Bioresource Technology 99 (2008) 8156–8163

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Production of bio-ethanol from soybean molasses by *Saccharomyces cerevisiae* at laboratory, pilot and industrial scales

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ARTICLE INFO

Article history:
Received 13 January 2008
Received in revised form 8 March 2008
Accepted 11 March 2008
Available online 15 May 2008

Keywords:
Soybean molasses
Bio-ethanol
Pilot scale
Industrial scale
Saccharomyces cerevisiae

ABSTRACT

The aim of this work was to develop an economical bioprocess to produce the bio-ethanol from soybean molasses at laboratory, pilot and industrial scales. A strain of *Saccharomyces cerevisiae* (LPB-SC) was selected and fermentation conditions were defined at the laboratory scale, which included the medium with soluble solids concentration of 30% (w/v), without pH adjustment or supplementation with the mineral sources. The kinetic parameters – ethanol productivity of 8.08 g/L h, $Y_{P/S}$ 45.4%, $Y_{X/S}$ 0.815%, m 0.27 h^{-1} and μ_X 0.0189 h^{-1} – were determined in a bench scale bioreactor. Ethanol production yields after the scale-up were satisfactory, with small decreases from 169.8 L at the laboratory scale to 163.6 and 162.7 L of absolute ethanol per ton of dry molasses, obtained at pilot and industrial scales, respectively.

1. Introduction

Natural energy resources such as petroleum and coal have been consumed at high rates over the last decades. The heavy reliance of the modern economy on these fuels is bound to end, due to their environmental impact (and the corresponding pressure of society) and to the fact that they might eventually run out. Therefore, alternative resources as ethanol are becoming more important. Bio-ethanol is one of the most important renewable fuels contributing to the reduction of negative environmental impacts generated by the worldwide utilization of the fossil fuels (Cardona and Sánchez, 2007). Some biological processes have rendered possible routes for producing ethanol in large volumes using the cheap substrates (Gunasekaran and Raj, 1999).

The worldwide production of bio-ethanol (all grades) reached around 51 billion liters in 2006, of which 17 billion were produced in Brazil from sugarcane. The United States produced around 18 billion liters from maize. Studies on bio-ethanol production from the cellulosic materials are being financed by the US Department of Agriculture (RFA, 2008).

Ethanol can be used directly as a fuel, but most often it is blended with gasoline to yield gasohol (Staniszewski et al., 2007). The Brazilian National Bio-Fuel Program, initiated in 1975, stimulated the substitution of gasoline for sugarcane alcohol for automobile use, and intensified the use of a mixture of ethanol and gasoline as fuel for common cars (Soccol et al., 2005). Anhydrous ethanol is added to gasoline at a 20–26% proportion in volume (Cortez et al., 2003). Today, about 3 million automobiles run on 100% alcohol, and about 60% of all new motor vehicles produced in Brazil are "flex", i.e. they can run on any mixture of alcohol/gasoline, as well as on 100% alcohol (Grad, 2006).

A worldwide interest in the utilization of bio-ethanol as energy source has stimulated studies on the cost and efficiency of industrial processes for ethanol production. Intense research has been carried out for obtaining efficient fermentative organisms, low-cost fermentation substrates, and optimal environmental conditions for fermentation to occur (Cysewski and Wilke, 1978). Even though the fermentative process for ethanol production is well known, the production costs are still the key impediment for the wide use of ethanol as fuel. Therefore, the development of fermentation processes using economical carbon sources is important for the ethanol production in a commercial scale (Cazetta et al., 2007).

The Brazilian production of soybean is estimated in 56.6 million tons (2006/2007), which represent around 30% of the global production (IBGE, 2007). Soybean molasses is a co-product generated in the production of protein-concentrate soybean meal. The protein-concentrate is a soy bran with around 70% protein (in dry basis), obtained by the extraction of sugars from de-oiled soybean meal using a mixture of water/ethanol as solvent (Fig. 1), its major

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doi:10.1016/j.biortech.2008.03.037

Nomenclature

°Brix	percentage (w/v) of soluble solids	r_S	sugar consumption rate, dS/dt (g/L h)
S_0	initial sugar concentration (g/L)	r_X	biomass formation rate, dX/dt (g/L h)
S_f	final sugar concentration (g/L)	r_P	product formation rate, dP/dt (g/L h)
X_0	initial biomass concentration (g/L)	μ_S	specific sugar consumption rate, $r_S \cdot X^{-1}$ (h^{-1})
X_f	final biomass concentration (g/L)	μ_X	specific biomass production rate, $r_X \cdot X^{-1}$ (h^{-1})
P_0	initial ethanol concentration (g/L)	μ_P	specific ethanol production rate, $r_P \cdot X^{-1}$ (h^{-1})
P_f	final ethanol concentration (g/L)	$Y_{X/S}$	biomass yield from sugar, r_X/r_S (g/g)
m	maintenance coefficient (h^{-1})	$Y_{P/S}$	ethanol yield from sugar, r_P/r_S (g/g)

application field being the animal feed industry. An important Brazilian soybean-processing company produces 600 tons per day of protein-concentrate, generating 220 tons per day of molasses (Siqueira, 2006).

Yeasts are the most commonly used microorganisms for ethanol fermentation. Anaerobic cultivation of *Saccharomyces cerevisiae* generates, besides ethanol, carbon dioxide, glycerol and cell biomass as the most significant byproducts. Carbon dioxide is an inevitable fermentation product, but the off-gas can be sold as a high-quality raw material and is, therefore, more of a logistic problem. Glycerol can be produced as a compatible solute during osmotic stress (Brandberg et al., 2007).

The main objective of this work was to develop an economical bioprocess to produce bio-ethanol from soybean molasses by a selected strain of the yeast *S. cerevisiae*, through experiments at laboratory, pilot and industrial scales. Fermentative assays evaluated the effects of initial soluble solids concentration, supplementation with mineral sources, pH, operational mode and addition of antifoam and dispersant agents.

2. Methods

2.1. Characterization of the soybean molasses

The soybean molasses was received from a soybean-processing company in the concentrated form (75–80% soluble solids); this material is stable, and was stored at room temperature, being diluted with distilled water to concentrations of 15–30% soluble solids prior to fermentation tests. The percentage (w/v) of soluble solids (°Brix) was determined with a portable refractometer for sugar – Instrutherm, model RT-30 ATC (Singh et al., 1996).

The carbohydrate composition of soybean molasses was determined by HPLC (High Performance Liquid Chromatography; see Section 2.2). Protein concentration was determined by the Kjeldahl method. Lipids concentration was determined by gravimetric analysis after solvent extraction with hexane (Soxhlet method). Ashes were quantified by gravimetric analysis after burning samples at 550 °C for 5 h. Fibers concentration was calculated by difference. Moisture content was determined by gravimetric analysis after drying at 105 °C to constant weight.

2.2. Determination of biomass, sugars and ethanol concentrations

Biomass concentration in cells/mL was quantified with a Neubauer counting chamber; biomass concentration in g/L was determined by gravimetric analysis after drying to constant weight. The viability of yeast cells was determined by methylene-blue staining (Alfenore et al., 2002).

Individual sugars and ethanol were quantified by HPLC (Varian Liquid Chromatography: solvent delivery module 240; column valve module 500; RI Detector 350; Workstation software 5.0) using a Shodex KS-801 column, that separates sugars by molecular size, at

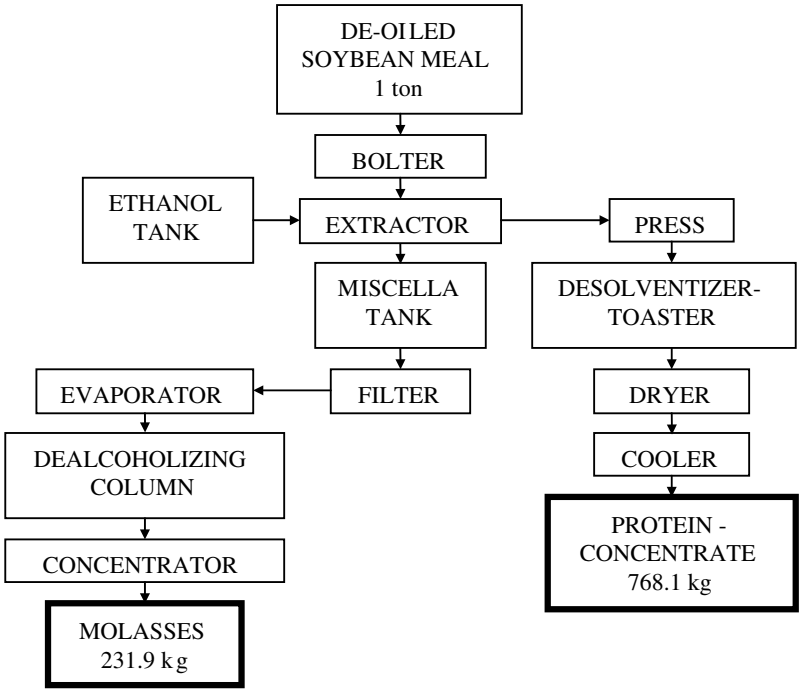

Fig. 1. Production process of the soybean protein-concentrate.

a flow rate of 1.0 mL/min, mobile phase ultra pure H_2O, temperature 80 °C. Samples were diluted (10-fold) with ultra pure water and filtered with hydrophilic PVDF membranes (0.22 μm pore size, 13 mm diameter, Millipore). Standards were Stachyose tetrahydrate (Acros Organics), D(+)-Raffinose pentahydrate (Acros Organics), Sucrose (Synth), D(+)-Glucose anhydrous (Acros Organics), D(+)-Fructose anhydrous (Vetec), D(+)-Galactose (Acros Organics) and absolute ethanol (Merck, 99%+). All reagents were of analytical grade. Concentrations were calculated by means of standard curves relating individual concentration to peak area. Total sugars concentration was calculated by the sum of individual sugars' concentrations.

2.3. Selection of the yeast

Ten different *S. cerevisiae* strains (LPB 1–6, LPB-SC, LPB-MA, LPB-JP and LPB-FR), from the Culture Collection of the Bioprocess Engineering and Biotechnology Division/Federal University of Paraná (DEBB/UFPR), were tested for ethanol production from soybean molasses. The six strains LPB 1–6, stored in PDA agar slants at 4 °C, were reactivated in sterile YM broth, incubated in shaker at 30 °C, 100 rpm, for 24 h. This broth was transferred, at the proportion of 10% (v/v), to the inoculum medium (soybean molasses with 15% soluble solids). The other four strains, available in the form of pressed yeast and stored at 4 °C, were reactivated directly in the molasses medium. Inocula for fermentation assays were incubated in shaker at 100 rpm, 30 °C, for 24 h. Volumes transferred to the fermentation media were calculated so that initial biomass concentration was 1×10^8 viable cells/mL.

Selection of the yeast strain was performed in molasses medium with 30% soluble solids, non-sterilized, without nutrients supplementation or pH adjustment, in Erlenmeyer flasks incubated in shaker at 100 rpm, 30 °C, for 24 h.

2.4. Fermentation tests at laboratory scale

Fermentation assays using the selected strain were conducted in Erlenmeyer flasks, incubated in shaker (Innova, model 4080) at 30 °C, 100 rpm. Inoculum (yeast biomass 10 g/L, with 57% moisture and a minimum of 95% viable cells) was previously cultivated in diluted molasses (15% soluble solids, not sterilized) at 30 °C, 100 rpm, for 12 h. Media were then concentrated by addition of molasses 75 °Brix to the desired final concentration. Biomass concentration at the beginning of fermentation was adjusted to 1×10^8 viable cells/mL. Fermentations were conducted until CO_2 liberation was over (visual analysis). The effects of initial °Brix, addition of inorganic salts as sources of magnesium ($MgSO_4.7H_2O$, Reagen) and nitrogen (NH_4NO_3, Nuclear) (Machado, 1999) and initial pH were tested.

Fermentations to determine kinetic parameters were conducted in bioreactor (8 L, MDL B.E. Marubishi), filled with 6 L of non-sterilized medium. The bioreactor had agitation and temperature controlled.

2.5. Fermentation tests at pilot scale

The process developed at laboratory scale was transferred to a pilot scale plant. Fermentations were carried out in two bioreactors with a total capacity of 1 m^3 each. Molasses was collected directly with 20–30 °Brix, before the concentration step to 80 °Brix. One of the tanks was used for storing the must and the other for inoculum preparation and fermentation. The process was carried out as batch and fed-batch. For the batch process, biomass (pressed yeast) was diluted with molasses and, after homogenization, the tank was filled with molasses to 80% of its capacity. For the fed-batch process, biomass was diluted in water and molasses was fed at the flow rate of 200 L/h (feeding time 4 h).

Fermentations were conducted until there was no liberation of CO_2 bubbles. Enough agitation was provided by the liberation of CO_2 during fermentation and by circulation of the broth through the plate heat exchangers. Temperature was maintained at 30 °C. New inoculum (30 g/L of pressed yeast LPB-SC) was added at the beginning of each fermentation. Antifoam and dispersant agents tested were AE-2002 and AD-1009, respectively. These products consist of a mixture of silicon oil, solvents, emulsifying waxes and tensioactive agents. Dispersant agent was added at the beginning of feeding to avoid foam formation and antifoam was added to break the formed foam after feeding. Best concentrations were determined considering effect on foam reduction and cost. The fermented broth was discarded after fermentation and treated as a common effluent.

2.6. Fermentation tests at industrial scale

After the results obtained at laboratory scale were reproduced at the pilot plant and the necessary parameters were defined, the process was scaled-up again, being transferred to an industrial plant with a production capacity of 10 m^3 ethanol per day. This plant consists of eight 20 m^3 fermentation tanks, a system for biomass recovery that includes one biomass centrifuge and an agitated tank for biomass acid treatment and a distillation device composed of four columns (depurator, exhauster, concentrator and rectifier) that work at atmospheric pressure.

Molasses was collected directly with 20–30 °Brix. The concentration was adjusted to 30 °Brix, when necessary, by the addition of concentrated molasses (80 °Brix), in an agitated tank of 5 m^3. Fermentations were conducted as fed-batches. Biomass was diluted in water in an agitated tank and transferred to one pair of fermentation tanks. Air was injected at 1 VVM to allow biomass multiplication and molasses was fed at the flow rate of 2 m^3/h in each tank for 4 h, until tanks were half-filled. Then, half of the content of each tank was transferred to a pair of subsequent tanks and the procedure for biomass multiplication was repeated. The first pair of tanks was fed again with molasses at 4 m^3/h, until tanks were filled to 80% of their capacities, and fermentation was conducted, with no aeration, to the end of CO_2 liberation. This procedure was repeated for the other pairs of tanks. Dispersant agent was added at the beginning of feeding and antifoam was used to break the formed foam after feeding. Temperature control (at 30 °C) and agitation were assured by circulation of the broth through plate heat exchangers.

When fermentation was finished, biomass was separated by continuous centrifugation, the yeast cream containing around 60% solids was treated with acid (H_2SO_4 to pH 2.2 for 2 h), in order to avoid flocculation and inactivate the weak cells, in a 5.5 m^3 agitated tank, and then transferred to the fermentation tanks to be used as inoculum. Biomass concentration at the beginning of fermentation was adjusted to at least 3×10^8 viable cells per mL, the inoculum volume being around 30% of the total fermentation volume. The wine was distilled for ethanol recovery.

2.7. Determination of fermentation yields and kinetic parameters

The maximum theoretical ethanol yield from sugar was calculated according to the stoichiometric relation represented by Eq. (1), i.e., 100 g of hexose produce 51.1 g of ethanol and 48.9 g of CO_2. Ethanol yields over total initial sugars (Y_1) and consumed sugars (Y_2) were calculated according to Eqs. (2) and (3).

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \quad (1)$$

$$Y_1\ (\%) = \frac{[p_f - p_0, g/L] \times 100}{[S_0, g/L] \times 0.511} \quad (2)$$

$$Y_2\ (\%) = \frac{[p_f - p_0, g/L] \times 100}{[S_0 - S_f, g/L] \times 0.511} \quad (3)$$

A mathematical model (polynomial equation of third order) was adjusted to experimental data, representing concentrations as time functions. The maintenance coefficient was determined by solving the mass balance for substrate consumption represented by Eq. (4). By-products formation was not considered. CO_2 formation was calculated in relation to produced ethanol, according to Eq. (1). Conversion yields, specific rates and the maintenance coefficient were determined either using experimental data or the values predicted by the model.

$$\frac{ds}{dt} = \frac{dX}{dt} + \frac{dP}{dt} + \frac{dCO_2}{dt} + mX \quad (4)$$

3. Results and discussion

3.1. Characterization of soybean molasses

The composition of soybean molasses is shown in Table 1. Carbohydrates, representing 57.3% of molasses' dry mass, include mainly sucrose (28.4%), which is a fermentable disaccharide for yeasts, and also stachyose (18.6%) and raffinose (9.68%), complex tetra- and trisaccharides, respectively, that are not fermentable by many microorganisms due to the presence of α-1,6 bonds (Lan et al., 2007). The molasses contains also significant amounts of proteins (9.44%) and lipids (21.2%).

3.2. Selection of the yeast

Table 2 shows the results of the microorganism screening test. Analysis of variance at the level of 5% demonstrated that at least one average yield is statistically different. According to the Tukey test, the ethanol yield presented by the strain LPB-SC (41.5% of the theoretical from total initial sugars) was significantly higher than the other averages, except for strains LPB 1, 4 and 5. Since the strain LPB-SC was available in the form of pressed yeast (thus facilitating inoculum preparation – see Section 2.3), it was chosen for the subsequent fermentation tests with soybean molasses.

3.3. Definition of fermentative conditions at laboratory scale

3.3.1. Effect of initial concentration of soybean molasses in bio-ethanol production

Results presented in Table 3 show that the only significant drop in fermentation yield occurs for the initial concentration of 35 °Brix (38.53%), the highest value being 42.57%, obtained at 25 °Brix. As it was expected, fermentation time increases in direct proportion to initial soluble solids concentration. However, decrease in productivity was observed only at 35 °Brix. Several studies have reported that high substrate concentrations inhibit growth and fermentation of yeasts in industrial ethanol production as the result of high osmotic pressure (Takeshige and Oushi, 1995). On the other side, low dilution rates represent an economy of equipment and process costs (e.g., distillation costs). In this way, for industrial applications, it was established that the concentration of 30 °Brix would be more appropriate.

Table 1
Composition of soybean molasses

Component	% in dry basis	Standard deviation
Glucose	0.243	0.06860
Fructose	0.127	0.06576
Galactose	0.254	0.03250
Sucrose	28.4	2.069
Lactose	–	–
Raffinose	9.68	1.287
Stachyose	18.6	2.828
Total carbohydrates	57.3	1.381
Proteins	9.44	1.160
Lipids	21.2	2.680
Fibers	5.7	2.675
Ash	6.36	1.387

Average of three different samples.

Table 2
Ethanol production yields over total initial sugars (Y_1) after 24 h of fermentation for different *Saccharomyces cerevisiae* strains

Strain	Total sugars (g/L)	Ethanol (g/L)	Y_1 (%)	Standard deviation
N.f.m.[a]	222.1	–	–	–
LPB 1	135.2	38.9	34.3	1.57
LPB 2	125.0	35.9	31.6	0.0567
LPB 3	131.5	37.0	32.6	1.29
LPB 4	135.6	42.9	37.8	2.04
LPB 5	134.9	37.5	33.0	1.82
LPB 6	118.3	33.2	29.3	1.68
LPB-SC	126.9	47.1	41.5	2.07
LPB-MA	145.0	30.6	27.0	0.551
LPB-JP	121.7	30.5	26.9	0.905
LPB-FR	127.9	30.3	26.7	2.13

Initial concentrations were 30 °Brix for the soybean molasses (non-supplemented, natural pH of 5.5) and 1×10^8 cells/mL for biomass. Average of two experiments.
[a] N.f.m. – non-fermented medium.

3.3.2. Effect of nutrients supplementation to the soybean molasses medium in bio-ethanol production

Results presented in Table 4 show that the addition of nutrients to the medium did not improve ethanol production and process yield. The highest yield (48.86%) was obtained when the medium was not supplemented with salts. The soybean molasses is rich in mineral salts (6.36% ashes) and contains a considerable amount of proteins that can be used as nitrogen source by the yeast. Effect of nutrients addition was tested in a medium with 20% soluble solids to avoid inhibition by osmotic pressure. For other substrates such as beet molasses, addition of nitrogen source (ammonium sulfate 0.2 g/L) can improve ethanol production in about 10% (Nahvi et al., 2002). Because of the digestible nitrogen deficiency of sugar beet molasses, ammonium phosphate, ammonium dihydrogen phosphate and ammonium sulfate are usually added to the fermentation medium for better productivity (Ergun and Mutlu, 2000).

3.3.3. Effect of initial pH in bio-ethanol production

There was no significant difference among the initial pH tested (4.3, 5.0 and 5.5) regarding ethanol production, comparing with non-supplemented soybean molasses diluted to 30 °Brix. The medium fermented at the natural pH of soybean molasses (5.5) presented an average yield over total initial sugars (Y_1) of 50.39% (2.92 standard deviation) after 25 h of fermentation. Yields obtained in this experiment were high in comparison to the ones presented in Table 3 (average 42.08% for the same conditions). This difference may be due to changes in the molasses' sugar composition.

3.3.4. Kinetics of bio-ethanol production at laboratory scale

The fermentation development was adjusted according to the desired condition at industrial scale, a 6 h fermentation step. For that, 30 g/L of pressed yeast had to be added at the beginning of fermentation, providing an initial concentration of 3×10^8 viable cells/mL (6 g cells/L in dry basis). Table 5 shows the kinetics of bio-ethanol production from soybean molasses under optimized conditions in bioreactor. The maximum ethanol productivity (10.8 g/L h) occurred after 3 h of fermentation. A product yield ($Y_{P/S}$) of 45.4% from consumed substrate represents 88.8% of the theoretical maximum. Considering only the initial substrate

Table 3
Effect of media initial soluble solids concentration (°Brix) on ethanol productivity and yield over total initial sugars (Y_1)

°Brix	Fermentation time (h)	Total sugars (g/L)	Ethanol (g/L)	Average productivity (g/L h)	Y_1 (%)	Average yield (%)	Standard deviation
20	0	169.5	0		–		
	20	87.4	38.0	1.820	43.87	42.08	1.266
	20[a]	93.4	34.9		40.29		
25	0	207.3	0		–		
	25	108.2	46.6	1.804	43.99	42.57	1.001
	25[a]	107.8	43.6		41.16		
30	0	267.5	0		–		
	30	138.3	52.1	1.867	38.11	40.97	2.019
	30[a]	134.3	59.9		43.82		
35	0	311.6	0		–		
	40	152.3	63.5	1.530	39.88	38.53	0.9546
	40[a]	151.3	59.2		37.18		

Media fermented without nutrients supplementation and pH adjustment. Initial biomass concentration was 1×10^8 cells/mL.
[a] Represents the duplicate experiment.

concentration (182 g/L), ethanol yield was 45.8% of the theoretical maximum. This is equivalent to 134.1 kg or 169.8 L of absolute ethanol per ton of dry molasses, since one ton of dry molasses contains 573 kg of sugar (Table 1).

Experimental data (presented in Fig. 2) were used to derive third order polynomials, representing concentrations (S, X and P) as functions of fermentation time (t). These are Eqs. (5)–(7) presented below. Kinetic parameters based on experimental data and on the values predicted by the model are presented in Table 6.

$$S(t) = 0.536t^3 - 5.74t^2 - 0.631t + 182, \quad R^2 = 0.999 \tag{5}$$

$$X(t) = -0.0036t^3 + 0.0367t^2 + 0.0296t + 6.00, \quad R^2 = 0.998 \tag{6}$$

$$P(t) = -0.244t^3 + 2.57t^2 + 0.468t + 0.338, \quad R^2 = 0.998 \tag{7}$$

Table 4
Ethanol yields over total initial sugars (Y_1) after 20 h of fermentation

Sample	Total sugars (g/L)	Ethanol (g/L)	Y_1 (%)	Average yield (%)	Standard deviation
M	156.1	0	–	–	–
M[a]	151.9	0	–		
A	73.3	39.1	49.69	48.86	0.5870
A[a]	65.2	37.8	48.03		
B	70.1	36.7	46.64	43.15	2.471
B[a]	70.2	31.2	39.65		
C	76.3	33.0	41.93	42.95	0.7112
C[a]	75.2	34.6	43.97		
D	72.5	34.7	44.09	44.28	0.1343
D[a]	75.8	35.0	44.47		

A, no addition of nutrients; B, addition of magnesium source ($MgSO_4$, 0.1 g/L); C, addition of nitrogen source (NH_4NO_3, 3.5 g/L); D, addition of magnesium and nitrogen sources; M, non-fermented medium. Initial concentrations were 20 °Brix for soybean molasses and 1×10^8 cells/mL for biomass.
[a] Represents the duplicate experiment.

Values representing yield in biomass ($Y_{X/S}$, 0.00815) and growth rate (μ_X, 0.0189) are very small in comparison to product yields (Table 6). These small values were expected since there is no oxygen supply and the soluble solids concentration is high (30%) and, consequently, dissolved oxygen concentration is low. Echegaray et al. (2000) reported values as high as 0.116 for $Y_{X/S}$ and ranging from 0.24 to 0.019 for μ_X for the anaerobic fermentation of sugarcane molasses by *S. cerevisiae* containing around 170 g/L of sugars (for this substrate, equivalent to 17% soluble solids). The decrease in μ_X along time was possibly due to product inhibition.

The calculated maintenance coefficient of 0.27 h^{-1} (Table 6) was similar to the value reported by Pirt (1975) for anaerobic fermentation by *S. cerevisiae* cultivated in glucose medium with 1 M NaCl (0.36 h^{-1}), which has a osmotic pressure comparable to that on the 30% molasses medium.

3.3.5. Consumption of individual sugars

Structural analysis of the main sugars present in soybean molasses (stachyose, raffinose and sucrose) shows that all of them have a β-1,2 bond, which is cleaved by the enzyme invertase (β-D-fructofuranoside fructohydrolase, E.C. 3.2.1.26). It is known that the yeast *Saccharomyces cerevisiae* produces intra and extracellular invertase. The extracellular invertase resides in the periplasmic space and is responsible for cleaving sucrose into glucose and fructose, monomers that are assimilated and converted into ethanol (Zech and Görisch, 1995). The other bonds, except for sucrose which is a disaccharide, are of the α-1,6 type, cleaved by the enzyme α-galactosidase (E.C. 3.2.1.22). This enzyme is probably not produced by the yeast LPB-SC, since there is such a high concentration of residual sugars after fermentation (Table 5).

The main sugars that remain after fermentation are tri- and disaccharides, whereas 48% of the soybean molasses' sugars are not fermented by the strain LPB-SC. Trisaccharides present in

Table 5
Kinetic study of bio-ethanol production from soybean molasses in bench scale bioreactor under optimized conditions: medium with 30% soluble solids, without pH adjustment or nutrients supplementation, initial biomass concentration 3×10^8 cells/mL

Time (h)	S (g/L)	X (g/L)	P (g/L)	r_S (g/L h)	r_X (g/L h)	r_P (g/L h)	$Y_{X/S}$ (%)	$Y_{P/S}$ (%)
0	182.0	6.00	0.400	–	–	–	–	–
1	176.1	6.05	3.20	5.9	0.05	2.8	0.847	47.5
2	163.6	6.18	8.80	12.5	0.13	5.6	1.04	44.8
3	140.5	6.31	19.6	23.1	0.13	10.8	0.563	46.7
4	123.9	6.49	27.0	16.6	0.18	7.4	1.08	44.6
5	102.1	6.59	36.4	21.8	0.10	9.4	0.459	43.1
6	87.6	6.72	43.0	14.5	0.13	6.6	0.897	45.5
Average				15.7	0.12	8.08	0.815	45.4

Concentrations represent the average of two experiments. Average standard deviations were 4.77% for sugar, 2.10% for biomass and 5.24% for ethanol concentrations.

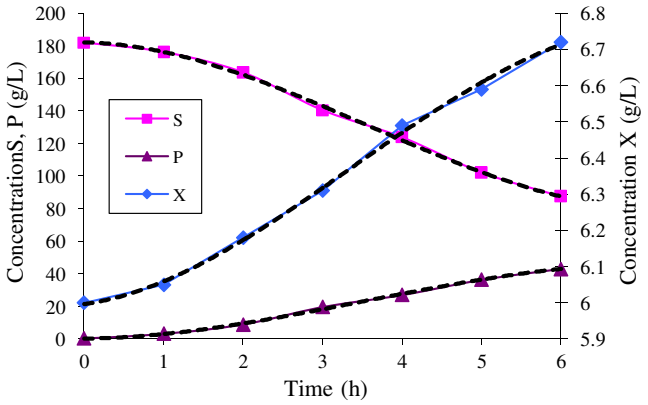

Fig. 2. Evolution of substrate (*S*), ethanol (*P*) and biomass (*X*) concentrations during bench scale fermentation of soybean molasses under optimized conditions, and the corresponding third order polynomial tendency lines.

Table 6
Fermentation kinetic parameters determined by using experimental data and by the resolution of the mathematical model (average values)

	$Y_{X/S}$	$Y_{P/S}$	m	μ_S	μ_X	μ_P
Experimental data	0.00815	0.454	0.270	2.47	0.0189	1.12
Mathematical model	0.00837	0.456	0.274	2.48	0.0189	1.12

soybean molasses are composed of raffinose and partially hydrolyzed stachyose, which is a tetrasaccharide. In the same way, disaccharides include sucrose and partially hydrolyzed raffinose. In this way, once it is known that sucrose is utilized by the yeast, it is supposed that the residual carbohydrates are oligomers linked by α-1,6 bonds. From stachyose and raffinose, only the terminal fructose and glucose units would be consumed, respectively.

Based on these considerations, it is possible to calculate the maximum theoretical yield in ethanol from the soybean molasses, counting only the fermentable sugars. Since glucose, fructose and galactose have the same molecular mass, the fourth and the third part of stachyose's and raffinose's mass concentrations, respectively, can be considered fermentable sugars. In this way, according to Table 1, the fermentable sugars' concentration in soybean molasses is 36.90% (dry basis), or 64.40% of the total amount of sugars. Therefore, the maximum theoretical yield in ethanol from soybean molasses' dry mass would be 18.86% (w/w) or 23.70% (v/w).

3.4. Bio-ethanol production at pilot scale

The main objective of scaling-up a process is to identify problems that were not significant at laboratory scale, and also check if the fermentation yield is maintained. For the first batch fermentation at pilot scale, a major problem identified was the high foam formation. The soybean molasses has a significant concentration of proteins and lipids that may contribute to increase viscosity and surface tension. Although molasses' viscosity should diminish while ethanol concentration increases, the intense production of CO_2 bubbles is a factor that strongly favors foam formation (Togrul and Arslan, 2004).

An alternative to control foam formation is the fed-batch process, which is considered one of the most useful systems for economical ethanol fermentation (Roukas, 1996). Controlled substrate feeding, besides avoiding intense CO_2 production, prevents inhibition and catabolite repression, improving productivity of fermentation by maintaining a low substrate concentration (Prasad et al., 2007). Addition of antifoam and dispersant agents was also necessary to prevent foam formation.

Table 7 shows the results of 11 fermentation cycles conducted at the pilot scale plant. Effects of antifoam and dispersant agents addition are shown in Table 8. An average product yield of 44.13% (standard deviation 1.323) over the total initial sugars (Table 7), represents an ethanol yield of 129.2 kg or 163.6 L of absolute ethanol per ton of dry molasses. The average productivity for the fed-batch process was 7.882 g/L h (standard deviation 0.8560).

Roukas (1996) reported a maximum productivity value of 3.8 g/L h for the fed-batch fermentation of beet molasses with initially 250 g/L sugars and 3.7×10^8 cells/mL. These conditions are similar to those of the eleventh fermentation cycle (Table 7), when a productivity of 6.916 g/L h was achieved. However, it is important to remark that the sugarbeet molasses is composed mainly of sucrose (Vicik et al., 1990), while soybean molasses contains almost 50% of non-fermentable sugars, consequently, ethanol concentration is lower and product inhibition may be less expressive.

The dispersant agent added at 5 mL/m^3 showed good efficiency in preventing the foam formation during the feeding. A minimum concentration of 35 mL/m^3 of antifoam agent had to be added after feeding to dissolve the formed foam and prevent its formation during the last 2 h of "batch" fermentation (Table 8). After the results obtained at lab scale were reproduced at the pilot plant and the necessary parameters were defined, the process was transferred to an industrial plant for ethanol production, with a production capacity of 10 m^3 ethanol per day.

Table 7
Results of pilot scale fermentations for bio-ethanol production from soybean molasses at various °Brix using an initial biomass concentration of 3×10^8 cells/mL

Cycle nr./op. mode[a]	°Brix	Initial sugar (g/L)	Final sugar (g/L)	Ethanol (g/L)	Time (h)	Productivity (g/L h)	Yield[b] (%)
1/B[c]	20.0	116.6	54.00	26.10	6	4.350	43.80
2/B	30.0	251.8	118.2	58.60	6	9.767	45.54
3/FB[d]	21.0	166.9	59.1	39.28	7	5.611	46.06
4/FB	22.0	168.9	79.2	41.92	5	8.384	48.57
5/FB	23.0	185.0	89.9	43.72	5	8.744	46.25
6/FB	21.0	182.1	83.3	40.01	4	10.00	43.00
7/FB	21.0	189.5	76.9	40.50	6	6.750	41.82
8/FB	21.5	190.7	85.0	40.81	6	6.802	41.88
9/FB	22.0	180.8	83.2	41.14	5	8.228	44.53
10/FB	23.0	173.2	80.0	38.00	4	9.500	42.93
11/FB	30.0	230.7	74.37	48.41	7	6.916	41.06
Average productivity/yield						7.882[e]	44.13

[a] Operational mode.
[b] Yield over total initial sugars.
[c] B, batch.
[d] FB, fed batch.
[e] For the fed-batches only.

Table 8
Effect of antifoam (R$ 4.40/kg) and dispersant (R$ 11.50/kg) agents addition on foam control and production cost for fed-batch fermentations at pilot scale

Cycle number	Anti-foam (mL/m^3)	Dispersant (mL/m^3)	Foam reduction	Ethanol (L/m^3)	Additional cost (R$/L)
4	100	100	+	49.72	0.0309
5	30	30	+	53.06	0.0092
6	30	30	+	52.34	0.0091
7	40	20	+	55.34	0.0068
8	20	20	+	50.65	0.0063
9	40	20	+	51.27	0.0081
10	40	10	+	54.30	0.0053
11	35	5	+	51.66	0.0041

+ Represents sufficient foam reduction.

3.5. Implementation of the process at industrial scale

For the industrial scale fermentations, the molasses concentration was adjusted to 30% soluble solids, in order to provide an ethanol concentration in the fermented broth of 40–50 g/L. This is the minimum concentration required for an economically feasible distillation process (Siqueira, 2006). Foam formation was easily controlled by the techniques developed at pilot scale.

A major problem identified after starting-up the plant was the contamination with bacteria. Microscopy analysis indicated the presence of gram-positive bacilli. This problem was expected since the biomass is recycled along the process and may lose activity, allowing the development of opportunistic contaminants. The problem was solved by the addition of appropriate antibiotic – commonly used in industrial ethanol fermentations, does not affect yeast metabolism – together with the previous treatment of soybean molasses (removal of solids), in order to facilitate the separation of yeast biomass at the end of fermentation and the inoculum acid treatment. The pre-treatment may also preserve the equipments (tanks, pipelines, distillation columns).

As presented in Fig. 3, the mass balance based on industrial data showing all products obtained from soybean processing, the fermentation yield (18.39 kg of absolute ethanol per ton of soybean), representing a yield of 43.89% of the theoretical over the total initial sugars, or 162.7 L of absolute ethanol per ton of dry soybean molasses, was maintained after the second scale-up.

Because of environmental problems, the treatment of the residue generated by the distillation process, called vinasse, is one of the most significant and challenging issues in the industrial production of ethanol. A typical distillery with a daily ethanol production of 100 m^3 has a vinasse discharge of 1300 m^3 having a high pollution load, with BOD values ranging from 30 to 60 gO_2/L (Navarro et al., 2000). The BOD of the vinasse produced by the fermentation of soybean molasses was determined (LCK 555 kit, Dr. Lange) and presented a value of 77.2 gO_2/L.

Siqueira (2006) reported that enzymatic and acid hydrolyses are alternatives to increase ethanol yield from soybean molasses' sugars and, consequently, reduce the BOD of the vinasse. Enzymatic hydrolysis of the molasses by the enzyme α-1,6-galactosidase provided an increase of 20% in ethanol yield from total sugars. However, the yield improvement did not compensate for the additional cost. The acid hydrolysis of the vinasse to release fermentable sugars was also tested and provided an increase of 17% in ethanol yield, but due to the severe conditions demanded (HCl at 10% v/v, pH 0.1, 100 °C, 60 min), the process demonstrated to be economically unfeasible (data not shown).

Currently, the vinasse produced at the industrial plant is being concentrated and incinerated to produce energy, since it cannot be treated as a common wastewater due to its high organic charge. The utilization of the vinasse as raw material for other fermentative processes is being studied.

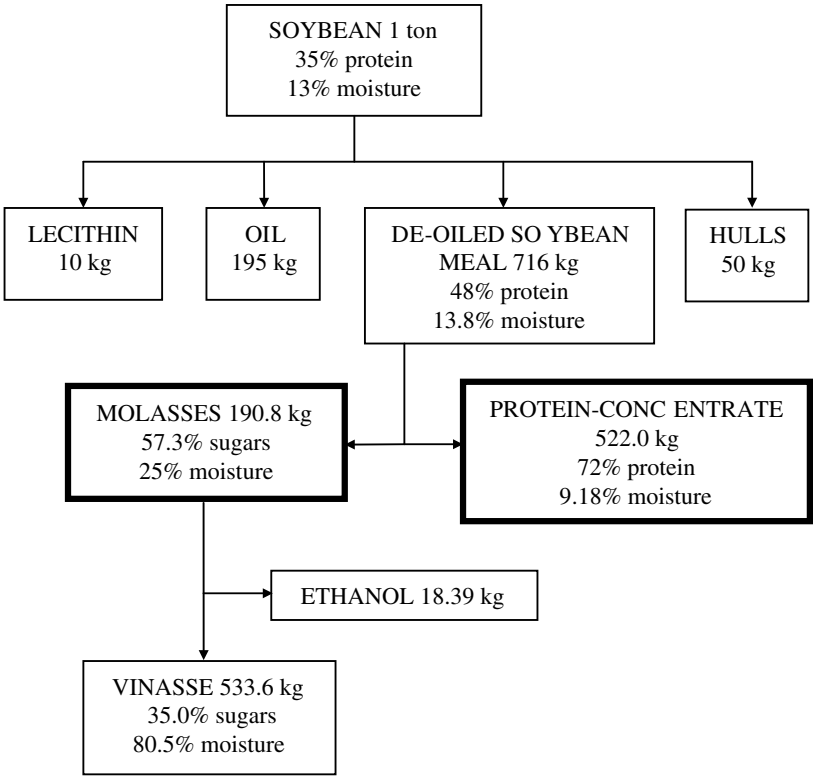

Fig. 3. Mass balance: from soybean to ethanol. Protein and sugar percentages in dry basis.

4. Conclusions

From the results, it could be concluded that soybean molasses is an attractive raw material for the production of bio-ethanol. Since it provided the necessary nitrogen and magnesium and the appropriate hydrogen balance for the fermentation, there was no need for supplementing these additionally or making any pH adjustment. The laboratory process was scaled-up with promising results. The residue of bio-ethanol production, called soybean vinasse, did not present any environmental problem since it was employed as raw material for energy co-generation.

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