerol (g/L)	Ethanol (g/L)	Yield over total sugars (%)	Yield kg abs. et./ton (%)*
LPB 1.0h	219.9	-	5.0	-	-
LPB 1.24h	135.2	6.3	43.9	34.6	15.7
LPB 2.0h	232.1	-	7.7	-	-
LPB 2.24h	130.6	6.9	45.2	31.6	16.1
LPB 3.0h	220.3	-	6.6	-	-
LPB 3.24h	131.5	5.6	43.6	32.9	15.5
LPB 4.0h	225.0	-	4.9	-	-
LPB 4.24h	137.6	7.2	47.8	37.3	17.0
LPB 5.0h	214.7	-	5.9	-	-
LPB 5.24h	130.2	6.0	42.2	33.1	15.1
LPB 6.0h	234.5	-	7.1	-	-
LPB 6.24h	124.9	5.0	42.2	29.3	15.1

*Yield in kg of absolute ethanol (100%) per ton of dry soybean molasses.

Saccharomyces sp. strain number four (LPB 4) showed the best result among the wild strains tested. In 24h of fermentation, this strain achieved a yield of 37.3% over the initial sugars present in molasse.

4.2.3 *Saccharomyces cerevisae* – four commercial strains

A – Levasaf
B – Mauri
C – JP1
D – Fermol

Fermentation conditions: Molasses at 30ºBrix, temperature of 30ºC, fermentation in shaker at 100 rpm, 24h. Inoculum previously adapted in molasse.

Table 13 – Ethanol production from *Saccharomyces cerevisae* (commercial strains).

Sample	Total sugars (g/L)	Glycerol (g/L)	Ethanol (g/L)	Yield over total sugars (%)	Yield kg abs. et./ton (%)*
A.0h	209.2	-	2.4	-	-
A.6h	248.2	-	8.2	5.4	-
A.24h	143.1	5.1	46.8	41.5	16.6
B.0h	222.9	-	5.4	-	-
B.6h	191.2	-	17.8	10.9	-
B.24h	145.0	4.6	36.0	26.9	12.9
C.0h	233.4	-	5.6	-	-
C.6h	216.6	-	7.0	1.2	-
C.24h	134.5	5.3	37.6	26.8	15.5
D.0h	219.1	-	5.1	-	-
D.6h	225.6	-	6.6	1.3	-
D.24h	127.9	5.6	35.4	27.0	16.5

*Yield in kg of absolute ethanol (100%) per ton of dry soybean molasses

The best results using commercial strains of *Saccharomyces cerevisae* were obtained with strain A (Levasaf) and D (Fermol).

An additional experiment (table 4.2.5) aimed to compare galactose and fructose consumption in an enriched medium with these sugars, testing the best commercial strains (Levasaf, Fermol and JP1, which is a strain capable of metabolizing galactose to produce ethanol). The medium was enriched with commercial sugars.

Table 14– Ethanol production from commercial strains to test their capacity of galactose and fructose consumption.

Strain		Total sugar (g/L)	Ethanol (g/L)	Yield* (%)
Initial (medium)		97.8	1.8	-
Levasaf	19h	60.8	19.3	35.0
	29h	35.1	25.1	46.6
Fermol	19h	85.1	7.8	12.0
	29h	49.4	19.5	35.4
JP1	19h	60.1	17.7	31.8
	29h	39.4	32.0	60.4

*yield over initial sugar

The different commercial strains were able to convert galactose and fructose into ethanol; however, they spent more time for fermentation. The commercial strain JP1 showed the best yield in ethanol production. Nevertheless, galactose and fructose conversion in fresh molasses was not efficient, even with a longer fermentation time.

The commercial strain A (Levasaf) was selected (among the other strains tested) for the development of this work, because this strain does not require inoculum preparation in sterile conditions (which is necessary for the wild strains) and it is also less expensive (6 fold cheaper) than the other commercial strain (Fermol). Another important parameter was the strain capacity of consuming galactose and fructose. These sugars are present in important amounts in soybean molasse and their assimilation is more difficult than glucose consumption.

4.3 OPTIMIZATION OF FERMENTATIVE CONDITIONS IN LABORATORY SCALE

4.3.1 Addition of nutrients to the medium

A – molasse 15ºBrix
B – molasse 17ºBrix
C – molasse 20ºBrix
1 – addition of magnesium ($MgSO_4$)
2 – addition of nitrogen (NH_4NO_3)
3 – addition of magnesium and nitrogen

Fermentation conditions: Inoculum 10 g/L of yeast (Levasaf), temperature of 30ºC, in shaker at 120 rpm (12h, half-filled flask), then static (8h, full flask).

Table 15 – Ethanol production and process yield.

Sample	Total sugars (g/L)	Glycerol (g/L)	Ethanol (g/L)	Yield (%)	Yield kg abs. et./ton (%)*
A.0h	104.1	1.8	1.5	-	-
A.8h	48.9	2.9	26.1	46.2	15.7
A1.8h	46.7	2.1	24.5	43.2	14.7
A2.8h	44.2	5.6	22.0	38.5	13.1
A3.8h	61.7	3.6	29.8	53.2	18.1
B.0h	143.5	2.3	1.5	-	-
B.8h	70.4	3.8	33.8	44.0	18.0
C.0h	151.9	5.1	1.9	-	-
C.8h	65.2	5.4	37.8	46.3	16.7
C1.8h	70.2	5.6	31.2	37.7	13.7
C2.8h	75.2	3.5	34.6	42.1	15.3
C3.8h	75.8	5.6	35	42.6	15.4

*Yield in kg of absolute ethanol (100%) per ton of dry soybean molasses

The results present in table 4.3.1 showed that the addition of nutrients in the medium did not improve ethanol production and yield of process.

4.3.2 Effect of soluble solids concentration in ethanol production from soybean molasse

A – 20ºBrix
B – 22ºBrix
C – 24ºBrix
D – 34ºBrix

Fermentation conditions: Inoculum 10 g/L of yeast (Levasaf), temperature of 30ºC, shaker 100 rpm, 15h.

Table 16 – Ethanol production and yield over total sugars.

Sample	Total sugars (g/L)	Glycerol (g/L)	Ethanol (g/L)	Yield (%)	Yield kg abs. et./ton (%)[#]
A.0h	169.5	3.1	-	-	
A.15h	87.4	6.0	38.0	43.9	17.7
A.15h*	93.4	7.3	34.9	40.3	16.3
B.0h	196.9	2.9	-	-	
B.15h	101.4	7.7	38.2	38.0	16.1
B.15h*	98.5	6.8	43.9	43.6	18.5
C.0h	207.3	2.2	1.2	-	
C.15h	108.2	6.9	46.6	44.0	17.4
C.15h*	107.8	6.6	43.6	41.2	16.3
D.0h	311.6	2.4	-	-	
D.15h	152.3	10.2	63.5	39.9	16.6
D.15h*	151.3	9.6	59.2	37.2	15.5

*duplicate
[#]Yield in kg of absolute ethanol (100%) per ton of dry soybean molasses

Yields obtained for different concentrations of molasses were practically the same, with a small decrease for 34ºBrix. The concentration of 30ºBrix was chosen, in order to provide a minimum concentration of ethanol in the fermented broth (8ºGL).

4.3.3 Effect of initial pH

A – No adjustment
B – pH adjusted to 5.0
C – pH adjusted to 4.3 without previous separation of proteins
D – pH adjusted to 4.3 without proteins (separated by centrifugation)

Fermentation conditions: Molasses at 20ºBrix, temperature of 30ºC, inoculum 30 g/L of yeast (Levasaf), shaker 100 rpm, 6h.

Separation of proteins: pH 4.3 adjusted with H_2SO_4 10 N (isoelectric point of the proteins), 4.000 rpm, 30 min.

Table 17 – Ethanol production over total sugars.

Sample	Total sugars (g/L)	Glycerol (g/L)	Ethanol (g/L)	Yield (%)	Yield kg abs. et./ton (%)
A.0h	113.2	0	0.9	-	-
A.6h	64.5	2.6	30.5	51.17	14.2
A'.6h	54.1	3.5	27.8	46.50	13.0
B.0h	113.5	0	0	-	-
B.6h	52.9	1.5	28.3	48.79	13.2
B'.6h	50.8	1.6	29.0	50.00	13.5
C.0h	122.8	0	0	-	-
C.6h	60.0	2.3	28.9	46.05	13.5
C'.6h	71.9	4.3	26.3	41.91	12.3
D.0h	123.6	0	1.2	-	-
D.6h	48.1	2.9	26.7	40.37	12.1
D'.6h	52.4	1.3	30.8	46.87	14.0

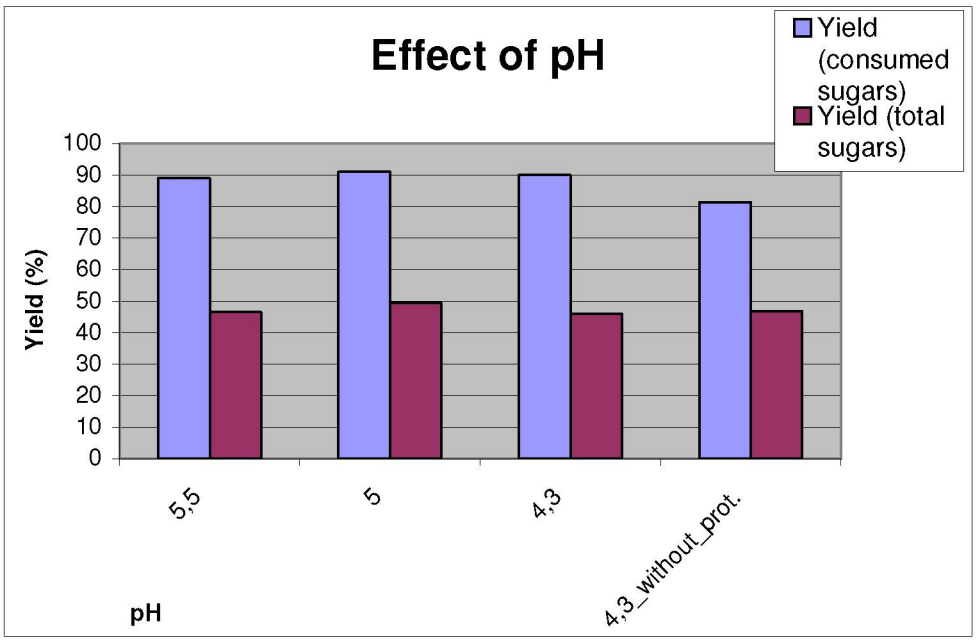


Figure 5 - pH *versus* yield.

There was no significant difference among the initial pH tested (4.3 – 5.5) in relation to ethanol produced in the first fermentation cycle. Therefore, it is possible that the influence of pH becomes more significant after many cycles, when contamination problems and changes in the metabolic pathway may occur.

4.3.4 Effect of preliminary protein separation

The centrifugation of soybean molasse at the isoelectric point of the proteins (pH 4.3) was considered as a possibility of recovering the proteins before fermentation. In this condition, the proteins should precipitate and the sugars should remain at the supernatant.

Two centrifugation conditions were evaluated:

1 – Molasses 20ºBrix, pH adjusted to 4.3 with H_2SO_4, 5.000 rpm, 20 min.
2 – Molasses 25ºBrix, pH adjusted to 4.3 with H_2SO_4, 20.000 rpm, continuous.

The concentration of proteins through precipitation was not efficient (from 9,44% in the molasse to 10,9% in the precipitate – condition 1, and from 8,66% to 12,7% – condition 2, with percentages in dry weight). Besides, centrifugation caused the precipitation of some sugars, which represented a loss of around 3.0% in ethanol yield per ton of dry molasse. Therefore, the separation of proteins before fermentation was discarded, at least in the conditions mentioned above.

4.3.5 Determination of optimal fermentation time

The objective of this experiment was to determine the optimal time necessary to conclude one batch. Fermentation kinetics was monitored by the difference in weight of the system (using an Erlenmeyer flask coupled with a gas outlet). The difference in weight is caused by CO_2 production and release. Different conditions were applied: 20 and 30ºBrix, pH 3.5, 4.5 and 5.5. The samples were inoculated with 10 g/L of yeast (Levasaf), and the temperature of 30ºC was controlled in a shaker (100 rpm).

The results are shown in graphics below. Individual values are listed in tables (Appendix 3).

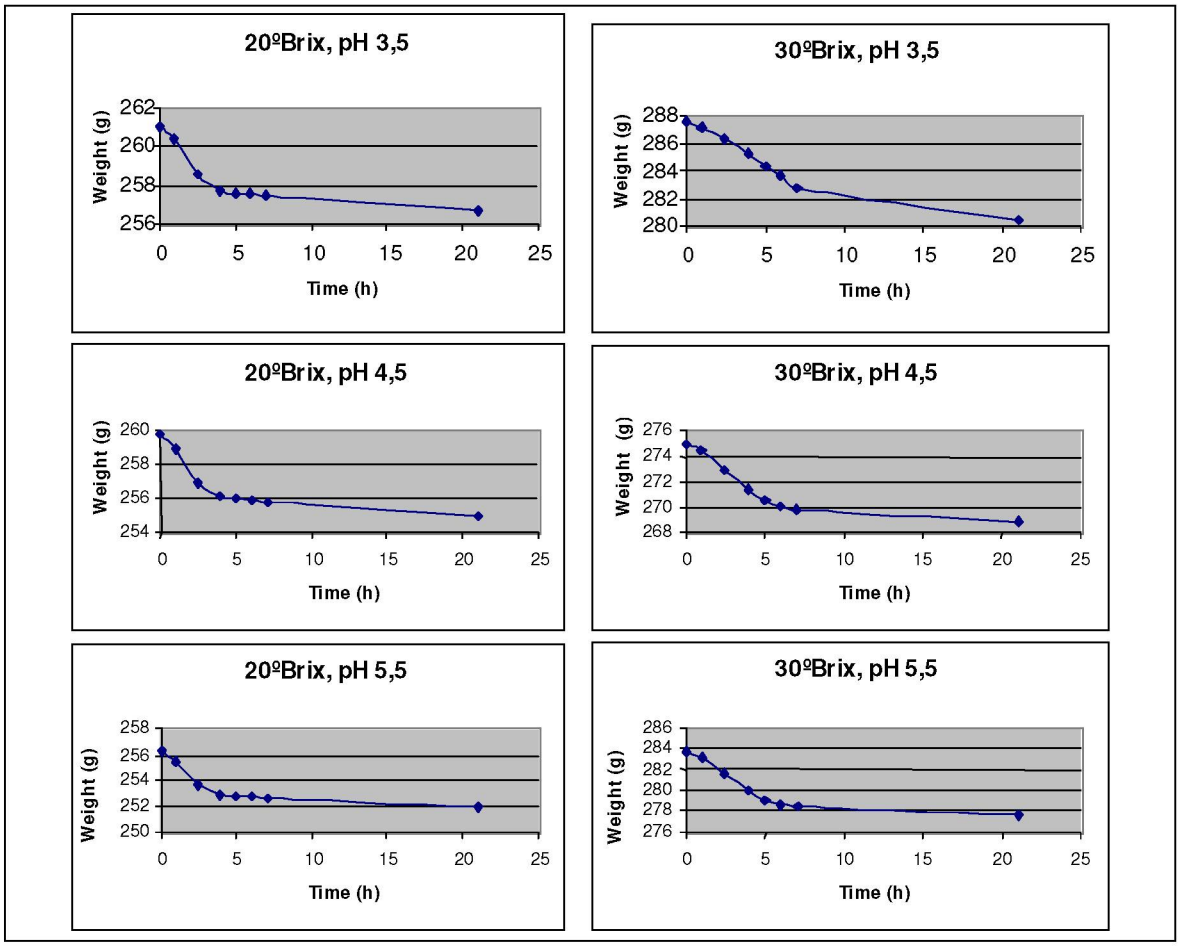


Figure 6 - Weight variation in the system at different conditions: 20/30°Brix; pH 3.5, 4.5 and 5.5.

The fermentations at 20ºBrix and 30ºBrix were finished after around 5 and 7 hours, respectively. A turbulent stage (intense release of CO_2) was noticed during the fermentation. After this stage, ethanol could be produced.

Regarding to the different values of pH tested, it was not found a relevant influence over the time of fermentation.

4.3.6 Effect of biomass recycling

An industrial process for ethanol fermentation would not be profitable if new inoculum (commercial yeast) had to be added at the beginning of each fermentation. The common practice is to use the biomass produced in one preliminary fermentation as inoculum for the subsequent fermentations. The biomass is separated from the fermented broth by centrifugation, treated with acid (H_2SO_4, pH 2.5, 2h), in order to destroy the "weak" cells, and then it can be used as inoculum. Sometimes, high aeration rates are necessary to multiply this biomass, and eventually, the addition of a nitrogen source is required. Subsequently, the inoculum is transferred to the fermentation tanks, which are then fed with molasse. The process is carried out with no aeration and slow agitation.

The biomass recirculation was tested in different conditions:

- Different concentrations of soluble solids (20 and 30ºBrix)
- Different values of pH (4.3 and 5.5 – not adjusted)
- Pre-treatment of the molasses (previous separation of proteins, sterilization)

The results are shown in the following figures. The tables with all the results are listed in Appendix 3.

The first experiment was performed with molasses at 20ºBrix. The previous removal of proteins and the sterilization of the medium were investigated.

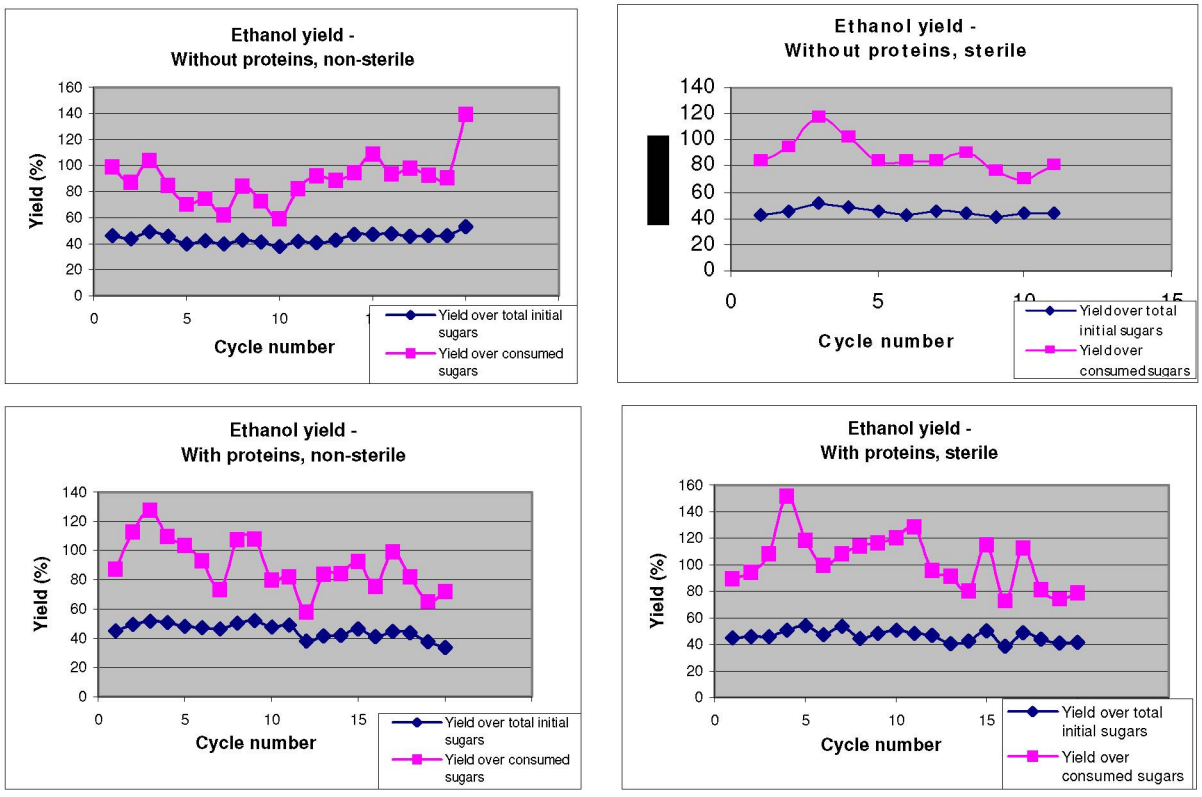


Figure 7- Yields obtained after 20 cycles of fermentation.

The fermentations conducted with non sterilized molasses and without proteins separation (most economic condition) showed a loss of around 5% in ethanol yield from the 12th cycle on. In the other three conditions, the yield remained constant, yet the cell viability decreased. An increase in acids production, which is indicative of bacterial contamination, was noticed in all four conditions studied. The presence of proteins in the medium did not affect the yield of the fermentation, but the separation of the biomass at the end of the fermentation was easier in the absence of proteins.

A second experiment was carried out with soybean molasses at 30ºBrix, without previous removal of proteins, pH adjustment or sterilization of the medium. Nutrient solution and antibiotic (Kamoran HJ) were added from the 15th cycle on.

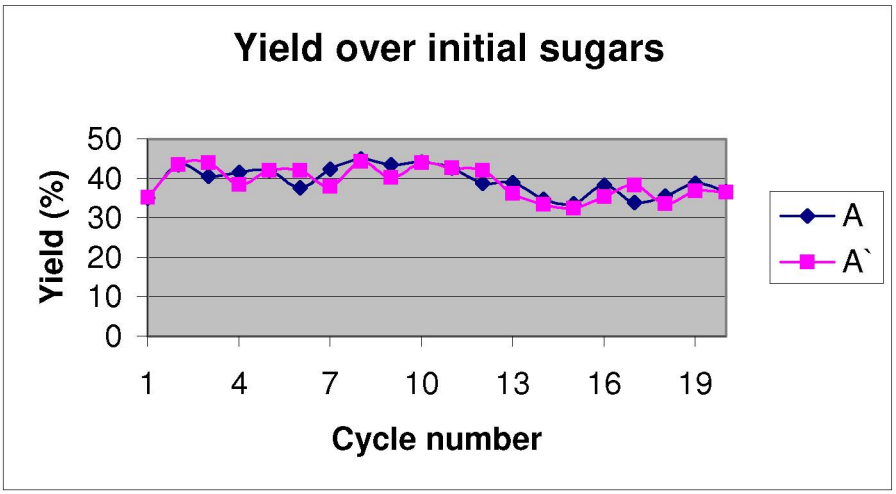


Figure 8 - Practical yields in ethanol throughout 20 cycles (A' is the duplicate experiment).

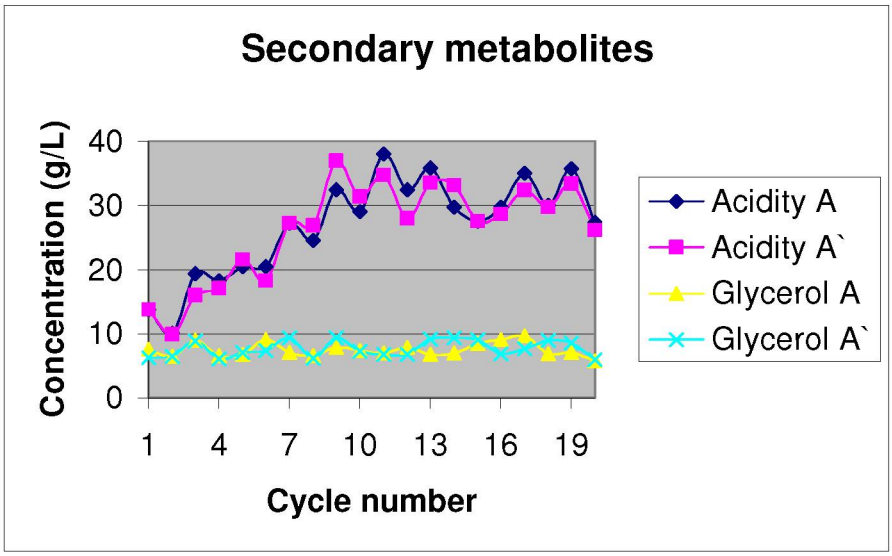


Figure 9 -Production of secondary metabolites throughout 20 cycles.

A significant drop in fermentation yield was observed from the 12th cycle on, as well as an increase in acids production. The addition of nutrient solution and antibiotic did not show a positive effect in cell viability and ethanol yield.

During the accomplishment of the biomass recirculation experiment, a problem which was capable of reducing significantly the ethanol yield of the fermentation was identified: the contamination with bacteria.

The yeasts are naturally selected throughout the fermentation cycles becoming adapted to the medium. The same condition may occur with some opportunistic bacteria.

The sterilization of the substrate (121ºC, 20 min) did not solve the problem, as concluded from the experiment described above, since bacteria was growing in the inoculum which could not be sterilized. Therefore, different strategies were applied in order to control bacterial growth.

4.3.7 Inhibitory study of bacterial contamination in ethanol fermentation

In order to inhibit the growth of bacteria during fermentation, two strategies were assayed: fermentation at lower pH (4.3) and use of antibiotic (Kamoran HJ, 3 ppm). The reduction of pH showed no effect on controlling contamination. The contaminants might be lactic acid bacteria, which are acid-tolerant. The antibiotic was also ineffective, and showed a negative effect in yeast cell viability.

Table 18 - Fermentation with antibiotic: chromatographic results.

Samples	Total sugar (g/L)	Glycerol (g/L)	Ethanol (g/L)	Yield (%)	
				Over consumed sugar	Over total sugar
0h	260.1	6.1	12.0	-	-
2h	228.2	6.6	12.9	5.5	0.7
4h	207.0	6.4	23.0	40.5	8.3
6h	172.9	6.5	32.3	45.6	15.3
9h	164.4	8.6	40.9	66.0	21.7
12h	152.0	5.9	36.0	43.4	18.1
24h	142.5	6.8	30.5	30.8	13.9

Table 19 - Fermentation without antibiotic: chromatographic results.

Samples	Total sugar (g/L)	Glycerol (g/L)	Ethanol (g/L)	Yield (%)	
				Over consumed sugar	Over total sugar
0h	226.9	4.1	9.3	-	-
2h	221.9	6.6	11.1	70.3	1.6
4h	190.5	5.5	17.8	45.7	7.3
6h	174.2	6.3	25.3	59.4	13.8
8h	160.0	5.7	35.3	76.0	22.4
10h	159.7	6.4	29.3	58.2	17.2
12h	159.5	5.9	38.8	85.7	25.4
24h	143.2	8.5	46.4	86.7	32.0

Table 20 - Fermentation with pH 4.3: chromatographic results.

Samples	Total sugar (g/L)	Glycerol (g/L)	Ethanol (g/L)	Yield (%)	
				Over consumed sugar	Over total sugar
0h	220.8	3.8	8.7	-	-
4h	186.3	4.8	15.8	40.3	6.3
6h	181.5	5.7	23.2	91.8	12.9
8h	165.8	6.5	30.2	76.5	19.1
10h	159.2	6.4	33.4	78.5	21.9
12h	157.1	5.6	33.2	65.6	21.7
24h	139.4	5.3	39.2	85.0	27.0

As it can be seen in the results shown above, the yield over initial sugars was higher when the molasse was fermented without antibiotic and pH adjustment. However, this value, 32%, is lower than the maximum value obtained with non-recycled inoculum (46,5%). As it is shown in the figure below, there was an expressive contamination with bacteria, which probably caused this reduction in fermentation yield.

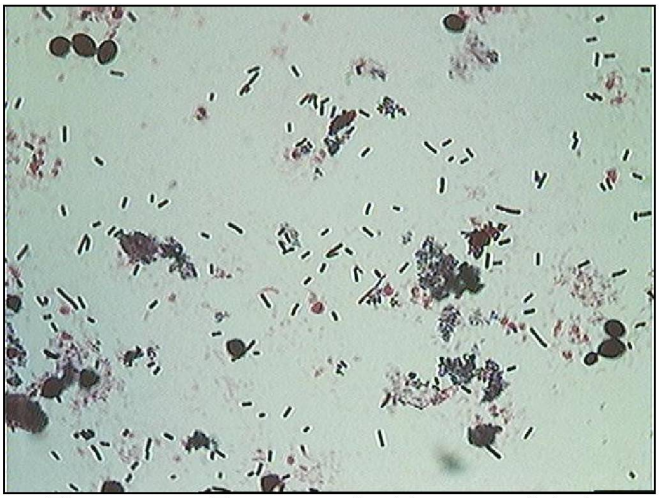


Figure 10 - Fermented without antibiotic.

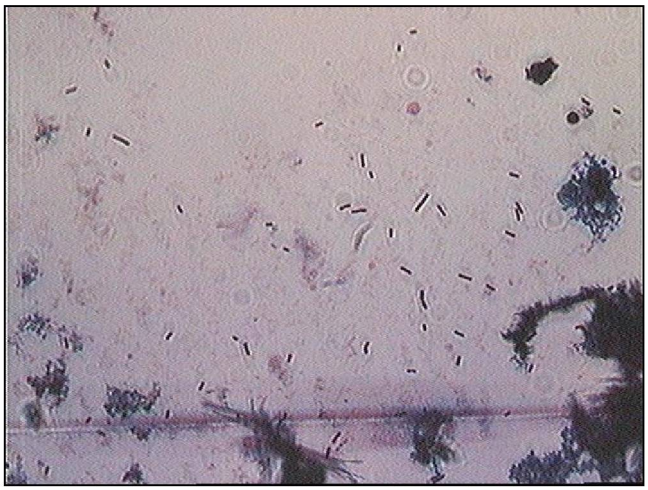


Figure 11 - Fermented with antibiotic.

Figures 4.3.6 and 4.3.7 are pictures of Gram stained samples of fermented broth. The contaminants are Gram-positive rods, which might be latic acid bacteria. The addition of antibiotic inhibited the bacterial proliferation, however, the contamination persisted, at least in the concentration applied. An increase in antibiotic concentration or the choice of other antibiotics should solve this problem, since it does not affect the yeast cell viability.

4.4 DETERMINATION OF KINETIC PARAMETERS

The objective of this study was to define the main kinetic parameters of the soybean molasses fermentation to produce ethanol. The following variables were considered:

- Theoretical yield
- Oxygen demand
- Aeration time
- Air volume per medium volume
- C/N relation and control at different process stages
- Specific Growth Rate of the yeast in this medium
- Specific Substrate Consumption Rate
- Specific Product Formation Rate

Definition of terms:

Table 21 – Definition of cell culture yields.

$Y_{X/S}$	Mass or moles of biomass produced per unit (mass or mol) of consumed substrate.
$Y_{P/S}$	Mass or moles of formed product per unit (mass or mol) of consumed substrate.
$Y_{P/X}$	Mass or moles of formed product per unit (mass or mol) of produced biomass.
RQ	Moles of formed carbon dioxide per mol of consumed oxygen. This yield is called respirometric coefficient.
r_S	Substrate consumption rate (g/L.h)
Q_S	Specific substrate consumption rate (h^{-1})
r_P	Product formation rate (g/L.h)
Q_P	Specific product formation rate (h^{-1})

4.4.1 Cell growth kinetics

The variation of cell growth rates depend on the growth stage. During the lag stage, immediately after the inoculation, the growth rate is practically inexistent. Cells use the lag stage to adapt themselves at the new medium, producing new enzymes or structural components. After that, cell growth starts in the acceleration stage and continues during the growth and decline stages. Whether the growth is exponential, the growth stage (log phase) appears like a straight line. As the medium nutrients are consumed or inhibiting products are accumulated, the growth decreases and the decline stage begin. After this transition period, the stationary stage is reached, where there is no cell growth. Some strains present the cell death stage, where the cells lose viability or suffer lyses.

During the growth and decline stages, the cell growth rate is expressed by:

$$r_X = dX/dt = \mu X \quad \text{(eq. 4.4.1)}$$

where r_x is the volumetric rate of biomass production, X is the viable cells concentration, μ is the specific growth rate.

In a closed system, where growth is the only factor that affects cell concentration, the integration of the equation 4.4.1 provides a new equation for X in function of time. Whether the specific growth rate is constant, what generally occurs during the exponential stage, we have the following equation:

$$X = X_0 e^{\mu t} \quad \text{(eq. 4.4.2)}$$

Applying natural logarithms:

$$\ln X = \ln X_0 + \mu t \quad \text{(eq. 4.4.3)}$$

When a graphic of ln X *versus* time is plotted, a straight line is obtained, and the slope or angular coefficient represents the specific growth rate.

Cell growth rates, generally, are expressed in terms of cell duplication time (T_d), as the following equation:

$$T_d = \frac{\ln 2}{\mu} \quad \text{(eq. 4.4.4)}$$

4.4.2 Inoculum production – adapted yeasts

The yeasts used in the experiments of fermentative parameters determination were previously adapted to soybean molasses, in order to obtain better results when scaling up the process.

The commercial yeast (Levasaf) was inoculated in a dilute medium (molasse 5°Brix, 50 mL) and incubated for 24h. Then, this broth was transferred to a medium with higher concentration of solids (15°Brix, 500 mL) and incubated for 24h. This pre-inoculum was transferred to the fermentor and the volume was completed to 2 L with molasse 30°Brix. The cells were activated with high agitation and aeration rate (200 rpm, 2 VVM) for 12h, until they reached the end of the exponential stage. Then, the fermentor was filled with more 4 L of molasse 30° Brix for fermentation (no aeration, slow agitation – 70 rpm). The biomass produced in one fermentation was decanted (4°C, 24h), treated with acid (H_2SO_4 pH 2.5, 2h) and used as inoculum for subsequent fermentations.

In an industrial scale, the biomass produced in one batch is reactivated and used in the next batch. Thus, in a real fermentation process, yeasts which are more adapted to the substrate are naturally selected.

(A) (B) (C)

Figure 12. (A) Aeration stage (B) Fermentation stage (C) Decanted biomass, excessive flocculation indicates contamination problems.

4.4.3 Oxygen demand and theoretical yields

The mass balance used to calculate the theoretical oxygen demand was performed considering glucose as main carbon source, applied in the first calculation, and sucrose in a second calculation. In this study, ethanol production was not taken into account, once the equilibrium was dislocated to biomass production.

Glucose as carbon source:

$$C_6H_{12}O_6 + aO_2 + bNH_3 \rightarrow cCH_{1,8}O_{0,5}N_{0,2} + dCO_2 + eH_2O \quad (1)$$

$$C: 6 = c + d \quad (2)$$

$$H: 12 + 3b = 1{,}8c + 2e \quad (3)$$

$$O: 6 + 2a = 0{,}5c + 2d + e \quad (4)$$

$$N: b = 0{,}2c \quad (5)$$

- d/a = 1,06 (respirometric coefficient or RQ of *S. cerevisae,* retrieved from literature)

$$d = 1.06a$$

- Solving the equation:

$$C_6H_{12}O_6 + 2.6549.O_2 + 0.6372.NH_3 \rightarrow 3.1858.CH_{1,8}O_{0,5}N_{0,2} + 2.8142.CO_2 + 4.0885.H_2O$$

- Therefore, each mol of consumed glucose requires 2.65 moles of oxygen. Or, for each 180 g of glucose, it is necessary 59.5 L of O_2 (283.2 L of air, 21% O_2).

- Theoretical biomass yield:

Taking as molecular mass:

C = 12gmol
H = 1gmol
O = 16gmol
N = 14gmol

1mol glucose → 3.1858 mol biomass
180g glucose → 78.3707g biomass

- $Y_{X/S\ Max}$ = (X/S) * 100, where $Y_{X/S\ Max}$ is the maximum theoretical yield, X is the biomass and S is the substrate.

$$Y_{X/S\ Max} = 43.54\%$$

- It means that with 180g of glucose, it would be possible to achieve 78.3707g of biomass at most.

Sucrose as carbon source:

$$C_{12}H_{22}O_{11} + aO_2 + bNH_3 \rightarrow cCH_{1,8}O_{0,5}N_{0,2} + dCO_2 + eH_2O \quad (1)$$

$$C: 12 = c + d \quad (2)$$
$$H: 22 + 3b = 1{,}8c + 2e \quad (3)$$
$$O: 11 + 2a = 0{,}5c + 2d + e \quad (4)$$
$$N: b = 0{,}2c \quad (5)$$

- d/a = 1.06 (respirometric coefficient RQ of *S. cerevisae* retrieved from literature)

$$d = 1.06a$$

- Solving the equation:

$$C_6H_{12}O_6 + 5.3097.O_2 + 1.2743.NH_3 \rightarrow 6.3716.CH_{1,8}O_{0,5}N_{0,2} + 5.6283.CO_2 + 7.1770.H_2O$$

- Therefore, each mol of consumed sucrose requires 5.31 moles of oxygen. Or, for each 342 g of sucrose, it would be necessary 118.9 L of O_2 (566.4 L of air, 21% O_2).

- Theoretical biomass yield:

Taking as molecular mass:

C = 12gmol
H = 1gmol
O = 16gmol
N = 14gmol

1mol glucose → 6.3716 mol biomass
342g glucose → 156.7414g biomass

- $Y_{X/S\ Max}$ = (X/S) * 100, where $Y_{X/S\ Max}$ is the theoretical maximum yield, X is the biomass and S is the substrate.

$$Y_{X/S\ Max} = 45.83\%$$

- It means that with 342g of sucrose, it would be possible to obtain 156.7414g of biomass at most.

4.4.4 Determination of specific growth rate

The specific growth rate during the exponential stage is the same as the maximum specific growth rate when all the nutrients in the medium are in excess, which is a characteristic of metabolism.

In order to determine the specific growth rate of the yeast, the parameters of cell concentration *versus* time were plotted in a graphic. Data were obtained in different cultivation conditions. A straight line is obtained by applying the natural logarithm of X (cell concentration) *versus* time and the slope or angular coefficient of the line corresponds to the specific growth rate.

4.4.5 Aeration rate: air volume per medium volume

Fermentations were carried out with different aeration rates to verify experimentally the need of oxygen supply to the cell growth.

The adapted biomass which was stored at the refrigerator (4ºC) presented 6 hours of lag stage, and more 6 hours of log stage.

Table 22 – Aeration rates variation.

Condition	Specific growth rate	Biomass increase after 12 h	Duplication time (hours)
0,5 VVM	0.2767	14.04 x	2.50
1 VVM	0.3418	12.93 x	2.03
2 VVM	0.403	18.47 x	1.72
3 VVM	0.2966	17.63 x	2.34

The best results were obtained with an aeration rate of 2 VVM.

4.4.6 Carbon/Nitrogen ratio

The assimilated carbon content present in the soybean molasses was calculated over metabolized sugar composition (glucose and fructose).

1 g hexose → 0.4 g carbon

The organic nitrogen content was determined over the protein content of soybean molasses.

1 g nitrogen → 6.25 g protein

Results:

Percentual average of metabolized sugars (hexoses): 33.5
Percentual protein content: 9.44

33.5 g hexoses → 13.4 g carbon
9.44 g protein → 1.51 g nitrogen

C/N = 8.87

The ideal C/N ratio for biomass production was considered to be around 10. Whether all the organic nitrogen present in the molasse were metabolized by the yeast, representing a C/N ratio of 8.87, the substrate supplementation wouldn't be necessary. However, part of this nitrogen source is composed by complex proteins which are not assimilated by the cells. Therefore, an inorganic nitrogen source (NH_4OH – 18 mL/L) was tested and the influence of this supplementation over the specific growth rate and biomass production was investigated.

Table 23 – Influence of nitrogen supplementation over □.

Condition	Specific growth rate	Biomass increase after 12 h	Duplication time (hours)
With nitrogen source, 2 VVM	0.4208	31.23 x	1.65
Without nitrogen source, 2 VVM	0.2271	8.267 x	3.05
Without nitrogen source, 2 VVM, with antibiotic	0.2840	14.27 x	2.44

The supplementation of the medium with an inorganic, directly metabolizable nitrogen source improved the biomass productivity significantly.

4.4.7 Substrate consumption and product formation (specific rate determination)

Fermentation conditions: Molasse at 30°Brix, pH without adjustment and medium without antibiotic. Total volume: 6L.

Table 24 – Evolution of different kinetic parameters of fermentation.

Time (h)	X (g/L)	S (g/L)	P (g/L)	Yield kg abs. et./ton*
0	9.29	226.9	9.30	
2	12.0	221.9	11.1	
4	12.7	190.5	17.8	
6	22.1	174.2	25.3	
8	22.0	160.0	35.3	
10	22.0	159.7	37.0	
12	21.9	159.5	38.8	
24	16.0	143.2	46.4	16.8

*Yield in kg of absolute ethanol (100%) per ton of dry soybean molasses

Table 25 – Evolution of substrate consumption and product formation rates

Time (h)	r_S (dS/dt)	r_P (dP/dt)	Q_S (h^{-1})	Q_P (h^{-1})
0				
2	2.50	0.900	0.208	0.075
4	15.7	3.35	1.24	0.264
6	8.15	3.75	0.369	0.170
8	7.1	5.00	0.323	0.227
10	0.15	0.850	0.00682	0.0386
12	0.10	0.900	0.00457	0.0411
24	1.36	0.633	0.0850	0.0396

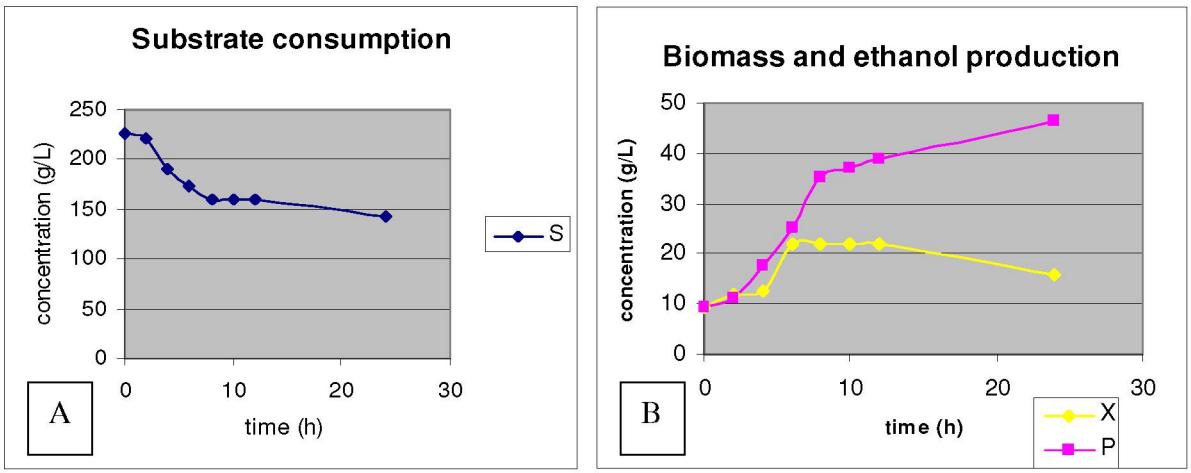


Figure 13 - Fermentation process kinetics.
(A) Substrate consumption.
(B) Biomass and product formation.

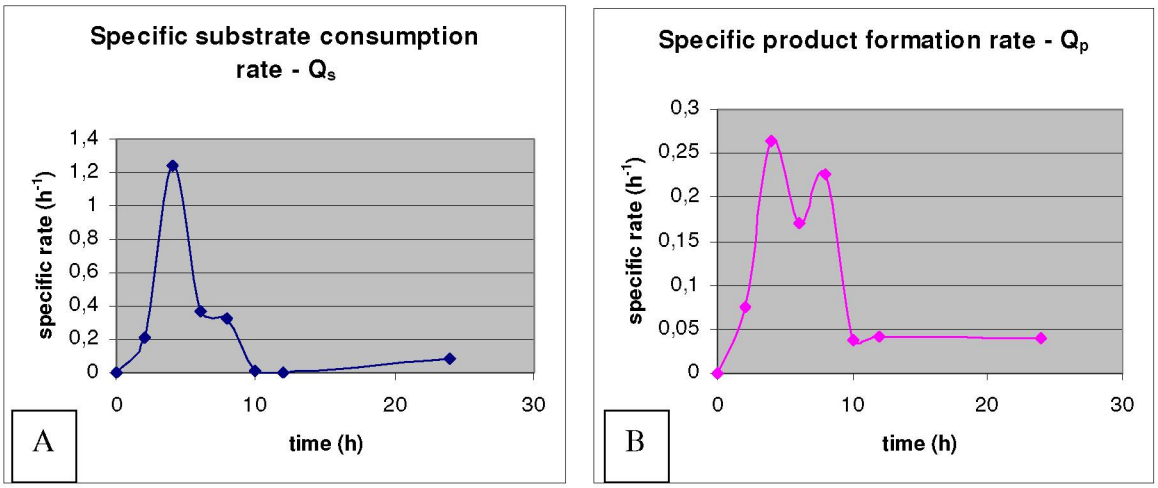


Figure 14 - Evolution of fermentative parameters.
(A) Specific substrate consumption rate.
(B) Specific product formation rate.

According to figures 4.4.2 and 4.4.3, the maximum rates of substrate consumption and ethanol formation occur during the exponential growth phase, which means that ethanol is a primary metabolite.

Maximum productivity of biomass: between 4 and 6 hours

$$\frac{dX}{dt} = \frac{22.1 - 12.7}{2} = 4.7 \text{ g/L.h}$$

Total productivity of biomass: until 12 hours

$$\frac{dX}{dt} = \frac{21.9 - 9.29}{12} = 1.05 \text{ g/L.h}$$

Maximum rate of substrate consumption: between 2 and 8 hours

$$\frac{dS}{dt} = \frac{221.9 - 160.0}{6} = 10.3 \text{ g/L.h}$$

Maximum productivity of ethanol: between 2 and 8 hours

$$\frac{dP}{dt} = \frac{35.3 - 11.1}{6} = 4.03 \text{ g/L.h}$$

Total productivity of ethanol: until 24 hours

$$\frac{dP}{dt} = \frac{46.4 - 9.30}{24} = 1.55 \text{ g/L.h}$$

Overall yield of biomass formation:

$$Y_{X/S} = \frac{\Delta X}{\Delta S} = \frac{16.0 - 9.29}{226.9 - 143.2} = 0.0802 \text{ g biomass per g sugar}$$

Overall yield of substrate conversion into product:

$$Y_{P/S} = \frac{\Delta P}{\Delta S} = \frac{46.4 - 9.30}{226.9 - 143.2} = 0.443 \text{ g ethanol per g sugar}$$

Comparison between theoretical yield for ethanol and obtained yield:

0.511 g ethanol per g sugar (hexose)

$$\text{Obtained yield} = \frac{0.443}{0.511} = 0.867$$

This means that 86.7 % of the consumed sugars were used for ethanol formation. The remaining amount, 13.3%, was used for biomass formation, cell maintance and also consumed by contaminants.

4.5 IMPLEMENTATION OF THE PROCESS IN PILOT SCALE

The process developed in laboratory scale was transferred to pilot scale. The pilot plant was designed according to parameters previously optimized.

The main objective of scaling-up a process is to identify problems that were not significant at the laboratory scale, and also check if the yield of the fermentation is maintained.

A major problem identified after starting-up the plant was the high foam formation. This problem was solved with the addition of anti-foam and dispersant agents, and by conducting the fermentation as fed-batch.

The yield of the fermentation was maintained after the scale-up (around 37.0 g/L of absolute ethanol from a broth with 20ºBrix of soybean molasses).

The fermentations in pilot scale were performed according to the following conditions:

- Inoculum: 30 g/L of pressed yeast
- Time of fermentation: 6 hours
- Antifoam agent: Serquímica SQ-2002, 30.0 mL/m^3
- Dispersant agent: Serquímica SQ-1009, 5.0 mL/m^3

PICTURES OF THE PILOT PLANT (1 m^3/DAY)

Figure15 - (A) Preparing the must – diluting the soybean molasses from 75 to 30ºBrix.
(B) Transferring the broth to the second tank.

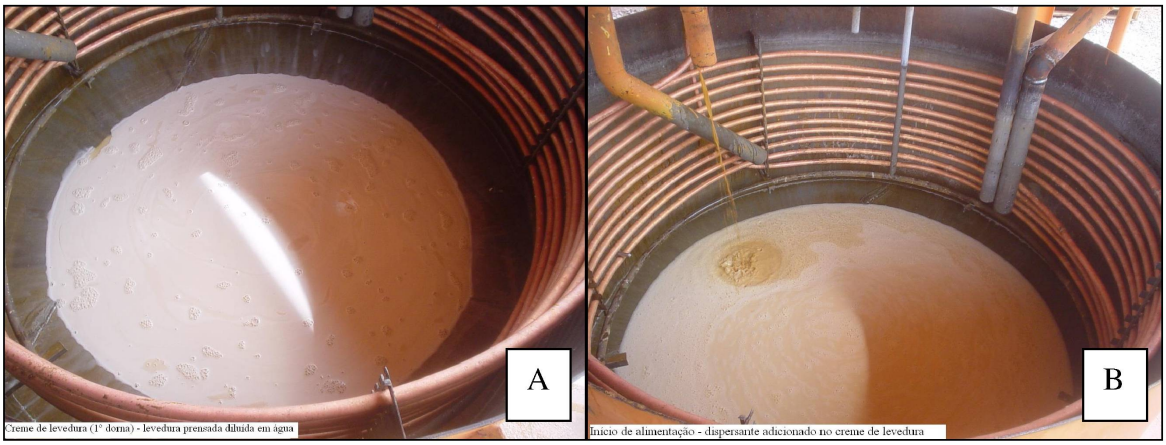


Figure 16- (A) Yeast cream (tank 1) – pressed yeast diluted in water.
(B) Feeding with soybean molasses.

Figure 17 - (A) After four hours – end of feeding.
(B) After six hours – end of fermentation.

Figure 18 - Fermented wine.

After the results obtained in laboratory scale were reproduced in the pilot plant (1 m^3/day), the process was scaled-up again, being transferred to a plant with a production capacity of 10 m^3/day. This plant was purchased from an industry that processed sugarcane molasses to produce ethanol. Depending on the results of the second scale-up, the process will be implemented in an industrial plant, with a production capacity of 70 m^3/day. Until now, the results obtained are satisfactory. These results are shown in table below:

Table 26– Ethanol production from soybean molasses in the pilot plant.

Date (day/month)	Production (L/day)	Date	Production (L/day)
08/April	0	06/May	4777
09/April	1500	07/May	3740
10/April	1000	08/May	3397
11/April	0	09/May	3528
12/April	1400	10/May	2204
13/April	1150	11/May	4098
14/April	750	12/May	4082
15/April	1000	13/May	4409
16/April	0	14/May	6086
17/April	0	15/May	4197
18/April	1300	16/May	2697
19/April	250	17/May	4111
20/April	0	18/May	1479
21/April	868	19/May	3858
22/April	1840	20/May	2454
23/April	1554	21/May	6114
24/April	0	22/May	5598
25/April	0	23/May	4822
26/April	208	24/May	6802
27/April	3316	25/May	8602
28/April	998	26/May	7987
29/April	1670	27/May	8560
30/April	1286	28/May	8149
01/May	0	29/May	7461
02/May	568	30/May	4490
03/May	1993	31/May	4902
04/May	1934	1/June	10180
05/May	3414		

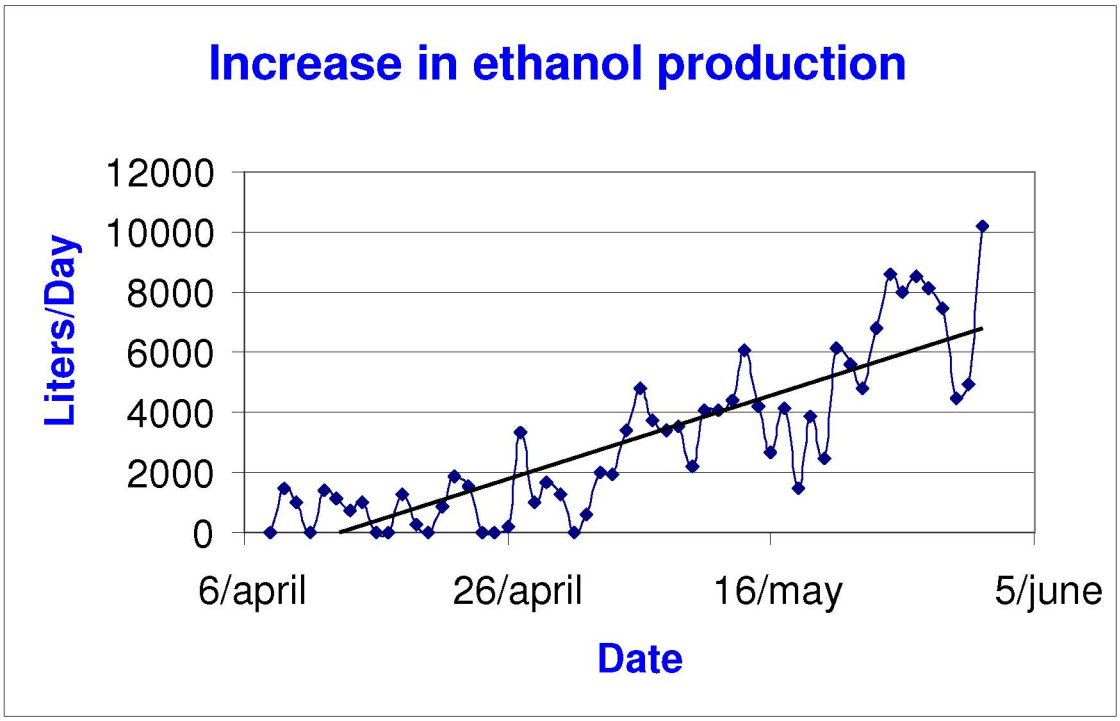


Figure 19 - Increase in ethanol production from soybean molasses at the pilot plant.

PICTURES OF THE PILOT PLANT (10 m^3/DAY)

Figure 20 - Frontal view.

Figure 21 - Upside view.

Figure 22 - Fermentation tanks.

Figure 23 - Distillation tower.

FLOWCHART: PILOT PLANT 1 m^3/DAY

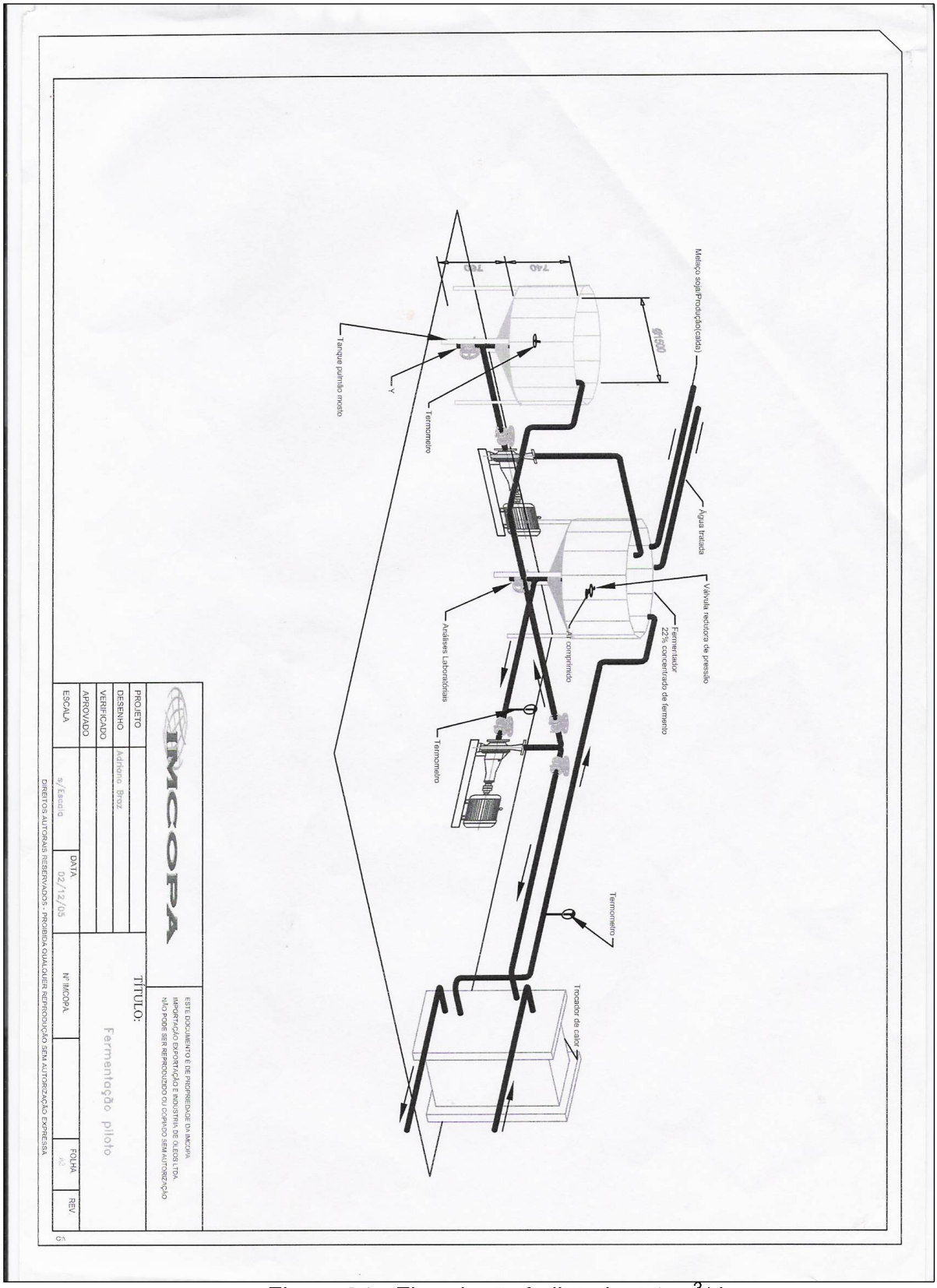


Figure 24 - Flowchart of pilot plant 1 m^3/day.

FLOWCHART: PILOT PLANT 10 m^3/DAY

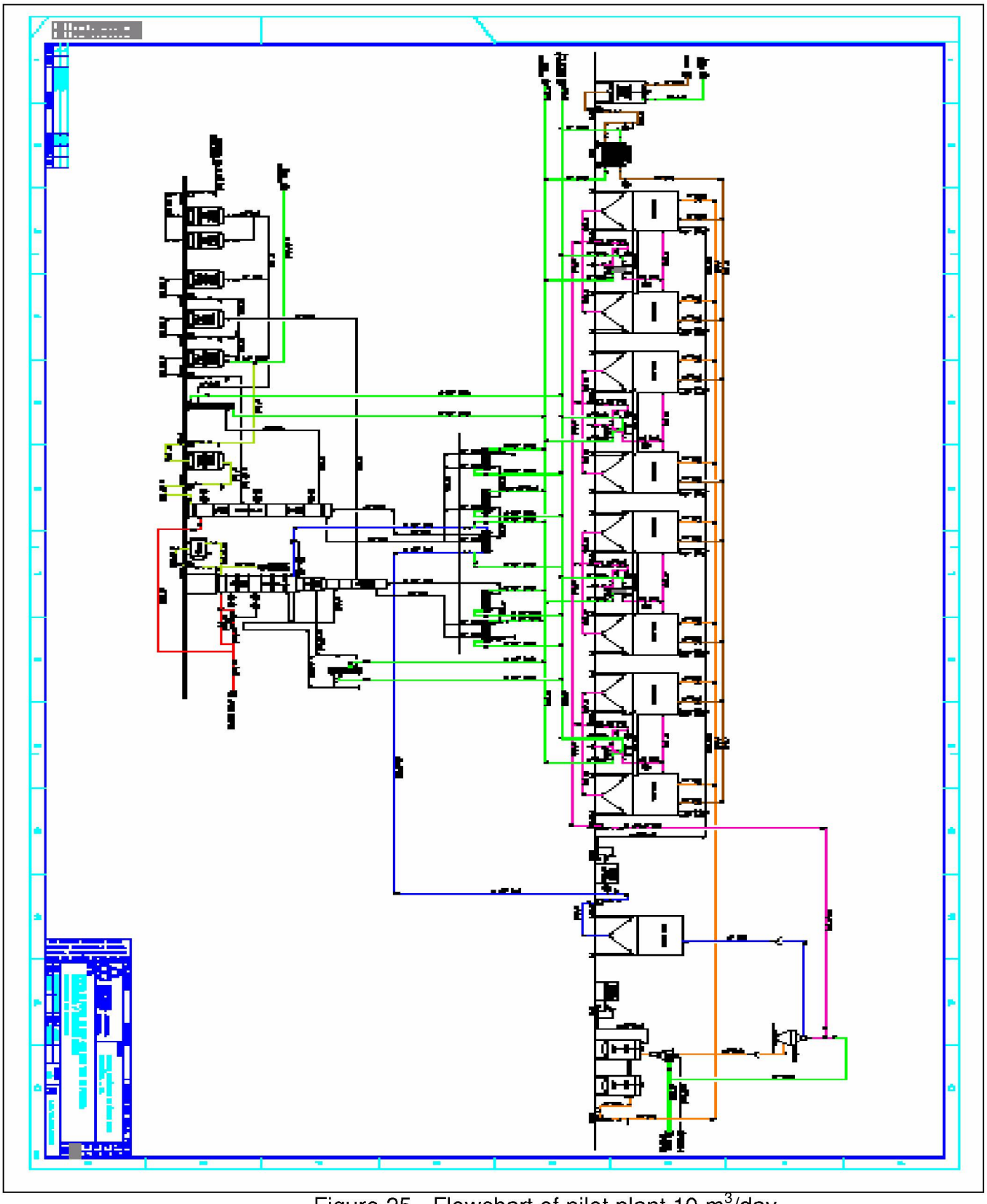


Figure 25 - Flowchart of pilot plant 10 m^3/day.

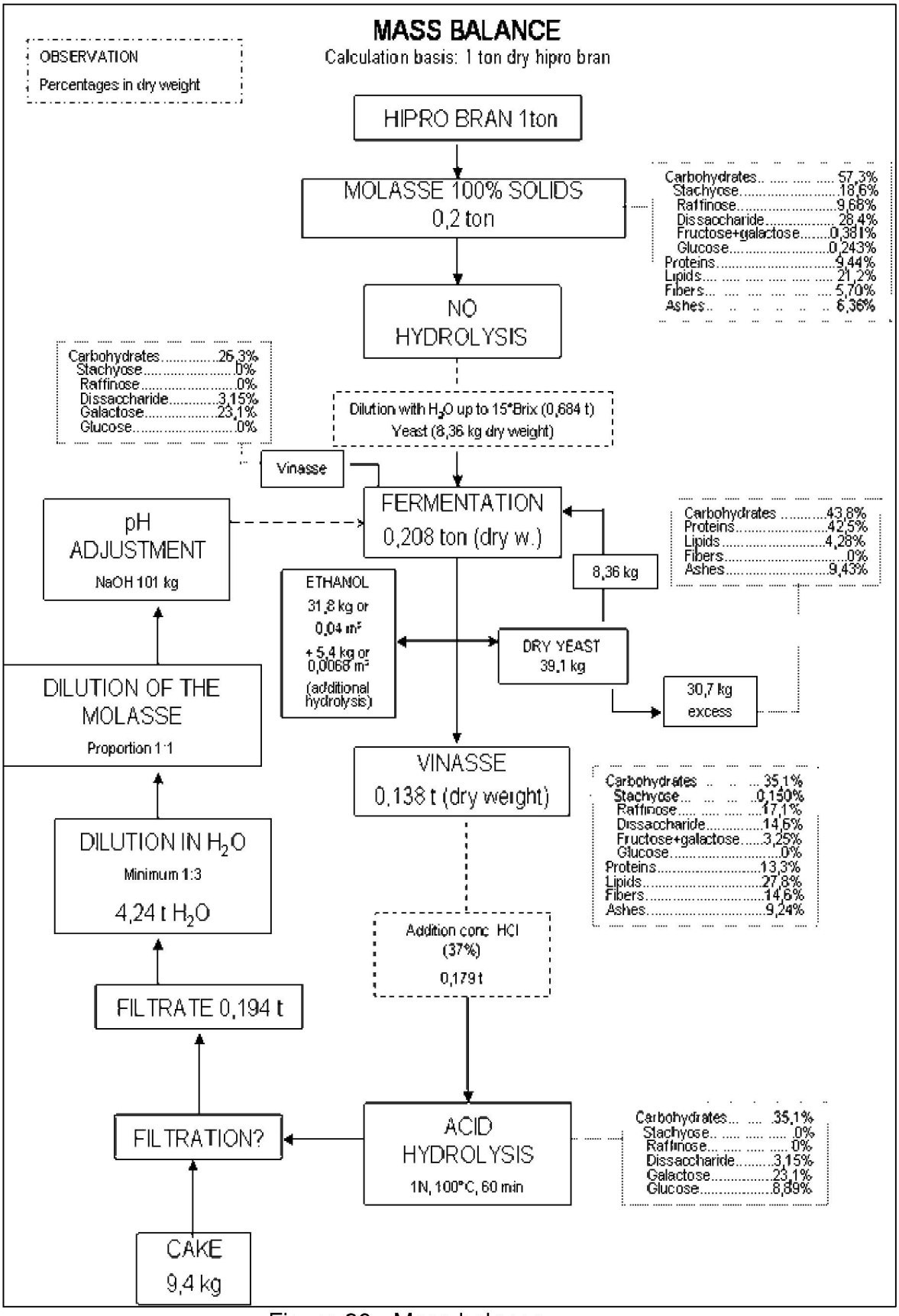


Figure 26 - Mass balance.

5 CONCLUSIONS

Hydrolysis

The hydrolysis of the soybean molasses, in appropriated conditions that did not damage the culture medium (120ºC, 20 min, pH 3,5 adjusted with H_2SO_4), was not only efficient in breaking the □-1,2 bindings of sucrose, but also on removing one monosaccharide unit from stachyose and raffinose. However, these bindings are cleaved as well by the enzyme invertase, which is produced by the yeast. Therefore, the hydrolysis in such conditions did not improve ethanol yield of the fermentation, in spite of the increase in reducing sugars concentration.

The optimized enzymatic hydrolysis (500g of enzyme per ton of soy bean molasses, pH 5.0, 55°C, 24h), using the alpha-galactosidase AEO 301 (Prozyn), released the reducing sugars, which were not able to be fermented before. The enzymatic hydrolysis of soybean molasses converted 75% of the non-reducing sugars into fermentable sugars with an increase of 20% in ethanol yield from the fermentation. Nevertheless, the process was not considered as economic and feasible due to the high cost of the enzyme (US$ 130.00/kg). Other enzymes tested were not efficient in breaking the alpha-1,6 bindings.

The acid hydrolysis of the vinasse using HCl 1N, at 100ºC for 60 min, provided the release of fermentable reducing sugars (by breaking the □-1,6 bindings) with an efficiency of 91%, increasing in 17% the overall yield of the fermentation. However, the high amounts of acid (10% V/V) and alkali for neutralization produced a broth with high concentration of salts, which required a dilution of 4 fold to allow cell growth. Besides, only glucose units released by hydrolysis were converted into ethanol. The galactose units, which represented 2/3 of the total amount of the released sugars, were not efficiently metabolized and remained as residue. It is possible that the hydrolysis in severe conditions damaged the sugar molecules which could not be metabolized by yeast cells. Nevertheless, the acid hydrolysis of vinasse was an interesting alternative to increase the ethanol yield of the process.

Selection of the strain

The different strains of *Zymomonas mobilis* assayed did not show a good yield in ethanol production from soybean molasses, as it was expected, once these bacteria are more selective to the types of metabolizable sugars. These bacteria preferentially consume glucose and fructose, which are present at a low rate in the molasses.

Levasaf and Fermol yeasts presented the best yields among the commercial strains; however, Levasaf was chosen considering the lower cost (US$ 2.20/kg against US$ 13.00 of the other commercial strain).

One of the wild strains (LPB 4) presented a better yield than the commercial yeasts, producing 170 kg of absolute ethanol per ton of dry molasses, against 166 kg per ton obtained by Levasaf. Given that the difference of ethanol production was not substantial, the strain selection was made according to the procedure used to work with these strains. The wild strains request more unit operations (in order to get a pure strain), hence the commercial strain was chosen for the present work. The wild strain was stored for future studies.

Regarding to the concentration of reducing sugars, a high rate of reducing sugars that were not metabolized during fermentation (fructose and, mainly, galactose) was noticed. JP1 strain and the wild strains are capable of galactose

conversion into ethanol; however, a longer duration of fermentation time would be necessary. This conversion was not efficient in soybean molasses, for unknown reasons until now.

Optimization of the fermentative process

In the fermentative process, the supplementation of the medium with nitrogen and magnesium sources and pH adjustment were not necessary, at least in the first cycle of fermentation. It was possible to ferment the soybean molasses at 30ºBrix, without significant losses in ethanol yield.

The previous separation of the molasses proteins was not efficient in the studied conditions (5000 rpm, 20 min or 20.000 rpm, continuous). Moreover, some sugars precipitated with the proteins, which represented a decrease of around 3.00% in ethanol yield.

The recycle of biomass (which consisted in the use of the biomass from previous fermentations as inoculum for subsequent fermentations) was tested with soybean molasses in different concentrations. More expressive contamination problems and losses in cell viability were found in the fermentation of soybean molasses at 30ºBrix in comparison with 20ºBrix. However, the fermentation process at 30ºBrix was chosen because the final ethanol concentration was higher. This represents an economy in the distillation process. Therefore, the growth of bacteria needs to be controlled in order to assure a satisfactory ethanol yield, since these microorganisms consume the reducing sugars and produce organic acids that decrease the cell viability.

The antibiotic tested in our experiments (Kamoran HJ) and the reduction of the medium pH were not effective in inhibiting bacterial growth. The main contaminants are probably lactic acid bacteria, which are acid tolerant. Consequently, a more effective antibiotic will be necessary in order to control contamination and maintain a good ethanol yield.

On the other hand, in pilot scale, the acid treatment of the inoculum provided a better ethanol yield in fermentation. Recently, the pre-treatment of the molasses (removal of insoluble solids) has been studied. The pre-treatment may preserve the equipments (tanks, pipelines, distillation columns) and avoid bacterial proliferation.

Scale-up

The major problems identified after scaling-up the process were foam formation and contamination with bacteria. The first problem was solved by carrying out the process as fed-batch and using antifoam and dispersant agents. The second problem was solved by the previous treatment of soybean molasses (removal of solids by centrifugation or filtration), in order to facilitate the separation of the yeast biomass at the end of the fermentation and the acid treatment. These solid particles can act as “support” for contaminants. The yield in ethanol production was maintained after scale-up.

6 PERSPECTIVES

The main by-product generated by the alcoholic fermentation is the waste produced after distillation and ethanol recovery. This residue (called *vinasse*) has a high concentration of organic compounds, essentially non-reducing sugars. The vinasse can not be treated as a common wastewater, because of the high BOD and COD amount.

Currently, this residue is being concentrated up to 80ºBrix, and then burned in the industrial boiler generating energy for the industrial activities. However, projects are being developed in order to find other destinations for this by-product.

The waste is also being tested as substrate for production of two other products of high commercial value: xanthan gum and lactic acid.

Xanthan gum is a long chain polysaccharide composed by the sugars glucose, mannose, and glucuronic acid. The backbone is similar to cellulose, with added side chains of trisaccharides (three sugars in a chain); however, the trisaccharide side chains of mannose and glucuronic acid make the molecule rigid, and allow it to form a right-handed helix. These features make it interact with itself and with other long chain molecules to form thick mixtures and gels in water. It is a slimy gel produced by the bacterium *Xanthomonas campestris*, which causes black rot on cruciferous vegetables such as cauliflower and broccoli. The slime protects the bacterium from virus attack, and prevents it from drying out.

Xanthan gum is used as a thickener in sauces, as an agent in ice cream preventing the formation of ice crystals, and as a fat substitute that add the "mouth feel" of fat without the calories. It is used in canned pet food to add "cling". In pastry fillings, it prevents "weeping" (*syneresis*) of the water in the filling, protecting the crispness of the crust. It has a very high viscosity (thickness) even when very little is used. When mixed with guar gum or locust bean gum, the viscosity is higher than when using either one separately, so a lower quantity of each gum can be used.

Lactic acid, $CH_3CHOHCO_2H$, is a colorless liquid organic acid. It is miscible with water or ethanol. Lactic acid is a fermentation product of lactose (milk sugar), being present in sour milk, koumiss, leban, yogurt, and cottage cheese. The protein in milk is coagulated (curdled) by lactic acid. Lactic acid is also produced in the muscles during intense activity. Calcium lactate, a soluble lactic acid salt, is used as a source of calcium in the diet. Lactic acid is produced commercially for use in pharmaceuticals and foods, in leather tanning and textile dyeing, and in making bio-degradable plastics, solves, inks, and lacquers. Although it can be prepared by chemical synthesis, the production of lactic acid by fermentation of glucose and other substances is a less expensive method. Chemically, lactic acid occurs as two optical isomers, a dextro- and a levo-form; only the levo-form takes part in animal metabolism. The commercial lactic acid is usually an optically inactive racemic mixture of the two isomers.

Polylactic acid (PLA) is a biodegradable polymer derived from lactic acid. It is a highly versatile material and is made from 100% renewable resources like corn, sugar beets, wheat and other starch-rich products. Polylactic acid exhibits many properties that are equivalent to or better than many petroleum-based plastics, which makes it suitable for a variety of applications.

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APPENDICES

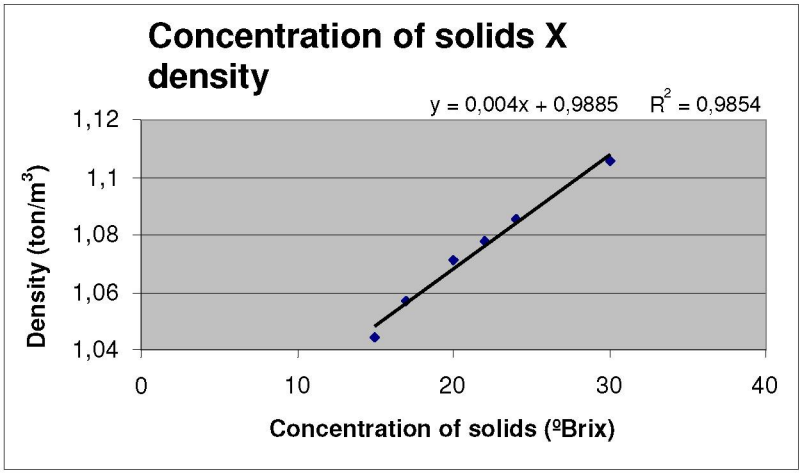
APPENDIX 1: PHYSICAL AND CHEMICAL PROPERTIES OF SOYBEAN MOLASSES

Density of the molasses:

Soluble solids concentration (ºBrix)	Density (ton/m^3)
15	1,0445
17	1,0571
20	1,0714
22	1,0777
24	1,0853
30	1,1058

Linear equation:

Density = 0,004.[concentration of solids] + 0,9885



Mineral composition of the molasses:

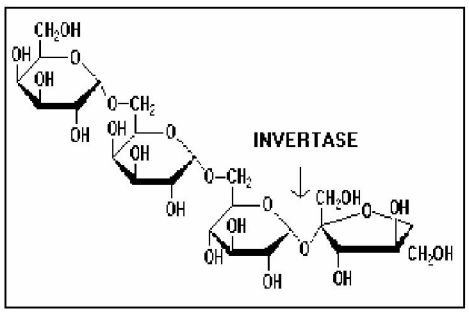
Components	Concentration (mg/100g dry weight)
Aluminum	10,645
Calcium	38,032
Cobalt	0,25807
Copper	7,4839
Iron	2,5807
Phosphorus	492,81
Magnesium	3,3871
Manganese	0,54839
Potassium	5.582,3
Sodium	242,55
Zinc	4,7742

Essential minerals required for yeast growth:

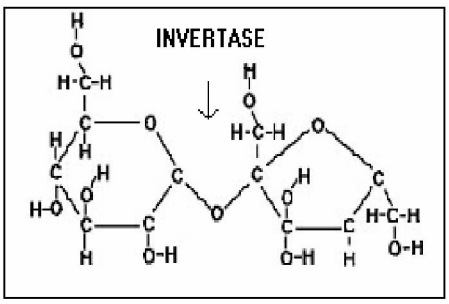
Components	Concentration (g/L)
$(NH_4)_2SO_4$	3,54
$MgSO_4$	0,061
$CaCl_2$	0,022
KH_2PO_4	0,1

Structural formula of the predominant sugars in soybean molasses:

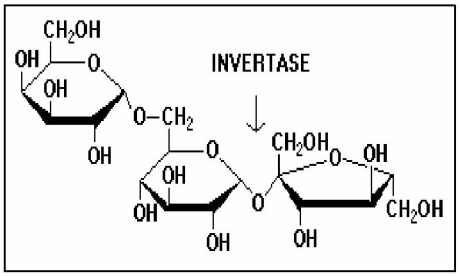
Stachyose: 32,5%



Sucrose: 49,6%



Raffinose: 16,9%



APPENDIX 2: ETHANOL YIELDS

Estimation of absolute ethanol yield per ton of dry molasses:

Considerations:

- The yield was calculated on the basis of ethanol concentration (g/L) obtained from molasses with a determined concentration of soluble solids (ºBrix).
- Densities of the molasses in different concentrations of soluble solids were evaluated (see appendix 1).
- When the density of the molasses with a defined solids concentration was unknown, the following linear equation was used:

$$y = 0{,}004\ x + 0{,}9885;\ R^2 = 0{,}9854$$

where y is the density of the molasses (ton/m^3) and x is the concentration of soluble solids (ºBrix).

Example:

After the fermentation of soybean molasses with 30ºBrix, an ethanol concentration of 58.0 g/L was produced. The density of the molasses at 30ºBrix is 1.1058 ton/m^3, or 1.1058 kg/L. Calculate the yield in absolute ethanol per ton of dry molasses.

1L of molasses at 30ºBrix – 58.0 g of absolute ethanol
1.1058 kg/L of molasses at 30ºBrix – 58.0 g of absolute ethanol
Thus:
52.45 kg of absolute ethanol per ton of molasses at 30ºBrix

Correcting to dry basis:

Molasses with 30% of solids – 52.45 kg/ton
Molasses with 100% of solids – X

X = 174.8 kg/ton or 17.48%

Ethanol yield over total initial sugars concentration:

Considerations:

- Calculated on the basis of the stoichiometric relation:
  100 kg sugar → 51.1 kg absolute ethanol + 48,9 kg CO_2
- So, a yield of 51.1% over total initial sugars concentration represents a yield of 100% over maximum theoretical value.
- In this work, yields over total initial sugars concentration were expressed in terms of percentage over maximum theoretical value.

Example:

After the fermentation of soybean molasses with 30ºBrix, with an initial concentration of sugars of 264.2 g/L, an absolute ethanol concentration of 58.0 g/L was produced. Calculate the yield of the fermentation over total initial concentration of sugars.

Maximum theoretical yield:

$$264.2 \text{ g/L} \times 51.1\% = 135.0 \text{ g/L}$$

Obtained yield:

$$135.0 \text{ g/L} - 100\% \text{ yield}$$
$$58.0 \text{ g/L} - X$$

$$X = 42.96\% \text{ yield}$$

Ethanol yield over consumed sugars:

Considerations:

- Calculated on the basis of the stoichiometric relation:
  $$100 \text{ kg sugar} \rightarrow 51{,}1 \text{ kg absolute ethanol} + 48{,}9 \text{ kg } CO_2$$
- So, a yield of 51,1% over the amount of consumed sugars represents a yield of 100% over the maximum theoretical value.
- In this work, yields over consumed sugars concentration were expressed in terms of percentage over maximum theoretical value.

Example:

After the fermentation of soybean molasses with 30ºBrix, with an initial concentration of sugars of 264.2 g/L, it was produced an absolute ethanol concentration of 58.0 g/L. At the end of the fermentation, the concentration of sugars in the broth was 125.4 g/L. Calculate the yield in ethanol over the consumed sugars concentration.

$$\text{Consumed sugars} = 264.2 \text{ g/L} - 125.4 \text{ g/L} = 138.8 \text{ g/L}$$

Maximum theoretical yield:

$$138.8 \text{ g/L} \times 51.1\% = 70.93 \text{ g/L}$$

Obtained yield:

$$70.93 \text{ g/L} - 100\% \text{ yield}$$
$$58.0 \text{ g/L} - X$$
$$X = 81.77\% \text{ yield}$$

APPENDIX 3: TABLES

Table 1. Experiment: kinetics of fermentation at 20ºBrix.

Time (h)	Weight (g) pH 3.5	Weight (g) pH 4.5	Weight (g) pH 5.5
0	261.05	259.71	256.32
1	260.44	258.87	255.48
2.5	258.58	256.94	253.65
4	257.68	256.15	252.90
5	257.57	255.94	252.78
6	257.53	255.87	252.72
7	257.47	255.77	252.65
21	256.66	254.93	251.90

Table 2. Experiment: kinetics of fermentation at 30ºBrix.

Time (h)	Weight (g) pH 3.5	Weight (g) pH 4.5	Weight (g) pH 5.5
0	287.57	275.01	283.76
1	287.12	274.47	283.12
2.5	286.31	272.96	281.57
4	285.29	271.47	280.07
5	284.39	270.47	279.06
6	283.61	270.01	278.60
7	282.77	269.81	278.44
21	280.49	268.88	277.64

Table 3. Experiment: biomass recirculation 1 – Results of fermentation

Cycle	Initial sugar (g/L)	Residual sugar (g/L)	Ethanol (g/L)	Yield over initial sugar (%)	Yield over consumed sugar (%)	Acidity	Glycerol (g/L)
1 A	169.2	81.6	39.1	45.22	87.35	44.9	6.0
1 B	165.5	83.0	37.8	44.70	89.63	44.9	7.1
1 C	189.9	101.7	44.6	45.96	98.96	51.5	5.6
1 D	183.0	89.3	40.1	42.88	83.75	50.7	6.5
2 A	153.3	86.1	38.7	49.40	112.7	39.2	5.7
2 B	149.8	77.3	35.0	45.72	94.42	41.5	4.8
2 C	170.8	85.2	38.3	43.88	87.59	41.5	5.0
2 D	164.6	84.8	38.3	45.53	93.96	42.6	5.2
3 A	151.9	90.7	40.0	51.53	127.8	41.5	4.9
3 B	149.0	86.2	34.7	45.57	108.2	40.3	7.5
3 C	163.6	86.5	41.2	49.28	104.2	42.9	2.1
3 D	163.1	90.2	43.6	52.31	117.0	48.4	2.1
4 A	151.2	81.4	39.2	50.74	109.9	44.8	4.6
4 B	147.9	98.6	38.2	50.55	151.8	46.7	8.8
4 C	164.7	76.0	38.4	45.63	84.75	42.6	2.1
4 D	164.4	84.7	41.4	49.28	101.7	49.5	2.0

5 A	147.8	79.4	36.3	48.06	103.8	42.6	1.7
5 B	147.5	80.3	40.7	54.00	118.5	45.3	1.6
5 C	172.6	75.0	35.0	39.68	70.21	48.4	2.8
5 D	172.2	77.8	40.2	45.68	83.36	49.5	0.1
6 A	148.1	72.6	35.9	47.44	93.00	40.3	2.0
6 B	147.8	77.9	35.8	47.40	99.93	42.6	4.1
6 C	172.2	75.4	37.0	42.05	74.82	40.3	0.6
6 D	171.4	84.0	37.1	42.36	83.07	41.5	1.5
7 A	147.9	55.0	34.9	46.18	73.48	41.0	2.8
7 B	148.1	75.1	40.4	53.38	108.2	42.6	1.5
7 C	173.2	62.3	35.1	39.66	61.95	49.5	1.2
7 D	172.2	79.2	39.5	44.90	83.14	51.4	0.6
8 A	147.9	78.8	38.00	50.28	107.5	42.6	2.8
8 B	148.2	90.9	33.40	44.10	114.1	43.8	5.2
8 C	172.9	85.5	37.70	42.67	84.37	51.1	0.9
8 D	172.6	88.6	38.50	43.65	89.74	53.7	1.2
9 A	148.1	76.9	39.40	52.06	108.2	43.3	1.8
9 B	148.4	87.1	36.50	48.13	116.5	46.1	6.3
9 C	172.9	75.2	36.40	41.20	72.88	51.1	0.4
9 D	172.6	79.3	36.30	41.16	76.17	50.2	3.4
10 A	148.0	61.0	36.00	47.60	80.06	41.9	1.5
10 B	148.7	86.5	38.30	50.40	120.4	42.6	2.6
10 C	173.0	63.0	33.20	37.55	59.05	44.9	1.6
10 D	173.3	66.8	38.50	43.47	70.08	46.8	0.6
11 A	147.8	59.7	37.00	49.00	82.18	44.9	2.2
11 B	148.5	92.6	36.70	48.36	128.5	46.1	6.0
11 C	173.7	86.0	37.00	41.68	82.54	53.7	0.8
11 D	173.3	78.7	39.20	44.27	81.07	53.4	0.2
12 A	164.9	55.8	32.2	38.21	57.76	41.5	2.5
12 B	165.9	85.2	39.6	46.71	96.00	42.1	2.1
12 C	152.2	85.0	31.7	40.76	92.34	46.5	6.4
12 D	contaminated						
13 A	164.5	83.5	34.8	41.40	84.08	54.0	2.0
13 B	163.7	91.7	33.7	40.29	91.60	56.9	5.0
13 C	154.6	80.0	33.9	42.91	88.93	56.0	2.6
14 A	163.1	81.8	35.0	41.99	84.25	56.9	1.9
14 B	163.1	77.2	35.3	42.35	80.42	57.3	4.3
14 C	155.3	78.1	37.3	47.00	94.55	53.4	2.4

15 A	163.1	82.0	38.5	46.19	92.9	54.1	1.5
15 B	162.1	91.9	41.4	49.98	115.41	56.9	2.6
15 C	152.3	86.6	36.5	46.90	108.72	65.6	3.8
16 A	164.5	75.1	34.6	41.15	75.70	50.7	2.0
16 B	164.9	77.4	32.5	38.57	72.69	51.8	4.3
16 C	135.8	67.0	32.9	47.40	93.54	-	1.1
17 A	161.4	89.2	36.7	44.51	99.51	62.9	2.9
17 B	161.4	91.9	40.1	48.63	113.0	57.6	2.4
17 C	136.3	72.7	31.9	45.82	98.23	66.3	4.0
18 A	162.2	76.2	36.1	43.55	82.15	63.3	1.7
18 B	162.1	75.0	36.3	43.83	81.59	66.8	1.5
18 C	136.7	69.1	32.1	45.94	92.87	65.6	3.9
19 A	156.2	66.3	29.9	37.46	65.09	55.2	3.7
19 B	162.1	72.4	34.1	41.18	74.42	64.1	1.5
19 C	131.2	64.6	30.9	46.08	90.77	72.9	1.0
20 A	158.3	83.9	27.4	33.88	72.12	60.1	4.6
20 B	165.3	78.1	35.1	41.56	78.81	64.1	2.2
20 C	130.6	82.4	34.3	52.88	139.2	80.2	1.8

From the 12th cycle on, experiment D was canceled because it was affected by contamination.

Table 4. Experiment: biomass recirculation 2 – Results of fermentation

Cycle	Initial sugar (g/L)	Residual sugar (g/L)	Ethanol (g/L)	Yield over initial sugar (%)	Yield over consumed sugar (%)	Acidity	Glycerol (g/L)
1 A	256.1	133.6	45.9	35.07	73.32	13.72	7.60
1B	256.1	126.2	46.2	35.30	69.60	13.72	6.30
2 A	256.1	129.25	57.01	43.56	87.95	10.12	6.50
2 B	256.1	130.03	56.90	43.48	88.33	9.89	6.50
3 A	256.1	134.80	53.21	40.66	85.85	19.34	8.94
3 B	256.1	155.11	57.52	43.95	111.45	15.97	8.83
4 A	256.1	138.05	54.29	41.48	90.00	18.21	6.62
4 B	256.1	140.81	50.43	38.53	85.61	17.09	6.07
5 A	256.1	138.95	55.00	42.03	91.88	20.46	6.74
5 B	256.1	137.73	55.00	42.03	90.92	21.59	7.07
6 A	256.1	144.07	49.24	37.63	86.01	20.46	9.05
6 B	256.1	145.73	54.98	42.01	97.48	18.21	7.40

7 A	256.1	139.45	55.47	42.39	93.05	27.21	7.07
7 B	256.1	139.34	49.61	37.91	83.15	27.21	9.39
8 A	240.4	150.29	55.32	45.03	120.13	24.55	6.63
8 B	240.4	145.23	54.38	44.27	111.82	26.79	6.19
9 A	240.4	140.30	53.44	43.50	104.48	32.42	7.86
9 B	240.4	142.07	49.46	40.26	98.43	36.92	9.41
10 A	240.4	148.58	54.28	44.19	115.69	29.04	7.41
10 B	240.4	154.11	53.95	43.92	122.36	31.29	7.30
11 A	240.4	148.67	52.47	42.71	111.95	38.04	6.97
11 B	240.4	141.47	52.47	42.71	103.80	34.67	6.83
12 A	240.4	146.77	47.56	38.72	99.41	32.42	7.85
12 B	240.4	148.98	51.65	42.05	110.55	27.92	6.75
13 A	240.4	147.12	47.93	39.02	100.55	35.79	6.75
13 B	240.4	143.58	44.39	36.14	89.71	33.54	9.19
14 A	271.7	152.12	48.20	34.72	78.87	29.75	6.96
14 B	271.7	150.11	46.49	33.48	74.83	33.13	9.41
15 A	271.7	145.57	46.60	33.56	72.30	27.5	8.52
15 B	271.7	146.90	45.17	32.53	70.83	27.5	9.08
16 A	271.7	157.93	53.27	38.37	91.62	29.75	9.08
16 B	271.7	144.97	49.06	35.34	75.76	28.63	6.87
17 A	271.7	144.09	47.07	33.90	72.18	34.98	9.64
17 B	271.7	147.01	53.14	38.27	83.40	32.36	7.64
18 A	271.7	148.54	49.44	35.61	78.56	29.98	6.86
18 B	271.7	152.03	46.69	33.63	76.35	29.74	8.96
19 A	271.7	159.04	53.94	38.85	93.69	35.69	7.09
19 B	271.7	157.27	51.28	36.93	87.70	33.31	8.53
20 A	271.7	153.25	51.01	36.74	84.27	27.36	5.75
20 B	271.7	153.36	50.79	36.58	83.99	26.17	5.98

Table 5. Experiment: biomass recirculation 2 – Results of cell viability

Cycle	Initial total cells (cells/mL)	Viability	Final total cells (cells/mL)	Viability
1 A	$3.19x10^8$	87.15%	$2.73x10^8$	45.05%
1 B	$3.27x10^8$	85.02%	$2.85x10^8$	44.91%
2 A	$1.95x10^8$	44.62%	$2.2x10^8$	56.82%
2 B	$2.03x10^8$	40.39%	$2.5x10^8$	63.6%
3 A	$2.47x10^8$	53.44%	$3.53x10^8$	50.99%
3 B	$2.09x10^8$	44.98%	$2.49x10^8$	55.02%
4 A	$2.44x10^8$	54.51%	$3.26x10^8$	60.74%
4 B	$1.64x10^8$	53.66%	$3x10^8$	63.67%
5 A			$3.12x10^8$	65.38%
5 B	$2.49\ x10^8$	51.81%	$3.32x10^8$	64.46%
6 A	$3.17x10^8$	65.62%	$3.03x10^8$	64.69%
6 B	$2.71x10^8$	66.79%	$3.15x10^8$	75.01%
7 A	$2.53x10^8$	69.64%	$2.78x10^8$	57.19%
7 B	$2.9x10^8$	76.17%	$3.38x10^8$	65.35%
8 A	$2.61x10^8$	55.86%	$3.2x10^8$	73.75%
8 B	$2.42x10^8$	61.57%	$3.05x10^8$	60.33%
9 A	$2.93x10^8$	58.7%	$3.21x10^8$	68.90%
9 B	$3.23x10^8$	56.01%	$3.3x10^8$	68.58%
10 A	$2.23x10^8$	3.71%	$2.46x10^8$	68.29%
10 B	$3.71x10^8$	73.09%	$1.98x10^8$	68.69%
11 A			$2.81x10^8$	74.02%
11 B			$2.33x10^8$	63.09%
12 A			$2.58x10^8$	56.20%
12 B			$2.02x10^8$	56.93%
13 A			$1.23x10^8$	62.6%
13 B			$1.59x10^8$	61.64%
14 A	$2.45x10^8$	45.71%	$2.37x10^8$	65.82%
14 B	$1.65x10^8$	30.91%	$2.29x10^8$	63.32%
15 A	$1.85x10^8$	52.43%	$2.24x10^8$	65.63%
15 B	$1.5x10^8$	62%	$1.96x10^8$	68.37%
16 A	$1.71x10^8$	75.44%	$1.25x10^8$	60%

16 B	$1.37x10^8$	58.39%	$1.1x10^8$	70.91%
17 A	$1.06x10^8$	69.81%	$1.97x10^8$	72.08%
17 B	$1.51x10^8$	72.19%	$2.18x10^8$	53.67%
18 A	$1.7x10^8$	65.29%	$2.26x10^8$	75.22%
18 B	$1.08x10^8$	54.63%	$1.79x10^8$	69.27%
19 A	$1.5x10^8$	60.11%	$2.06x10^8$	66.02%
19 B	$1.83x10^8$	57.33%	$2.18x10^8$	65.14%
20 A	$9.9x10^7$	77.78%	$1.85x10^8$	70.81%
20 B	$1.42x10^8$	70.42%	$2.13x10^8$	66.2%