SUSAN GRACE KARP

PRODUCTION OF L-LACTIC ACID FROM THE SOYBEAN VINASSE

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

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ABSTRACT

Lactic acid, the popular name of the 2-hydroxypropanoic acid ($C_3H_6O_3$), is the most widely occurring carboxylic acid in nature. L-lactic acid is classified as “Generally Recognized as Safe” by the Food and Drug Administration and finds several applications in four major categories: food and feed, cosmetics, pharmacy and chemistry. The most recent application of lactic acid is its polymerization to poly-lactic acid (PLA), which is a biocompatible and biodegradable plastic that finds many uses in the medical field. The main objective of this work was the development of an economical bioprocess for the production of L(+)-lactic acid using the soybean vinasse (residue of the alcoholic fermentation of the soybean molasses) as substrate. Experimental approach included selection of the strain for L-lactic acid production, optimization of fermentation medium’s composition (sugar concentration, addition of inorganic salts and yeast extract, concentration of calcium carbonate), optimization of fermentative conditions (inoculum’s age and size) and the definition of kinetic parameters. Among ten different strains assayed, *Lactobacillus agilis* LPB 56 was selected for lactic fermentation, due to its ability to metabolize the complex sugars of the vinasse, stachyose and raffinose. The vinasse provided the necessary nutrients for bacterial growth, so there was no need to supplement the medium with inorganic salts or yeast extract. The sugar content of the fermentation medium was increased by concentrating the vinasse, either by evaporation or by addition of concentrated molasses (65°Brix). Medium concentrated by evaporation to 20°Brix provided the best yield (90.86% over initial sugars), and a final lactic acid concentration of 73.04 g/L. The highest lactic acid concentration, 137.7 g/L, was reached in the medium concentrated with molasses to 30°Brix. Centrifugation of the medium before fermentation did not significantly improve fermentation yields. Calcium carbonate, a buffer agent, had to be added before fermentation at the minimum concentration of 6% (w/v), and the pH had to be controlled during fermentation by calcium hydroxide addition. The optimal inoculum’s ages were 24 h in MRS medium and 16 h in the vinasse. Inoculum added at the proportion of 50% resulted in higher productivity (130.7 g/L in 120 h, or 1.089 g/L.h), while the proportion of 25% provided higher concentrations and yields (139.1 g/L in 168 h and 81.82% over consumed sugars). Kinetic and yield parameters were determined for the fermentation of the vinasse, and the calculated values were 0.037 h^{-1} for maximum specific growth rate, 1.91 g/L.h for maximum lactic acid productivity, 1.43 g/L.h for average lactic acid productivity, 0.941% for biomass yield from substrate and 85.0% for product yield from substrate.

Keywords: L-lactic acid. Soybean vinasse. Soybean molasses. Lactic acid fermentation. Lactobacillus.

RESUMO

O ácido láctico, nome popular do ácido 2-hidroxipropanóico ($C_3H_6O_3$), é o ácido carboxílico mais abundante na natureza. O ácido L-láctico é classificado como "Generally Recognized as Safe" pelo Food and Drug Administration e encontra muitas aplicações em quatro grandes categorias: alimentação humana e animal, cosmética, farmácia e química. A mais recente aplicação do ácido láctico é a sua polimerização a ácido poli-láctico (PLA), um plástico biocompatível e biodegradável que encontra muitas aplicações na área médica. O principal objetivo deste trabalho foi o desenvolvimento de um bioprocesso econômico para a produção de ácido L(+)-láctico usando a vinhaça de soja (resíduo da fermentação alcoólica do melaço de soja) como substrato. A investigação experimental incluiu seleção da cepa para produção de ácido L-láctico, otimização da composição do meio de fermentação (concentração de açúcar, adição de sais inorgânicos e extrato de levedura, concentração de carbonato de cálcio), otimização das condições de fermentação (idade e tamanho do inóculo) e a definição de parâmetros cinéticos. Dentre dez diferentes cepas testadas, *Lactobacillus agilis* LPB 56 foi selecionada para a fermentação láctica, pela sua habilidade de metabolizar os açúcares complexos da vinhaça, estaquiose e rafinose. A vinhaça forneceu os nutrientes necessários para o crescimento bacteriano, de modo que não houve necessidade de suplementar o meio com sais inorgânicos ou extrato de levedura. O conteúdo de açúcar do meio de fermentação foi aumentado pela concentração da vinhaça, tanto por evaporação como por adição de melaço concentrado (65°Brix). O meio concentrado por evaporação até 20°Brix apresentou o melhor rendimento (90.86% sobre os açúcares iniciais), e uma concentração final de ácido láctico de 73.04 g/L. A maior concentração de ácido láctico, 137.7 g/L, foi atingida no meio concentrado com melaço até 30°Brix. A centrifugação do meio antes da fermentação não melhorou significativamente os rendimentos da fermentação. Carbonato de cálcio, um agente tamponante, precisou ser adicionado antes da fermentação a uma concentração mínima de 6% (m/v), e o pH teve que ser controlado durante a fermentação pela adição de hidróxido de cálcio. As idades do inóculo ótimas foram 24 h em meio MRS e 16 h na vinhaça. Inóculo adicionado a uma proporção de 50% resultou em maior produtividade (130.7 g/L em 120 h, ou 1.089 g/L.h), enquanto que a proporção de 25% forneceu maiores concentrações e rendimentos (139.1 g/L em 168 h e 81.82% sobre os açúcares consumidos). Parâmetros cinéticos e de rendimento foram determinados para a fermentação da vinhaça, e os valores calculados foram 0.037 h^{-1} para máxima velocidade específica de crescimento, 1.91 g/L.h para máxima produtividade em ácido láctico, 1.43 g/L.h para produtividade média de ácido láctico, 0.941% para rendimento em biomassa a partir do substrato e 85.0% para rendimento em produto a partir do substrato.

Palavras-chave: Ácido L-láctico. Vinhaça de soja. Melaço de soja. Fermentação ácido láctica. Lactobacillus.

LIST OF FIGURES

FIGURE 1 – MASS BALANCE: FROM SOYBEAN TO SOYBEAN VINASSE……. ……………………...19

FIGURE 2 – INDUSTRIAL PLANT FOR ETHANOL PRODUCTION FROM SOYBEAN MOLASSES (10 m³ ETHANOL PER DAY)……………………………. ……………………...20

FIGURE 3 – INCELTECH BIOREACTOR……………………………………….. ……………………..…22

FIGURE 4 – KINETICS OF BIOMASS PRODUCTION IN MRS MEDIUM……...... ……………………....37

FIGURE 5 – KINETICS OF LACTIC ACID PRODUCTION AND SUGAR CONSUMPTION IN THE VINASSE…………………………………………………. ………………………38

FIGURE 6 – SUGAR CONSUMPTION AND LACTIC ACID PRODUCTION KINETICS IN THE MEDIUM COMPOSED OF VINASSE+MOLASSES AT 30°BRIX USING THREE DIFFERENT INOCULATION VOLUMES: 10, 25 AND 50%.. ………………………40

FIGURE 7 – COMPARATIVE FERMENTATION YIELDS IN THE MEDIUM COMPOSED OF VINASSE+MOLASSES AT 30°BRIX USING THREE DIFFERENT INOCULATION VOLUMES: 10, 25 AND 50%.................................. ………………………40

FIGURE 8 – NATURAL LOGARITHM GRAPHIC OF BIOMASS CONCENTRATION REPRESENTING THE EXPONENTIAL GROWTH PHASE (0-36h)…………………………………………………………………………….. ………………………42

FIGURE 9 – STRUCTURAL FORMULA OF THE MAIN SUGARS PRESENT IN THE MOLASSES
(A) – STACHYOSE; (B) – RAFFINOSE; (C) – SUCROSE.................................... ………………………48

LIST OF TABLES

TABLE 1 – PROTOCOL FOR ENZYMATIC ASSAY (BIOSENTEC D/L-LACTATE KIT)……………………………………………………………………………………………..29

TABLE 2 – RESULTS OF LACTIC ACID PRODUCTION FOR DIFFERENT STRAINS GROWN IN SOYBEAN MOLASSES (SBM) AND SOYBEAN VINASSE (SBV) AT 15°BRIX……………………………………………………………………..31

TABLE 3 – EFFECT OF SALTS' ADDITION OVER LACTIC ACID AND BIOMASS PRODUCTION AND SUGAR CONSUMPTION AFTER 48 h OF FERMENTATION IN THE SOYBEAN VINASSE……………………………………………………………..32

TABLE 4 – EFFECT OF YEAST EXTRACT'S ADDITION OVER LACTIC ACID AND BIOMASS PRODUCTION AND SUGAR CONSUMPTION AFTER 48h OF FERMENTATION IN THE SOYBEAN VINASSE………………………………………..32

TABLE 5 – INFLUENCE OF PREVIOUS CENTRIFUGATION AND CONCENTRATION OF THE MEDIUM (BY EVAPORATION OR ADDITION OF MOLASSES) ON LACTIC ACID PRODUCTION AND SUGAR CONSUMPTION……33

TABLE 6 – INFLUENCE OF PREVIOUS CENTRIFUGATION AND CONCENTRATION OF THE MEDIUM: AVERAGE YIELDS OF LACTIC ACID PRODUCTION AND SUGAR CONSUMPTION ……………………………………………34

TABLE 7 – RESULTS OF THE STATISTICAL ANALYSIS……………………………………34

TABLE 8 – EFFECT OF SOLUBLE SOLIDS' CONCENTRATION (°BRIX) ON LACTIC ACID PRODUCTION AND SUGAR CONSUMPTION……………………………35

TABLE 9 – EFFECT OF SOLUBLE SOLIDS' CONCENTRATION (°BRIX): AVERAGE YIELDS OF LACTIC ACID PRODUCTION AND SUGAR CONSUMPTION…………………………………………………………………………………35

TABLE 10 – INFLUENCE OF CALCIUM CARBONATE CONCENTRATION OVER LACTIC ACID PRODUCTION AND SUGAR CONSUMPTION……………………………36

TABLE 11 – KINETICS OF BIOMASS PRODUCTION IN MRS MEDIUM…………………37

TABLE 12 – KINETICS OF LACTIC ACID AND BIOMASS PRODUCTION AND SUGAR CONSUMPTION IN THE VINASSE BY THE STRAIN *L. agilis* LPB 56……………38

TABLE 13 – EFFECT OF INOCULATION VOLUME ON LACTIC ACID PRODUCTION…………………………………………………………………………………39

TABLE 14 – EFFECT OF INOCULATION VOLUME ON SUGAR CONSUMPTION…………………………………………………………………………………39

TABLE 15 – EFFECT OF INOCULATION VOLUME: AVERAGE YIELDS OF LACTIC ACID PRODUCTION AND SUGAR CONSUMPTION……………………………39

TABLE 16 – KINETIC AND YIELD PARAMETERS FOR THE FERMENTATION OF THE SOYBEAN VINASSE BY THE STRAIN *L. agilis* LPB 56…………………………41

TABLE 17 – PROPERTIES OF THE LACTIC ACID…………………………………………47

TABLE 18 – COMPOSITION OF SOYBEAN MOLASSES AND SOYBEAN VINASSE……………………………………………………………………………… ……………………….48

TABLE 19 – CLASSIFICATION OF LACTIC ACID BACTERIA REGARDING FERMENTATIVE WAY AND PRODUCT FORM……………………………………… ……………………….49

TABLE 20 – LACTIC ACID BACTERIA SPECIES ABLE TO CONSUME THE VINASSE'S SUGARS.. …………………….49

LIST OF ABBREVIATIONS

ACS – American Chemical Society

AHA – alpha-hydroxy-acid

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

PLA – Poly Lactic Acid

UFPR – Universidade Federal do Paraná

USP – United States Pharmacopeia

v/v – volume/volume

w/v – weight/volume

w/w – weight/weight

LIST OF SYMBOLS

$C_3H_6O_3$ – Lactic acid (chemical formula)

% – 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

$CH_3CH(OH)COOH$ – Lactic acid (chemical formula)

mmHg – Mercury millimeter

J – Joule

kJ – Kilojoule

INDEX

1 INTRODUCTION 14
1.1 OBJECTIVES 14
1.1.1 Main objective 14
1.1.2 Secondary objectives 14
2 LITERATURE REVIEW 15
2.1 LACTIC ACID 15
2.2 LACTIC ACID BACTERIA 16
2.3 LACTIC ACID FERMENTATION 17
2.4 SOYBEAN VINASSE 18
3 MATERIALS AND METHODS 21
3.1 MATERIALS 21
3.1.1 Strains 21
3.1.2 Raw materials 21
3.1.3 Bioreactor 22
3.1.4 Media preparations 22
3.1.5 Standards for HPLC analysis 23
3.1.6 Accessories and mobile phase for HPLC analysis 23
3.2 EXPERIMENTAL METHODS 23
3.2.1 Storage of the strains 23
3.2.2 Selection of the strain 24
3.2.3 Addition of inorganic salts 24
3.2.4 Addition of yeast extract 25
3.2.5 Pre-treatment: centrifugation and concentration 25
3.2.6 Concentration of soluble solids 25
3.2.7 Concentration of calcium carbonate 26
3.2.8 Inoculum's age 26
3.2.9 Inoculum's size 27
3.3 ANALYTICAL METHODS 27
3.3.1 Soluble solids percentage (°Brix) quantification 27
3.3.2 Biomass quantification 28

3.3.3 Lactic acid and sugars quantification 28
3.3.4 Determination of D/L-lactate concentrations 28
4 RESULTS AND DISCUSSION 30
4.1 SELECTION OF THE STRAIN... 30
4.2 DEFINITION OF THE MEDIUM'S COMPOSITION... 31
4.2.1 Addition of inorganic salts 31
4.2.2 Addition of yeast extract.. 32
4.2.3 Pre-treatment: centrifugation and concentration 33
4.2.4 Concentration of soluble solids (°Brix) 35
4.2.5 Concentration of calcium carbonate.. 36
4.3 OPTIMIZATION OF THE FERMENTATIVE PROCESS 36
4.3.1 Inoculum's age.. 37
4.3.2 Inoculum's size 39
4.4 DETERMINATION OF KINETIC PARAMETERS.. 41
5 CONCLUSIONS 43
6 SUGGESTIONS FOR FURTHER RESEARCH.. 44
7 REFERENCES.. 45
APPENDICES 47

1 INTRODUCTION

This work presents the development of a bioprocess that employs an agro-industrial residue – soybean vinasse – in the production of an economically valuable product of increasing demand in the market, the L-lactic acid.

1.1 OBJECTIVES

1.1.1 Main objective

The main objective of this work was the development of an economical bioprocess for the production of L(+)-lactic acid using the soybean vinasse (residue of the alcoholic fermentation of the soybean molasses) as substrate.

1.1.2 Secondary objectives

- Selection of the strain for L-lactic acid production.
- Optimization of fermentation medium's composition (sugar concentration, addition of nutrients, concentration of calcium carbonate).
- Optimization of fermentative conditions (inoculum's age and size).
- Definition of kinetic parameters.
- Depending on the characteristics of the product, define possible applications.

2 LITERATURE REVIEW

2.1 LACTIC ACID

Lactic acid, the popular name of the 2-hydroxypropanoic acid ($C_3H_6O_3$), is the most widely occurring carboxylic acid in nature (NARAYANAN et al., 2004). Because it has a hydroxyl group adjacent to a carboxyl group, it is called an alpha hydroxy acid (AHA). The molecule has an asymmetrical carbon, and exists in the form of two optically active isomers, L(+)-lactic acid or S-lactic acid and D(-)-lactic acid or R-lactic acid.

Lactic acid was discovered by Scheele in 1780. In 1789, Lavoisier named it “acide lactique”, since it was believed that lactic acid was a milk component. Only in 1857, Pasteur discovered that the acid was not a milk component, but a product of the metabolism of some microorganisms. Industrial production of lactic acid was first performed in 1881, leaded by Charles Avery in Massachusetts (NARAYANAN et al., 2004).

Lactic acid can be produced either by chemical synthesis or fermentation. By the chemical way, the racemic mixture D/L is always produced, while optically pure isomers (D or L) can be obtained by fermentation when the appropriate microorganism is selected. L-lactic acid is classified as Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA) and can be used as food additive. However, the D-isomer is not metabolized by animals, causing acidosis and decalcification (WEE et al., 2006). About 90% of lactic acid is produced by the fermentative route (JOGLEKAR et al., 2006).

There are four major categories for the applications of lactic acid: food and feed, cosmetic, pharmaceutical and chemical applications. In the food industry, lactic acid can be used as acidulant, flavouring and pH buffering agent or as inhibitor of bacterial spoilage in a wide variety of processed foods. Esters of lactic acid are used as emulsifying agents. In the cosmetic and pharmaceutical industries, lactic acid is used in formulations of topical ointments, lotions, humectants, parenteral solutions and dialysis applications. Calcium lactate can be used for calcium deficiency therapy and as anti-caries agent. In other chemical applications, technical grade lactic acid is

used as acidulant in leather tanning industry and as finishing operant in other textile products. Lactic acid esters can be used as green solvents. The most recent application of lactic acid is its polymerization to poly-lactic acid (PLA), which is a biocompatible and biodegradable plastic that finds many applications in the medical field (NARAYANAN et al., 2004; WEE et al., 2006).

The worldwide production level of lactic acid is around 350,000 tons per year, and the market growth is believed to be around 12-15% per year (JOGLEKAR et al., 2006). The worldwide production of lactic acid is rapidly increasing, mainly as a result of the growing market for PLA. It is expected that this biodegradable polymer, produced from renewable resources, will replace various petrochemical industry based polymers in applications ranging from packaging to fibers (WEE et al., 2006). Dow Chemicals and Cargill have the largest PLA-producing company, with a production capacity of 140,000 tons per year (NARAYANAN, 2004).

2.2 LACTIC ACID BACTERIA

Lactic acid bacteria (LAB) are best known for their use as starter cultures in the manufacture of dairy products such as fermented milk, yogurt and cheese, and also in the processing of meats, vegetables and alcoholic beverages (CARR et al., 2002).

Generally, lactic acid bacteria can be characterized as Gram-positive, aerobic to facultative anaerobic, asporogenous rods and cocci which are oxidase, catalase and benzidine negative, lack cytochromes, do not reduce nitrate to nitrite, are unable to utilize lactate and have a low G-C content in their genomes (CARR et al., 2002).

The main genera of LAB include *Aerococcus, Carnobacterium, Enterococcus, Lactococcus, Lactobacillus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Weissella* and *Vagococcus* (LIU, 2003).

Lactic acid bacteria have a potential application in the health area, by providing a transient intestinal flora to compete with potentially harmful microorganisms and prevent diseases. Several LAB strains produce bacteriocins, which are protein or protein complexes with bactericidal activities directed against species which are closely related to the producer bacterium (KAWAMOTO and SHIMA, 2004).

Regarding the fermentative metabolism, LAB are classified in two groups: homofermentative or homolactic (produce more than 85% lactic acid from glucose) and heterofermentative (produce 50% lactic acid and considerable amounts of ethanol, acetic acid and carbon dioxide from glucose). Examples of homolactic LAB genera include *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus* and some species of *Lactobacillus*. Obligate heterofermentative LAB include *Leuconostoc*, *Oenococcus*, *Weissella*, and some species of *Lactobacillus* (TORO, 2005).

The desirable characteristics of microorganisms for industrial lactic fermentation are the ability to rapidly ferment raw materials with high yields in stereo-specific lactic acid and minimal requirement for nitrogenous compounds and vitamins (OH et al., 2005). The choice of the microorganism depends on the carbohydrate to be fermented. *Lactobacillus delbrueckii* subspecies *delbrueckii* is able to ferment sucrose. *Lactobacillus delbrueckii* subspecies *bulgaricus* is able to use lactose. *Lactobacillus helveticus* is able to use both lactose and galactose. *Lactobacillus amylophylus* and *Lactobacillus amylovirus* are able to ferment starch. *Lactobacillus lactis* can ferment glucose, sucrose and galactose. *Lactobacillus pentosus* has been used to ferment sulfite waste liquor (NARAYANAN, 2004). Besides lactic acid bacteria such as *Lactobacillus* and *Lactococcus*, fungal strains of *Rhizopus* are also used in industrial lactic acid production (SOCCOL, 1992).

2.3 LACTIC ACID FERMENTATION

The possible sources of carbohydrates for lactic acid fermentation include sucrose from sugarcane or beet, glucose and various sugars from molasses and whey. Sulfite waste liquor is a potential energy source. Pure sugars are generally costly, but facilitate the downstream processing. Alternative substrates such as molasses are low-priced sources, however the great amount of impurities can affect the downstream processing (NARAYANAN et al., 2004; JOGLEKAR et al., 2006).

The production of lactic acid during fermentation lowers the pH of the medium. The maintenance of pH values between 5.5 and 6.5 is important for the good development of many lactic acid bacteria (PEEVA and PEEV, 1997). The pH is usually controlled by the addition of calcium carbonate (in excess, at the beginning of the fermentation), producing calcium lactate as final product. The lactic acid is recovered by addition of sulfuric acid, producing calcium sulfate (gypsum) as residue

(SARHADDAR et al., 1997). The generation of gypsum in high amounts represents an environmental problem (WASEWAR et al., 2002). Other alkalis such as ammonium, calcium, sodium or potassium hydroxide and sodium carbonate can be used to control the pH; they should be recovered at the end of the process and recycled. Alternatively, lactic acid can be continuously removed from the fermentation broth by adsorption, extraction or membrane separation (JOGLEKAR et al., 2006).

2.4 SOYBEAN VINASSE

The soybean vinasse is the main by-product generated by the alcoholic fermentation of soybean molasses. It is the waste-product of the distillation process to recover ethanol. The residue has a high concentration of organic compounds, essentially non-reducing sugars. Because of the high biochemical and chemical oxygen demands, the vinasse can not be treated as a common wastewater.

The fermentation of soybean molasses to produce ethanol is a novel process developed by the Bioprocess Engineering and Biotechnology Division of the Federal University of Paraná, in partnership with the private Brazilian company named IMCOPA – Importação, Exportação e Indústria de Óleos. The molasses is a co-product generated by the industrial production of protein-concentrate soybean meal, as a result of the extraction of sugars from the de-oiled soybean meal. The molasses is a nutritive medium with high concentration of sugars – 57% in moisture free basis (m.f.b.) –, nitrogen and other macro and micronutrients. Before implementation of the ethanol plant, the molasses was being sold at a low price (US$ 3.00 per ton) for feed applications.

Currently, 10 m^3 of ethanol are being produced per day from the molasses at the industrial plant (FIGURE 2), generating a great amount of vinasse (around 220 m^3/day). The vinasse is concentrated up to 80ºBrix and then burned in the industrial boiler, generating energy for the industrial activities. The perspectives for the next years include the scale up of the process to an industrial plant with higher capacity (70 m^3/day).

The alcoholic fermentation of soybean molasses by *Saccharomyces cerevisiae* converts only 50% of the total amount of sugars into ethanol. Among these are sucrose, glucose, fructose and one monosaccharidic unit from stachyose and raffinose (released by the cleavage of the β-1,2 binding by the enzyme invertase).

The residual sugars are oligomers linked by an α-1,6 binding, which are hydrolyzed by the enzyme α-galactosidase (SIQUEIRA, 2006). Some lactic acid bacteria are capable of converting these oligomers into lactic acid, demonstrating the possibility of exploiting the soybean vinasse as substrate for lactic acid production. Figure 1 shows the mass balance, from soybean to soybean vinasse.

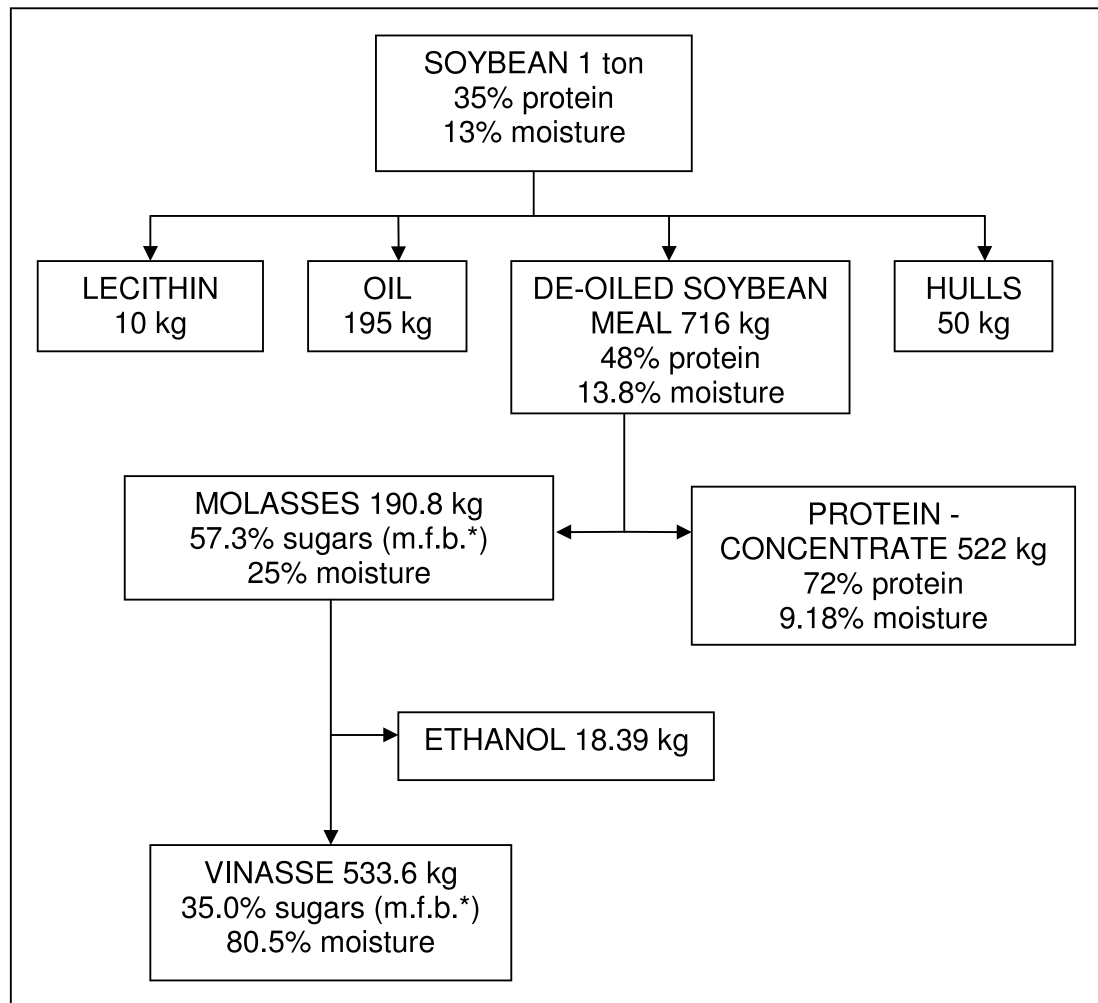

FIGURE 1 – MASS BALANCE: FROM SOYBEAN TO SOYBEAN VINASSE
SOURCE: Siqueira (2006)
[*m.f.b. – moisture free basis]

FIGURE 2 – INDUSTRIAL PLANT FOR ETHANOL PRODUCTION FROM SOYBEAN MOLASSES (10 m³ ETHANOL PER DAY)
SOURCE: Siqueira (2006)

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Strains

The following strains of lactic acid bacteria were tested:

Lactobacillus agilis LPB 56 (Bioprocess Engineering and Biotechnology Division/UFPR)
Lactobacillus delbrueckii (FAT 0846)
Lactobacillus delbrueckii (FAT 1344)
Lactobacillus delbrueckii (FAT 1377)
Lactobacillus pentosus (NRRL B-227)
Lactobacillus plantarum (NRRL B-4496)
Lactobacillus ruminis (NRRL B-14853)
Lactobacillus salivarius (NRRL B-1949)
LPB 2 (LAB, Bioprocess Engineering and Biotechnology Division/UFPR)
LPB 7 (LAB, Bioprocess Engineering and Biotechnology Division/UFPR)

3.1.2 Raw materials

The soybean vinasse and molasses were supplied by the company IMCOPA, at the soluble solids concentrations (per cent) of 15-20°Brix and 65°Brix, respectively. The molasses was stored at room temperature, and the vinasse was stored at -20°C.

3.1.3 Bioreactor

The bioreactor used for the fermentative assays was the INCELTECH LH SGi, total volume of 2 L (FIGURE 3). The equipment has temperature, agitation and pH controllers.

FIGURE 3 – INCELTECH BIOREACTOR
SOURCE: Toro (2005)

3.1.4 Media preparations

Portable refractometer for sugar (Instrutherm, model RT-30 ATC)
Lactobacillus MRS Broth (HiMedia, M 369)
Calcium carbonate P.A. (Alphatec)
Calcium hydroxide (Alphatec)
Potassium hydroxide 85.0% (Synth)
Diammonium-hydrogen-phosphate P.A. (Merck)
Ammonium sulfate P.A. – ACS (Synth)
Yeast extract (BioOrigin)

3.1.5 Standards for HPLC analysis

L-lactic acid 85.0% P.A. (Synth)
Stachyose tetrahydrate, 98.5% (Acros Organics)
D(+)-raffinose pentahydrate, 99+% (Acros Organics)
Sucrose P.A. – ACS (Ecibra)
D(+)-glucose, ACS, anhydrous (Acros Organics)
D(+)-fructose P.A. (Vetec)

3.1.6 Accessories and mobile phase for HPLC analysis

Sulfuric acid 95-97% P.A. (Merck)
Millex PVDF membranes 0.22 μm, diameter 13 mm (Millipore) – for samples
Cellulose-stearate membranes ME25 0.45 μm, diameter 47 mm (S&S) – for mobile phase

3.2 EXPERIMENTAL METHODS

3.2.1 Storage of the strains

Strains received in the lyophilized form were reactivated in Man-Rogosa-Sharp (MRS) medium and inoculated in MRS agar plates. Isolated colonies were again inoculated in MRS broth and agar tubes. After incubation (24h, at the optimal temperature of each strain), the agar tubes were stored in the fridge (4°C). Sterile glycerol 50% was added to the liquid cultures (1:1). This broth was distributed in sterile Eppendorf tubes and stored in the freezer (-20°C). All procedures requiring sterility were performed in a sterile cabin.

3.2.2 Selection of the strain

Two different media were used for strain selection: soybean molasses and soybean vinasse. Concentration of soluble solids (°Brix) was adjusted to 15% by addition of distilled water. Media were centrifuged at 4,700 g for 20 min. The pH was adjusted to 6.0, and $CaCO_3$ was added at 4% (w/v). Medium was supplemented with $(NH_4)_2HPO_4$ 0.25% (w/v). Erlenmeyer flasks were filled to 60% of their total capacity. Sterilization was performed at 121°C for 15 min. Inoculum was previously adapted in the same medium, diluted 1:3. Strains were reactivated in MRS medium, transferred to the inoculum (diluted) medium and incubated for 24 h at their optimal temperature, which is 45°C for *Lactobacillus delbrueckii* and 30°C for the other strains. Inoculum was then transferred to the fermentation media, at the proportion of 10% (v/v). Erlenmeyer flasks were incubated in shaker at 120 rpm, at the optimal temperature. Samples were taken every 24 h for biomass, sugars and lactic acid analyses.

3.2.3 Addition of inorganic salts

Effect of salts' addition was tested in the vinasse, with a soluble solids concentration of 15%. Vinasse was centrifuged at 4,700 g for 20 min. The pH was adjusted to 6.0 and $CaCO_3$ 4% (w/v) was added. Salts' addition was tested in three different conditions (in duplicates):

A – No addition of salts

B – $(NH_4)_2HPO_4$ 2.5 g/L

C – $(NH_4)_2HPO_4$ 1.8 g/L, $(NH_4)_2SO_4$ 3.0 g/L

Erlenmeyer flasks were filled to 80% of their total capacity, and sterilized at 121°C for 15 min. Inoculum of *Lactobacillus agilis* was grown in MRS medium for 24 h and transferred to the fermentation media at the proportion of 10% (v/v). Erlenmeyer flasks were incubated at 30°C, 120 rpm. Samples of 0 h and 48 h were taken for analyses.

3.2.4 Addition of yeast extract

Effect of yeast extract's addition was tested in the vinasse, with a soluble solids concentration of 15%. Vinasse was centrifuged at 4,700 g for 20 min. The pH was adjusted to 6.0 and $CaCO_3$ 4% (w/v) was added. Yeast extract's concentration was tested in three different conditions (in duplicates):

YE 0 – No addition of yeast extract

YE 0.5 – Yeast extract 0.5% (w/v)

YE 1.0 – Yeast extract 1.0% (w/v)

Erlenmeyer flasks were filled to 80% of their total capacity, and sterilized at 121°C for 15 min. Inoculum of *Lactobacillus agilis* was grown in MRS medium for 24 h and transferred to the fermentation media at the proportion of 10% (v/v). Erlenmeyer flasks were incubated at 30°C, 120 rpm. Samples of 0 h and 48 h were taken for analyses.

3.2.5 Pre-treatment: centrifugation and concentration

Vinasse medium was prepared in four different conditions, at two different soluble solids concentrations (20 and 30°Brix): centrifuged and non-centrifuged, concentrated by vacuum evaporation and by addition of molasses 65°Brix. Centrifugation conditions were 4,700 g, 20 min. Vacuum evaporation was performed at 70°C. Erlenmeyer flasks were filled to 80% of their total capacity. The pH was adjusted to 6.0 with KOH 10N, and 6% of $CaCO_3$ were added. Media were sterilized at 121°C for 15 min. Inoculum, grown in MRS medium, was added at the proportion of 10% (v/v). Erlenmeyer flasks were incubated in shaker at 120 rpm, 30°C. The pH was manually adjusted to 6.5, every twelve hours, by the addition of $Ca(OH)_2$ 25%. Fermentations were conducted until there was no change in pH.

3.2.6 Concentration of soluble solids

Two concentrations of soluble solids were tested: 30 and 35%. Previous centrifugation's effect was also tested. Medium was prepared with vinasse 15°Brix

concentrated with molasses 65°Brix to final concentrations of 45 and 55°Brix. Half of the volume of the media at 45 and 55°Brix was centrifuged at 4,700 g for 20 min. The pH was adjusted to 6.0 and 6% of $CaCO_3$ were added. Vinasse was used as inoculum medium (pH 6.0, 6% $CaCO_3$). Erlenmeyer flasks were filled with vinasse to 40% of their total capacity. Media were sterilized at 121°C for 15 min. Vinasse was inoculated with a 24h-culture grown in MRS medium, at the proportion of 10% (v/v), and incubated for 24 h at 30°C, 120 rpm. Concentrated medium was then added at the proportion of 1:1, resulting in final concentrations of 30 and 35°Brix. Erlenmeyer flasks were incubated at 30°C, 120 rpm. The pH was manually adjusted to 6.5, every twelve hours, by the addition of $Ca(OH)_2$ 25%. Fermentations were conducted until there was no change in pH.

3.2.7 Concentration of calcium carbonate

Medium was prepared with vinasse 15°Brix concentrated with molasses 65°Brix to a final concentration of 35°Brix. Erlenmeyer flasks were filled to 80% of their total capacity. The pH was adjusted to 6.0 with KOH 10N. Calcium carbonate was added at the following concentrations (w/v): 0%; 4%; 6% and 8%, in duplicates. The medium was sterilized at 121°C for 15 min. Inoculum grown in MRS medium was transferred to vinasse (15°Brix, pH 6) and incubated at 30°C, 120 rpm for 24 h. This broth was used as inoculum for the fermentations, added at the proportion of 10% (v/v). Erlenmeyer flasks were incubated in shaker at 120 rpm, 30°C. The pH was manually adjusted to 6.5, every twelve hours, by the addition of $Ca(OH)_2$ 25%.

3.2.8 Inoculum’s age

Optimal inoculum’s ages were considered the end of the exponential growth phase for MRS cultures and the phase of maximum lactic acid productivity for vinasse cultures. These parameters were determined by kinetic studies. Kinetics in MRS medium was performed in Erlenmeyer flasks, inoculated with 10% (v/v) of reactivated culture and incubated at 30°C, 120 rpm. Kinetics in the vinasse was performed in the 2L-bioreactor, filled with 1.5 L of vinasse (pH 6.0, 6% of $CaCO_3$).

The bioreactor was sterilized at 121°C for 15 min. Inoculum grown in MRS medium was added at the proportion of 10% (v/v). Fermentation conditions were 30°C, agitation of 150 rpm, pH automatically controlled at 6.0 with KOH 10N.

3.2.9 Inoculum's size

Effect of inoculum's size on fermentation kinetics was tested in three different conditions: 10%, 25%, 50%. Medium used for inoculum growth was the soybean vinasse at 15°Brix, centrifuged (4,700 g, 20 min), at pH 6.0 and with 6% of $CaCO_3$. This medium was transferred to Erlenmeyer flasks (proportions related to total volume mentioned above; final volume 80% of the flask's total capacity). Media for fermentation were prepared at 30, 35 and 45°Brix (vinasse concentrated with molasses) to reach a final concentration of 30°Brix, considering that the inoculum would dilute the medium. Media were centrifuged (4,700 g, 20 min), pH was adjusted to 6.0 with KOH 10N and 6% of $CaCO_3$ were added. Erlenmeyer flasks were sterilized at 121°C for 15 min. Inoculum's medium was inoculated with a 24h-culture grown in MRS medium at the proportion of 10% (v/v), and incubated at 30°C and 120 rpm for 16 h. Concentrated media were then added. The pH was manually adjusted to 6.5, every twelve hours, by the addition of $Ca(OH)_2$ 25%. Samples were taken every twenty four hours for biomass, lactic acid and sugars analyses. Fermentations were conducted until there was no change in pH.

3.3 ANALYTICAL METHODS

3.3.1 Soluble solids percentage (°Brix) quantification

The percentage of soluble solids (°Brix) was measured with a portable refractometer. Samples had to be diluted with distilled water to a final concentration of 0 – 30°Brix (reading range of the instrument).

3.3.2 Biomass quantification

Biomass was quantified by CFU counting in agar plates with MRS medium. The relation between number of cells per mL and g/L of biomass was determined in MRS medium.

3.3.3 Lactic acid and sugars quantification

Lactic acid and individual sugars were quantified by HPLC (High Performance Liquid Chromatography, Shimadzu Liquid Chromatograph), using the Aminex HPX-87 H column, mobile phase H_2SO_4 5 mM, 0.6 mL/min, 60℃. The samples were diluted with ultra purified water (milliQ) acidified with H_2SO_4, according to their concentrations (from 10 to 50-fold), centrifuged (10,000 g, 10 min) and filtered with 0.22 μm PVDF filters. Total sugars' concentration was calculated by the sum of the individual sugars' concentrations.

3.3.4 Determination of D/L-lactate concentrations

The concentrations of the isomers D and L were determined with the Biosentec D/L-lactate kit.

The principle of D and L optical isomers quantification is an enzymatic reaction, using the L-lactate dehydrogenase (L-LdH), D-lactate dehydrogenase (D-LdH) and glutamate-pyruvate-transaminase (GPT).

The samples were centrifuged and diluted with ultra purified water (milliQ) to a final concentration of 0.03 – 0.2 g/L lactic acid. The pH was adjusted to 8-9 with NaOH 0.1N

The reagents were added according to table 1.

TABLE 1 – PROTOCOL FOR ENZYMATIC ASSAY (BIOSENTEC D/L-LACTATE KIT)

	Blank	**Sample**
1 – Buffer pH 10 – L-glutamic acid	1 mL	1 mL
2 – NAD	0.2 mL	0.2 mL
3 – GPT	0.02 mL	0.02 mL
Water	1 mL	0.9 mL
Sample	0	0.1 mL
Agitate and read absorbance	OD 1	OD 1
4 – D-LdH	0.02 mL	0.02 mL
Agitate and read absorbance after 45 min	OD 2	OD 2
L-LdH	0.02 mL	0.02 mL
Agitate and read absorbance after 45 min	OD 3	OD 3

The spectrophotometric reading was performed at 340 nm, optical way of 1 cm, temperature of 20 – 37℃, against air.

Concentration of D/L-lactate:

For D-lactic acid: $\Delta OD_{D\text{-}lac} = [OD\ 2 - OD\ 1]_{sample} - [OD\ 2 - OD\ 1]_{blank}$

For L-lactic acid: $\Delta OD_{L\text{-}lac} = [OD\ 3 - OD\ 2]_{sample} - [OD\ 3 - OD\ 2]_{blank}$

Concentration $_{D\text{-}lac}$ [g/L] = 0.3203 x ΔOD

Concentration $_{L\text{-}lac}$ [g/L] = 0.3232 x ΔOD

4 RESULTS AND DISCUSSION

4.1 SELECTION OF THE STRAIN

Results of lactic acid production for the ten assayed strains, in the soybean vinasse and molasses, are shown in table 2. *Lactobacillus pentosus* showed the best lactic acid productivity in the molasses medium (around 30 g/L after 24h). The molasses contains a significant amount of sucrose (around 20% in m.f.b.), which is easily metabolized by many microorganisms. The vinasse, however, contains only complex sugars (stachyose and raffinose). The only strain that was able to metabolize these complex sugars was *Lactobacillus agilis* LPB 56, producing 27.76 g/L of lactic acid in 72h. Since the aim of this work was to use the vinasse as main raw material for the fermentation, the strain *Lactobacillus agilis* LPB 56 was chosen for the subsequent studies.

Considering the future applications of the product, it is necessary that the strain produces the L-form of lactic acid, a parameter required for food and feed applications. An enzymatic assay for D/L-lactate was performed in some randomly-taken samples of media fermented with *Lactobacillus agilis* LPB 56, in order to quantify the concentrations of L and D isomers. In the vinasse, the L-form represented 89-92% of the total lactic acid concentration. In the molasses, the concentration of L-lactate oscillated between 85 and 94%.

TABLE 2 – RESULTS OF LACTIC ACID PRODUCTION FOR DIFFERENT STRAINS GROWN IN SOYBEAN MOLASSES (SBM) AND SOYBEAN VINASSE (SBV) AT 15°BRIX

Strain	Medium	Lactic acid 24h (g/L)	Lactic acid 48h (g/L)	Lactic acid 72h (g/L)
Lactobacillus	SBM	29.38	31.25	31.55
pentosus	SBV	10.44	9.767	8.667
Lactobacillus	SBM	20.42	22.95	11.51
delbrueckii 0846	SBV	0	0	0
Lactobacillus	SBM	2.981	0	0
delbrueckii 1344	SBV	0.5930	0	1.067
Lactobacillus	SBM	5.602	1.067	3.351
delbrueckii 1377	SBV	1.075	0.4550	1.284
LPB 2	SBM	4.887	3.804	3.580
	SBV	0.633	0	0
LPB 7	SBM	16.19	26.52	31.44
	SBV	5.643	8.273	8.889
Lactobacillus	SBM	0	0	0
ruminis	SBV	0	0	0
Lactobacillus	SBM	2.276	7.530	22.27
salivarius	SBV	2.723	2.904	3.074
Lactobacillus	SBM	15.99	22.07	25.73
agilis LPB 56	SBV	9.644	22.29	27.76
Lactobacillus	SBM	17.52	18.35	21.63
plantarum	SBV	0.2690	10.99	13.83

4.2 DEFINITION OF THE MEDIUM'S COMPOSITION

4.2.1 Addition of inorganic salts

According to table 3, the addition of $(NH_4)_2HPO_4$ 2.5 g/L (experiment B) and $(NH_4)_2HPO_4$ 1.8 g/L plus $(NH_4)_2SO_4$ 3.0 g/L (experiment C) decreased lactic acid production from 42.21 g/L to 37.97 and 33.22 g/L, respectively (average values). The addition of salts may have inhibited fermentation, considering that the soybean

vinasse already contains a high concentration of salts. The effect over biomass production was the same.

TABLE 3 – EFFECT OF SALTS' ADDITION OVER LACTIC ACID AND BIOMASS PRODUCTION AND SUGAR CONSUMPTION AFTER 48 h OF FERMENTATION IN THE SOYBEAN VINASSE
A – No addition of salts; B – $(NH_4)_2HPO_4$ 2.5 g/L; C – $(NH_4)_2HPO_4$ 1.8 g/L, $(NH_4)_2SO_4$ 3.0 g/L;
' represents the duplicate experiment

	Lactic acid 48h (g/L)	**Total sugar 48h (g/L)**	**Biomass 48h (cells/mL)**
Non-fermented medium	-	58.61	8.9×10^{7} (initial)
A	41.55	10.85	1.0×10^{12}
A'	42.87	10.89	6.9×10^{11}
B	37.44	15.67	6.0×10^{11}
B'	38.51	15.53	9.1×10^{11}
C	33.39	20.62	7.0×10^{9}
C'	33.05	21.21	1.0×10^{10}

4.2.2 Addition of yeast extract

There was no significant effect of yeast extract addition over lactic acid production and sugar consumption (TABLE 4). The apparent negative effect on viable cells' concentration may indicate influence in cell growth kinetics and should be further investigated. The use of a medium without yeast extract prevents the decrease in optical purity caused by the presence of a small amount of DL-lactic acid in the yeast extract (OHARA and YAHATA, 1995).

TABLE 4 – EFFECT OF YEAST EXTRACT'S ADDITION OVER LACTIC ACID AND BIOMASS PRODUCTION AND SUGAR CONSUMPTION AFTER 48h OF FERMENTATION IN THE SOYBEAN VINASSE
YE 0 – No addition of yeast extract; YE 0.5 – Yeast extract 0.5% (w/v); YE 1.0 – Yeast extract 1.0% (w/v); ' represents the duplicate experiment

	Lactic acid 48h (g/L)	**Total sugar 48h (g/L)**	**Biomass 48h (cells/mL)**
Non-fermented medium	-	59.13	2.5×10^{9} (initial)
YE 0	41.97	12.13	2.9×10^{14}
YE 0'	43.67	12.58	8.9×10^{13}
YE 0.5	42.97	12.24	3.1×10^{13}
YE 0.5'	43.54	12.43	2.7×10^{13}
YE 1	41.97	13.33	8.9×10^{11}
YE 1'	41.57	13.94	2.0×10^{12}

4.2.3 Pre-treatment: centrifugation and concentration

The concentration of the vinasse was considered as a possibility of reaching higher concentrations of lactic acid at the end of fermentation. According to table 5, the highest concentrations of sugars in the non-fermented medium (186.9 g/L) and lactic acid in the fermented broth (137.7 g/L, average of the duplicates) were obtained in the vinasse concentrated by addition of molasses 65°Brix to a final concentration of 30°Brix, previously centrifuged. However, the fermentation time was the longest (168h).

TABLE 5 – INFLUENCE OF PREVIOUS CENTRIFUGATION AND CONCENTRATION OF THE MEDIUM (BY EVAPORATION OR ADDITION OF MOLASSES) ON LACTIC ACID PRODUCTION AND SUGAR CONSUMPTION
[*N. f. m. – non-fermented medium]; V / VM – vinasse / vinasse+molasses; 20 / 30 – percentage of soluble solids or °Brix; C / NC – centrifuged / non-centrifuged; ‘ represents the duplicate experiment

	Lactic acid (g/L)					Total sugar (g/L)				
	48h	96h	120h	144h	168h	48h	96h	120h	144h	168h
N. f. m.* V20			-					80.39		
V 20 C	60.81	71.79				20.19	3.760			
V 20 C’	64.93	71.18				21.55	5.979			
V 20 NC	61.78	72.67				16.18	5.945			
V 20 NC’	63.05	73.41				14.73	5.908			
N. f. m. V30			-					120.6		
V 30 C	64.61	89.87				68.28	14.67			
V 30 C’	65.24	90.88				50.98	17.37			
V 30 NC	73.41	97.19				40.15	7.302			
V 30 NC’	64.14	90.92				73.31	14.83			
N. f. m. VM20			-					124.6		
VM 20 C	81.09	102.3	109.3			52.64	3.869	3.621		
VM 20 C’	77.38	103.3	109.5			55.43	3.909	3.687		
VM 20 NC	76.74	104.8	103.6			43.94	3.682	3.725		
VM 20 NC’	78.71	103.7	105.0			40.57	3.701	3.655		
N. f. m. VM30			-					186.9		
VM 30 C	90.07	107.1	125.3	131.3	136.4	95.8	77.26	54.27	32.16	21.09
VM 30 C’	86.05	113.2	127.3	135.9	139.1	99.6	77.02	49.94	28.06	19.49
VM 30 NC	90.17	114.9	117.9	124.9	127.8	92.5	63.45	55.38	38.40	23.92
VM 30 NC’	90.35	117.1	128.0	135.6	137.2	98.6	59.76	48.11	31.97	20.15

According to the calculated values shown in table 6, the best yields of sugar conversion to lactic acid were obtained in the vinasse concentrated by evaporation to 20°Brix (98.09% over consumed sugars and 90.86% over initial sugars). The most expressive reduction in sugars’ concentration was achieved in the vinasse concentrated with molasses to 20°Brix (97.07%). The effect of centrifugation was non-significant.

TABLE 6 – INFLUENCE OF PREVIOUS CENTRIFUGATION AND CONCENTRATION OF THE MEDIUM: AVERAGE YIELDS OF LACTIC ACID PRODUCTION AND SUGAR CONSUMPTION
V / VM – vinasse / vinasse+molasses; 20 / 30 – percentage of soluble solids or °Brix; C / NC – centrifuged / non-centrifuged

Condition	Yield over consumed sugars (%)	Yield over total initial sugars (%)	Reduction in sugar concentration (%)
V 20 C	94.67	88.93	93.93
V 20 NC	98.09	90.86	92.63
V 30 C	86.41	74.93	86.72
V 30 NC	85.87	77.99	90.82
VM 20 C	90.45	87.80	97.07
VM 20 NC	86.26	83.71	97.04
VM 30 C	82.65	73.67	89.14
VM 30 NC	80.37	70.89	88.21

Data were submitted to a statistical Factorial Effect analysis, in order to verify the isolated and interactive effects of the variables. For yields over consumed and initial sugars, it was concluded that composition (V / VM) and °Brix (20 / 30) can not be analyzed separately, since there is a strong negative interaction between them. The previous treatment did not significantly influence the results (TABLE 7).

TABLE 7 – RESULTS OF THE STATISTICAL ANALYSIS
*Yield over consumed sugars; **Yield over initial sugars; C – "increase" in composition (V to VM); B – increase in °Brix (20 to 30); PT – pre-treatment (NC to C)

	Variable	Effect on yield 1*	Standard deviation	Effect on yield 2**	Standard deviation
Main effect	C	-6.33		-4.16	
	B	- 8.54		-13.5	
	PT	0.90		0.47	
Secondary effect	C B	-4.80	0.71	-8.51	0.95
	C PT	2.34		2.97	
	B PT	0.51		-0.61	
Tertiary effect	C B PT	-1.47		-0.05	

For industrial applications, a high concentration of lactic acid in the fermented broth is preferable. In order to keep the high concentration of lactic acid and improve fermentation yield, an experiment was conducted with the vinasse concentrated by addition of molasses to 30 and 35°Brix, however increasing inoculation volume from 10 to 50%.

4.2.4 Concentration of soluble solids (°Brix)

In comparison with the previous experiment, concentrations of sugars in non-fermented medium and lactic acid in fermented broth were lower (TABLE 8). This is probably due to changes in the raw material's composition. A significant difference between the duplicates was noticed in the non-centrifuged medium at 35°Brix. In the previous experiment, this was observed in the non-centrifuged medium at 30°Brix.

TABLE 8 – EFFECT OF SOLUBLE SOLIDS' CONCENTRATION (°BRIX) ON LACTIC ACID PRODUCTION AND SUGAR CONSUMPTION
[*N. f. m. – non-fermented medium]; 30/35 – percentage of soluble solids or °Brix; C / NC – centrifuged / non-centrifuged

	Lactic acid (g/L)			Total sugar (g/L)		
	96h	160h	208h	96h	160h	208h
N. f. m.* 30		-			151.1	
30 C	76.23	119.0	129.5	47.81	21.13	14.05
30 C'	82.85	117.7	124.9	52.39	29.30	16.67
30 NC	87.44	121.8	122.7	45.88	26.11	15.54
30 NC'	87.85	120.6	127.4	47.78	24.99	15.71
N. f. m. 35		-			179.5	
35 C	95.55	125.0	138.9	56.29	46.26	22.07
35 C'	96.26	117.7	137.9	62.64	49.04	25.59
35 NC	87.31	108.9	116.6	55.07	42.46	19.48
35 NC'	80.35	122.8	147.2	60.63	50.69	23.44

TABLE 9 – EFFECT OF SOLUBLE SOLIDS' CONCENTRATION (°BRIX): AVERAGE YIELDS OF LACTIC ACID PRODUCTION AND SUGAR CONSUMPTION
30/35 – percentage of soluble solids or °Brix; C / NC – centrifuged / non-centrifuged

Condition	Yield over consumed sugars (%)	Yield over total initial sugars (%)	Reduction in sugar concentration (%)
30 C	93.71	84.18	89.83
30 NC	92.34	82.79	89.65
35 C	88.91	77.10	86.72
35 NC	83.46	73.48	88.04

According to table 9, for the centrifuged medium at 30°Brix, the yields in lactic acid were improved from 82.65% and 73.67% (table 6) to 93.71% and 84.18%, over consumed sugars and initial sugars, respectively. A small decrease in yield was observed for the non-centrifuged media. It is possible that the lowest initial sugar concentration has contributed to the yield's improvement, not only the higher inoculum's size (50%). To solve this question, an experiment varying the inoculation volume from 10 to 50% was conducted in the centrifuged medium at 30°Brix.

The fermentation kinetics was notably slower in this experiment. It is possible that the storage of the strain in the fridge for long periods (in this case, more than one month) or the successive inoculations in agar medium affected its activity.

4.2.5 Concentration of calcium carbonate

Considering the possibility of working with higher soluble solids' concentrations in the fermentation medium (from 30 to 35°Brix), the initial concentration of calcium carbonate (buffer agent) was tested, since it is not accounted in the Brix measurement and could affect cellular metabolism. Results in table 10 show that calcium carbonate at 6% (w/v) is sufficient to maintain maximum lactic acid productivity, and must be added to provide buffering conditions in the fermentation. It is important to remark that, beside the addition of $CaCO_3$ at the beginning of fermentation, the pH was controlled by addition of calcium hydroxide. The same kinetic pattern of the previous experiment was observed, since the fermentation was not concluded after 160 h.

TABLE 10 – INFLUENCE OF CALCIUM CARBONATE CONCENTRATION OVER LACTIC ACID PRODUCTION AND SUGAR CONSUMPTION
[*Non-fermented medium]

	Lactic acid (g/L)			**Total sugar (g/L)**		
	72h	120h	160h	72h	120h	160h
N. f. m*.		-			180.9	
0%	45.40	62.17	74.97	147.7	107.8	89.43
0%'	42.66	56.91	72.26	128.1	94.71	77.63
4%	98.68	113.7	120.8	96.47	65.50	63.83
4%'	99.98	111.5	120.4	98.96	63.84	56.39
6%	112.8	127.4	135.8	87.97	55.81	48.89
6%'	108.4	125.6	133.0	93.27	55.03	45.68
8%	101.9	119.3	135.9	99.13	64.78	52.21
8%'	113.7	126.5	134.1	83.78	53.81	43.01

4.3 OPTIMIZATION OF THE FERMENTATIVE PROCESS

Because of the loss in cellular activity observed in the previous experiments, the biomass sample used in the following experiments was reactivated directly from the glycerol tubes stored in the freezer at -20°C.

4.3.1 Inoculum's age

4.3.1.1 Kinetics of biomass production in MRS medium

The best inoculum's age in MRS medium was considered the end of the exponential growth phase. According to table 11 and figure 4, this point corresponds to 24 h after inoculation.

TABLE 11 – KINETICS OF BIOMASS PRODUCTION IN MRS MEDIUM

	Biomass (CFU/mL)	Biomass (g/L)
0h	$1.0x10^8$	0.2035
4h	$2.2x10^8$	0.2274
8h	$3.2x10^8$	0.2473
12h	$4.0x10^8$	0.2632
16h	$4.7x10^8$	0.2771
20h	$9.1x10^8$	0.3647
24h	$2.5x10^9$	0.6811
28h	$1.5x10^9$	0.6821

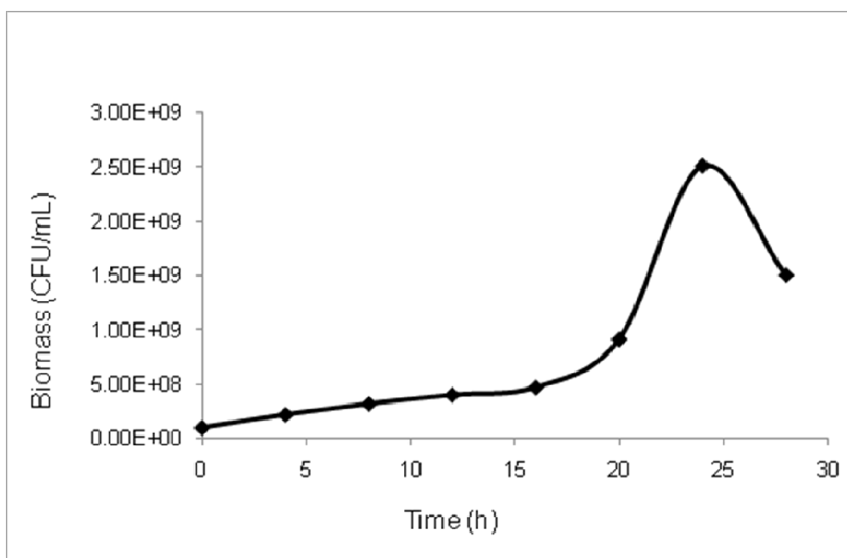

FIGURE 4 – KINETICS OF BIOMASS PRODUCTION IN MRS MEDIUM

4.3.1.2 Kinetics of lactic acid and biomass production and sugar consumption in the vinasse

The kinetic study of lactic acid and biomass production and sugar consumption in the soybean vinasse is presented in table 12 and figure 5. Since the vinasse is the medium used to inoculate the production medium, the best inoculum's age was considered the time of maximum lactic acid productivity, which is 1.91 g/L.h in 16 h (all calculated values are shown in table 16).

TABLE 12 – KINETICS OF LACTIC ACID AND BIOMASS PRODUCTION AND SUGAR CONSUMPTION IN THE VINASSE BY THE STRAIN *L. agilis* LPB 56

	Lactic acid (g/L)	**Total sugars (g/L)**	**Biomass (CFU/mL)**	**Biomass (g/L)**
0h	2.518	68.3	1.2 $x10^8$	0.2075
4h	3.537	67.54	3.1 $x10^8$	0.2453
8h	10.85	61.06	5.9 $x10^8$	0.3010
12h	17.62	55.14	$8.7x10^8$	0.3567
16h	25.25	47.69	$1.1x10^9$	0.4085
20h	29.96	40.8	$1.5x10^9$	0.4901
24h	35.06	33.03	$1.9x10^9$	0.5597
28h	42.14	24.7	$2.3x10^9$	0.6413
32h	47.84	17.94	$2.6x10^9$	0.6950
36h	49.27	16.1	$2.9x10^9$	0.7707
40h	51.17	13.8	$3.0x10^9$	0.7886
44h	52.01	10.29	$3.0x10^9$	0.7866
48h	50.28	6.883	$3.1x10^9$	0.7965

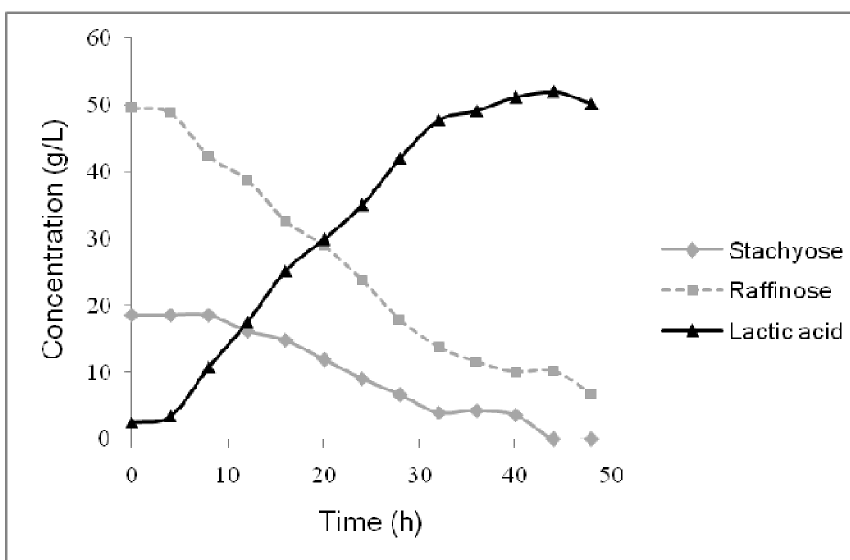

FIGURE 5 – KINETICS OF LACTIC ACID PRODUCTION AND SUGAR CONSUMPTION IN THE VINASSE

4.3.2 Inoculum's size

Results presented in tables 13 and 14 and figure 6 show that the inoculation volume of 50%, using a biomass sample reactivated from glycerol, reduced the fermentation time from 168 to 120 h. However, the highest concentrations of lactic acid and the best yields (TABLE 15 and FIGURE 7) were achieved when the inoculation volume of 25% was used.

TABLE 13 – EFFECT OF INOCULATION VOLUME ON LACTIC ACID PRODUCTION

	Lactic acid (g/L)								
	0h	24h	48h	72h	96h	120h	144h	168h	192h
10%	10.56	22.26	51.99	101.0	118.7	123.5	129.7	129.0	**128.4**
10%'		20.19	53.17	94.04	115.5	120.3	133.5	128.7	**140.8**
25%	16.23	41.83	84.28	111.7	127.6	129.5	138.0	**138.2**	-
25%'		49.46	86.61	111.0	126.8	128.3	132.4	**139.9**	-
50%	22.82	64.32	98.02	120.1	126.1	**129.8**	130.5	127.4	-
50%'		65.09	96.34	122.1	130.8	**131.6**	130.3	123.1	-

TABLE 14 – EFFECT OF INOCULATION VOLUME ON SUGAR CONSUMPTION

	Total sugars (g/L)								
	0h	24h	48h	72h	96h	120h	144h	168h	192h
10%	218.2	207.5	177.7	122.7	98.73	89.2	82.35	58.19	**46.25**
10%'		202.2	171.8	134.0	111.0	102.2	79.99	57.87	**46.44**
25%	195.5	166.4	124.1	102.9	76.04	72.49	58.01	**47.38**	-
25%'		162.4	127.5	106.9	78.32	71.01	55.05	**43.31**	-
50%	177.8	136.8	92.00	52.15	32.36	**30.02**	24.42	31.67	-
50%'		138.0	90.38	59.28	38.65	**31.47**	34.71	30.59	-

TABLE 15 – EFFECT OF INOCULATION VOLUME: AVERAGE YIELDS OF LACTIC ACID PRODUCTION AND SUGAR CONSUMPTION

Condition	**Yield over consumed sugars (%)**	**Yield over total initial sugars (%)**	**Reduction in sugar concentration (%)**
10%	72.16	61.69	78.76
25%	81.82	71.12	76.81
50%	73.34	60.67	82.71

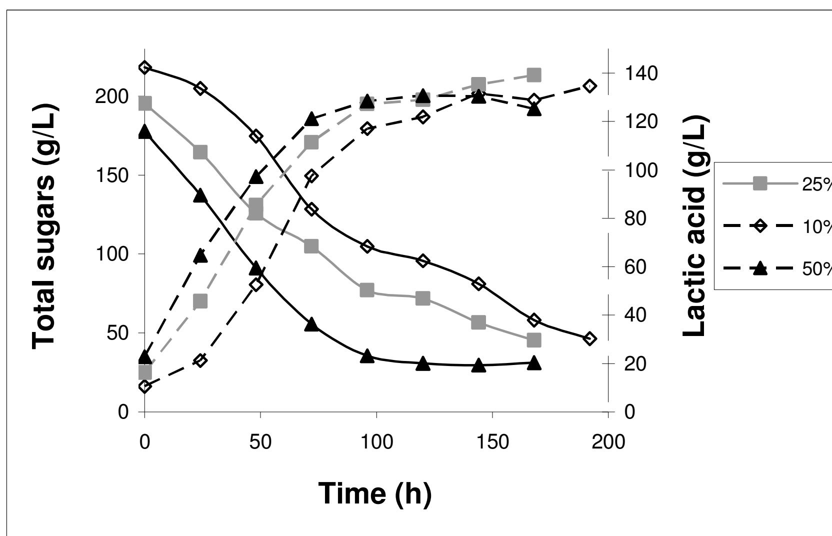

FIGURE 6 – SUGAR CONSUMPTION AND LACTIC ACID PRODUCTION KINETICS IN THE MEDIUM COMPOSED OF VINASSE+MOLASSES AT 30°BRIX USING THREE DIFFERENT INOCULATION VOLUMES: 10, 25 AND 50%

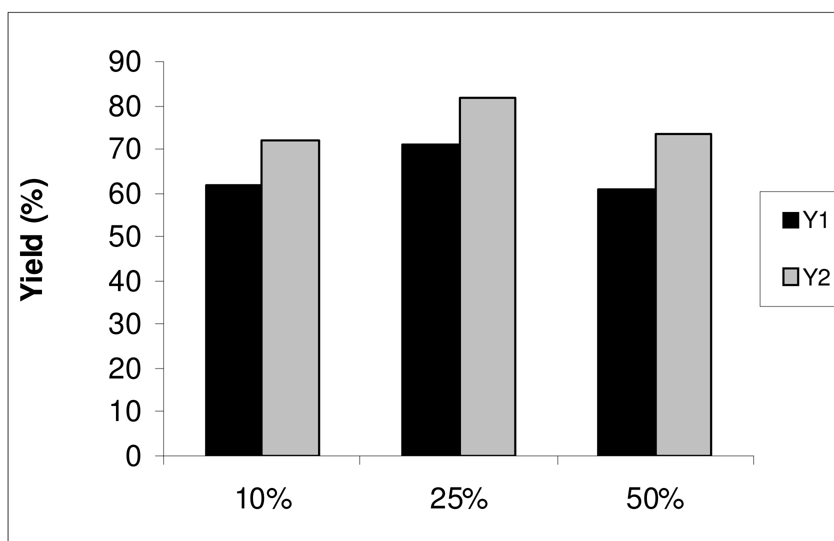

FIGURE 7 – COMPARATIVE FERMENTATION YIELDS IN THE MEDIUM COMPOSED OF VINASSE+MOLASSES AT 30°BRIX USING THREE DIFFERENT INOCULATION VOLUMES: 10, 25 AND 50%
Y1 – Average yield over total initial sugars; Y2 – Average yield over consumed sugars

The yields obtained in this experiment, using concentrated medium (vinasse + molasses), were not as high as the ones obtained with pure vinasse at 20°Brix (TABLE 6). However, considering future industrial applications, it was decided to maintain the high concentration of sugars to get high concentrations of product. Studies will be carried out in order to improve yields and keep high lactic acid concentration.

4.4 DETERMINATION OF KINETIC PARAMETERS

The kinetic parameters were determined for the fermentation of the soybean vinasse, using an inoculum's size of 10% (v/v). This was not performed in the concentrated medium because quantification of biomass was not reliable. The formation of pellets of biomass, calcium carbonate and probably other solids affected the homogenization of the sample, and there was significant variance between the duplicates for the CFU analysis.

Results presented in table 16 were calculated using data from table 12. Kinetics of biomass production is shown in figure 8. The slope of the curve lnX versus time, during the exponential growth phase, represents the maximum specific biomass growth rate.

TABLE 16 – KINETIC AND YIELD PARAMETERS FOR THE FERMENTATION OF THE SOYBEAN VINASSE BY THE STRAIN *L. agilis* LPB 56

Time (h)	r_X (g/L.h)	r_S (g/L.h)	r_P (g/L.h)	$Y_{P/S}$	$Y_{X/S}$
0	-	-	-	-	-
4	0.00945	0.19	0.255	1.34	0.0500
8	0.0139	1.62	1.83	1.13	0.00860
12	0.0139	1.48	1.69	1.14	0.00941
16	0.0129	1.86	1.91	1.02	0.00695
20	0.0204	1.72	1.18	0.683	0.0118
24	0.0174	1.94	1.27	0.656	0.00896
28	0.0204	2.08	1.77	0.850	0.00980
32	0.0134	1.69	1.43	0.843	0.00794
36	0.0189	0.46	0.357	0.777	0.0411
40	0.00447	0.575	0.475	0.826	0.00778
44	-0.0005	0.877	0.21	0.239	-0.0006
48	0.00247	0.852	-0.433	-0.51	0.00291
Average (4-36h)	0.0139	1.69	1.43	0.85	0.00941
Maximum (4-36h)	0.0204	2.08	1.91	1.34	0.05

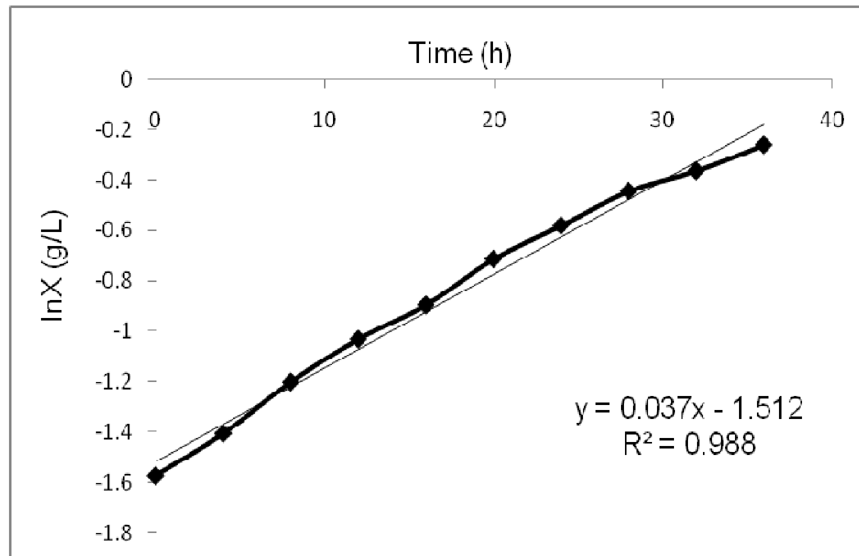

FIGURE 8 – NATURAL LOGARITHM GRAPHIC OF BIOMASS CONCENTRATION REPRESENTING THE EXPONENTIAL GROWTH PHASE (0-36h)

As it is expected for this fermentative process, around 85% of the substrate is directed to product formation. It is known that lactic acid production is associated to biomass growth and maintenance. However, energy required for product formation is much higher than the one required for biomass production.

5 CONCLUSIONS

This work demonstrated that it is possible to produce L-lactic acid using the soybean vinasse and molasses as raw materials. Among ten different strains assayed, *Lactobacillus agilis* LPB 56 was selected for lactic fermentation, due to its ability to metabolize the complex sugars of the vinasse, stachyose and raffinose.

The fermentation conditions were optimized focusing on high lactate concentration and fermentation yield. The vinasse provided the necessary nutrients for bacterial growth, so there was no need to supplement the medium with inorganic salts and yeast extract. The sugar content of the fermentation medium was increased by concentrating the vinasse, either by evaporation or by addition of concentrated molasses (65°Brix). Medium concentrated by evaporation to 20°Brix provided the best yield (90.86% over initial sugars), and a final concentration of lactic acid of 73.04 g/L. The highest lactic acid concentration, 137.7 g/L, was reached in the medium concentrated with molasses to 30°Brix. Centrifugation of the medium before fermentation did not significantly improve fermentation yields. Calcium carbonate, a buffer agent, had to be added before fermentation at the minimum concentration of 6% (w/v), and the pH had to be controlled during fermentation by calcium hydroxide addition.

The optimal inoculum's ages were determined, which were 24 h in MRS medium, representing the end of exponential growth phase, and 16 h in the vinasse, representing the time of maximum lactic acid productivity. Inoculum added at the proportion of 50% resulted in higher productivity (130.7 g/L in 120 h, or 1.089 g/L.h), while the proportion of 25% provided higher concentrations and yields (139.1 g/L in 168 h and 81.82% over consumed sugars). Kinetic and yield parameters were determined for the fermentation of the vinasse, and the calculated values were 0.037 h^{-1} for maximum specific growth rate, 1.91 g/L.h for maximum lactic acid productivity, 1.43 g/L.h for average lactic acid productivity, 0.941% for biomass yield from substrate and 85.0% for product yield from substrate.

6 SUGGESTIONS FOR FURTHER RESEARCH

The product developed using the soybean vinasse and molasses as raw materials, calcium lactate, has a great potential to be applied in the food and feed industries. The purification route to obtain pure lactic acid should not be followed, since the dark color of the fermented broth would demand many clarification steps. However, it was observed that the calcium lactate precipitates at room temperature after the end of fermentation, and the fermented broth becomes solid. After drying and grinding, a powder with 40-50% (w/w) calcium lactate is obtained, a product that could be used as additive in the animal feed industry, containing also lactobacillus biomass.

The process needs some adjustments before implementation at pilot scale. The minimum L-lactate content required for food applications is 95% of the total lactic acid concentration, and the product has around 90%. Two strains that should produce exclusively the L-form of lactic acid, *Lactococcus piscium* and *Lactococcus raffinolactis* (LE BLANC et al., 2005; CARR et al., 2002; LIU, 2003), will be tested in co-culture with *Lactobacillus agilis*, as a strategy to solve this problem. Monitoring the influence of fermentation conditions over L-isomer production is also necessary (OHKOUCHI and INOUE, 2006).

Improvement of fermentation yield and kinetics should also be the subjects of future studies. The conduction of the process at a pilot-scale plant, with automatic pH, temperature and agitation controls, would give essential information that could help to solve these problems.

This process has a great potential to become profitable, once the parameters of fermentation and downstream are finely adjusted. It adds commercial value to a residue that represents, currently, an environmental problem, and generates a product of increasing demand in the market.

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APPENDICES

APPENDIX A.. 47
APPENDIX B.. 48
APPENDIX C.. 49

APPENDIX A – PROPERTIES OF LACTIC ACID

TABLE 17 – PROPERTIES OF THE LACTIC ACID

Property	
Chemical name	2-hydroxypropanoic acid
Chemical formula	$C_3H_6O_3$ or $CH_3CH(OH)COOH$
Molecular mass	90.08 g/mole
CAS number	[50-21-5]
	L: [79-33-4]
	D: [10326-41-7]
	D/L: [598-82-3]
Melting point	L: 53 ℃
	D: 53 ℃
	D/L: 16.8 ℃
Boiling point	122℃ – 12 mm Hg
	82℃ – 0.5 mm Hg
Dissociation constant, 25℃	$1.37x10^4$
Heat of combustion	1,361 kJ/mole
Specific heat, 20℃	190 J/mole.℃

SOURCE: O'Neil et al. (1996)

APPENDIX B – COMPOSITION OF SOYBEAN VINASSE AND SOYBEAN MOLASSES

TABLE 18 – COMPOSITION OF SOYBEAN MOLASSES AND SOYBEAN VINASSE

Component	Molasses %, m.f.b.	Vinasse %, m.f.b.
Stachyose	18.6	11.09
Raffinose	9.68	22.07
Sucrose	28.4	0
Glucose	0.243	0
Fructose	0.127	0
Galactose	0.254	1.84
Total Carbohydrates	**57.3**	**35.0**
Proteins	**9.44**	**13.3**
Lipids	**21.2**	**27.8**
Fibers	**5.7**	**14.6**
Ash	**6.36**	**9.24**

SOURCES: Siqueira (2006); the author (2007)

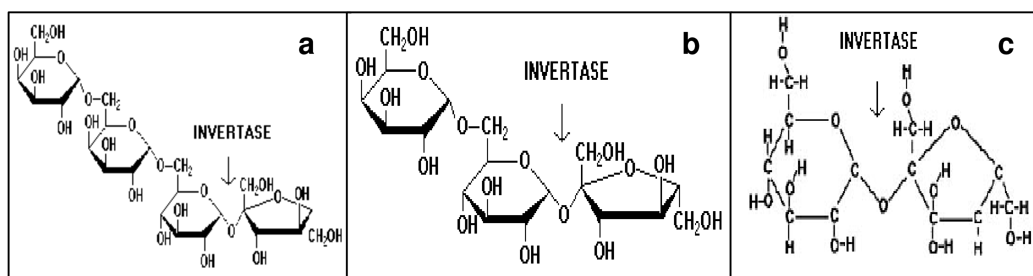

FIGURE 9 – STRUCTURAL FORMULA OF THE MAIN SUGARS PRESENT IN THE MOLASSES
(a) – STACHYOSE; (b) – RAFFINOSE; (c) – SUCROSE
SOURCE: The author (2007)

APPENDIX C – CHARACTERISTICS OF SOME LACTIC ACID BACTERIA

TABLE 19 – CLASSIFICATION OF LACTIC ACID BACTERIA REGARDING FERMENTATIVE WAY AND PRODUCT FORM

MICROORGANISM	PRODUCT FORM
HOMOFERMENTATIVE BACTERIA	
Aerococcus	L+
Enterococcus	L+
Streptococcus	L+
Tetragenococcus	L+
Vagococcus	L+
Lactococcus	L+
Pediococcus	D-, L+, DL
Lactobacillus	D-, L+, DL
Thermobacteria	D-, L+, DL
HETEROFERMENTATIVE BACTERIA	
Carnobacterium	L+
Betabacteria (Lactobacillus)	DL
Leuconostoc	D-
Oencoccus	D-
Weissella	L+, D-, DL

SOURCES: Carr et al. (2002); Liu (2003)

TABLE 20 – LACTIC ACID BACTERIA SPECIES ABLE TO CONSUME THE VINASSE'S SUGARS

GROUP	SPECIES	RAFFINOSE	MELIBIOSE
Streptobacteria	*Lactobacillus agilis*	✓	✓
	Lactobacillus hamsteri	✓	✓
	Lactobacillus murinus	✓	✓
	Lactobacillus pentosus	✓	✓
	Lactobacillus plantarum	✓	✓
	Lactobacillus barvaricus		✓
	Lactobacillus maltaromicus		✓
	Lactobacillus sake		✓
Thermobacteria	*Lactobacillus gallinarum*	✓	✓
	Lactobacillus kefiranofaciens	✓	✓
	Lactobacillus aviarius	✓	
	Lactobacillus salivarius	✓	✓
	Lactobacillus ruminis	✓	✓
Lactococcus	*Lactococcus piscium*	✓	✓
	Lactococcus raffinolactis	✓	✓
Pediococcus	*Pediococcus urinaeequi*	✓	

SOURCES: Carr et al. (2002); Liu (2003)