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**PRODUCTION OF BIOETHANOL BY SOYBEAN
MOLASSES FERMENTATION BY *ZYMOMONAS
MOBILIS***

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FERMENTATION BY *ZYMO MONAS MOBILIS***

Dissertação apresentada como requisito parcial para a obtenção do grau de Mestre pelo Programa de Pós-Graduação em Processos Biotecnológicos, Setor de Tecnologia da Universidade Federal do Paraná.

Orientador:
Prof. Dr. Carlos Ricardo Soccol

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Aos meus queridos pais
e ao meu querido irmão.

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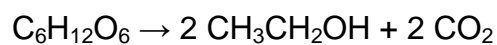
PRODUCTION OF BIOETHANOL BY SOYBEAN MOLASSES FERMENTATION BY *ZYMOMONAS MOBILIS*

1 INTRODUCTION

1.1 ETHANOL

Ethanol (C₂H₅OH), also known as ethylic alcohol, is a substance obtained from sugars fermentation, commonly used in alcoholic beverages, like beer, wine and brandy, and is used too in perfumery. In Brazil, such substance is also an important fuel of explosion motors, this way constituting a rising market to ethanol obtained from a renewable way. It is the establishment of a chemical based industry, sustained on utilization of biomass of agricultural origin and renewable.

Ethanol for use in alcoholic beverages, and the vast majority of ethanol for use as fuel, is produced by fermentation: when certain species of yeast (most importantly, *Saccharomyces cerevisiae*) metabolize sugar in the absence of oxygen, they produce ethanol and carbon dioxide. The overall chemical reaction conducted by the yeast may be represented by the chemical equation:



The process of culturing yeast under conditions to produce alcohol is referred to as brewing. Brewing can only produce relatively dilute concentrations of ethanol in water; concentrated ethanol solutions are toxic to yeast. The most ethanol-tolerant strains of yeast can survive in up to about 25% ethanol (by volume).

During the fermentation process, it is important to prevent oxygen getting to the ethanol, since otherwise the ethanol would be oxidized to acetic acid

(vinegar). Also, in the presence of oxygen, the yeast would undergo aerobic respiration to produce just carbon dioxide and water, without producing ethanol. In order to produce ethanol from starchy materials such as cereal grains, the starch must first be broken down into sugars. In brewing beer, this has traditionally been accomplished allowing the grain to germinate, or malt. In the process of germination, the seed produces enzymes that can break its starches into sugars. For fuel ethanol, this hydrolysis of starch into glucose is accomplished more rapidly by treatment with dilute sulfuric acid, fungal amylase enzymes, or some combination of the two.

The natural energy resources such as fossil fuel, petroleum and coal are being utilized at a rapid rate and these resources have been estimated to last over a few years. Therefore, alternative energy sources such as ethanol, methane, and hydrogen are being considered. Some biological processes have rendered possible routes for producing ethanol and methane in large volumes. A worldwide interest in the utilization of bio-ethanol as an energy source has stimulated studies on the cost and efficiency of industrial processes for ethanol production (CYSEWSKI, 1978). Intense research has been carried out for obtaining efficient fermentative organisms, low-cost fermentation substrates, and optimum environmental conditions for fermentation to occur.

1.2 SOYBEAN MOLASSES

Soybean molasses is a low cost material which imposes environmental disposal problems and therefore is used mainly as low-cost animal feed ingredient. The main constituents of soy molasses, soy sugars, consist of about

65% mono and disaccharide and about 35% oligosaccharides, mainly, raffinose (5-7%) and stachyose (30-32%).

The high percentage of mono and disaccharide present in soybean molasses make it a good substrate for growth of *Zymomonas mobilis*, and for production of bioethanol. If the oligosaccharides could be broken into simpler sugars, the productivity could be even greater.

The aim of this and others works is to change the view that soybean molasses is a byproduct, and rise it to a category of an important substrate for bioethanol production.

1.3 ZYMOMONAS MOBILIS

Traditionally, ethanol has been produced in batch fermentation with yeast strains that cannot tolerate high concentration of ethanol. This necessitated the strain improvement programme for obtaining alcohol-tolerant strains for fermentation process. *Zymomonas mobilis*, a gram-negative bacterium, is considered as an alternative organism in large-scale fuel ethanol production. Comparative laboratory- and pilot-scale studies on kinetics of batch fermentation of *Z. mobilis* versus a variety of yeast have indicated the suitability of *Z. mobilis* over yeasts due to the following advantages:

- i. its higher sugar uptake and ethanol yield,
- ii. its lower biomass production,
- iii. its higher ethanol tolerance,
- iv. it does not require controlled addition of oxygen during the fermentation, and
- v. its amenability to genetic manipulations.

The only limitation of *Z. mobilis* compared to the yeast is that its utilizable substrate range is restricted to glucose, fructose, and sucrose. *Z. mobilis* was originally isolated from alcoholic beverages like the African palm wine, the Mexican '*pulque*', and also as a contaminant of cider and beer in European countries. On the basis of evaluation using the modern taxonomic approaches, the genus *Zymomonas* (SWINGS, 1977) has only one species with two subspecies, *Z. mobilis* subsp. *mobilis* and *Z. mobilis* subsp. *pomaceae*. The ability to utilize sucrose as a carbon source distinguishes *Z. mobilis* from *Z. anaerobia* (SWINGS, 1984). It is one of the few facultative anaerobic bacteria which metabolizes glucose and fructose via the Entner–Deudoroff (E–D) pathway, which is usually present in aerobic microorganisms (MONTENECOURT, 1985). Under anaerobic conditions, *Z. mobilis* produces byproducts such as acetoin, glycerol, acetate, and lactate, which result in reduced production of ethanol from glucose. During growth of *Z. mobilis* in fructose, the formation of acetoin, acetic acid, and acetaldehyde was clearly more pronounced than when grown in glucose. However the cell yield was low during its growth in fructose.

In addition to ethanol fermentation, *Z. mobilis* has potential application in polymer production. Levan, a polymer of fructose units linked by β -2,6-fructosyl bond, is produced by *Z. mobilis* during its growth on sucrose medium. Microbial levan is of commercial importance and is used as a thickening, gelling, and suspending agent. In recent years, strategies to improve the yield of levan production by microorganisms attracted greater attention, but in this work, the production of other products than ethanol is not desirable.

Several reports about the utilization of “byproducts” to produce ethanol under *Zymomonas mobilis* have been presented. For example, in Japan, (Investigation of the Utility of Pineapple Juice and Pineapple Waste Material as Low-Cost Substrate for Ethanol Fermentation by *Zymomonas mobilis*, Tanaka, et al, 1999), pineapple juice and pineapple waste were investigated. They reported a production of 59 g/l of ethanol in undiluted pineapple juice without supplementation. In Thailand (Evaluation of Thai Agro-industrial Wastes for Bioethanol Production by *Zymomonas mobilis*, Ruanglek et al, 2006) Thai agroindustrial wastes were investigated. Ami-ami solution, autolysate of brewer’s yeast and hydrolysate of fish waste could support *Zymomonas mobilis* growth as effectively as 56, 61 and 96% when compared to a control medium. Also, in a work performed in Australia (Evaluation of *Zymomonas mobilis*-based ethanol production from a hydrolysed waste starch stream, Davis et al, 2006) it is shown that *Zymomonas mobilis* got superior fermentation kinetics when compared to *Saccharomyces cerevisiae* in three different media.

2 PRACTICAL OBJETIVES

2.1 MAIN OBJECTIVE

The main objective of this work is to optimize some variables to increase the production of ethanol by soybean molasses fermentation using *Zymomonas mobilis*.

2.2 SECONDARY OBJECTIVES

- To rise sucrose, glucose and fructose concentrations in soybean molasses by acid and enzymatic hydrolysis;
- To optimize the conditions of acid hydrolysis;
- To determine the profiles of sugar consumption and ethanol production;
- To compare *Zymomonas mobilis* and *Saccharomyces cerevisiae* yields of ethanol production;
- To compare the performance of the bacterium in static fermentation and bench batch fermentation.

3 MATERIALS AND METHODS

3.1 STRAINS REACTIVATION AND SEED CULTURE OBTAINION

The *Zymomonas mobilis* strains NRRL 806 e ATCC 35001 (provided by André Tosello Foundation - FAT) were used in a preliminary test. Both strains were reactivated from cultures stored in a freezer, in ZM medium (20 g/l sucrose, 10 g/l yeast extract e 10 g/l peptone) and cold protected with glycerol (50% v/v) in ependorfs of 2ml (utile volume of 1ml).

There were prepared assay tubes of 10 ml (util volume of 5ml) with ZM medium, to inoculate the strains from ependorfs. The tubes were maintained at the stove at 30 °C until biomass appearance (visual perception). The approximated time of waiting was of 5 for both strains. After confirmation of the strains purity (microscopy), the content of the tubes was used as seed culture for 125ml erlenmeyers (utile volume of 50ml) filled with ZM medium too. The erlenmeyers were maintained in shaker, at a rotation of 120 rpm, at 30 °C, for

24h. After the seed culture obtainment in erlenmeyers, successive transferences in diluted and centrifuged soybean molasses were carried out, in order to adapt the strains to this new environment. After at least 3 transferences (initially in 5 °BRIX soybean molasses and from the second transference and on, in 10 °BRIX soybean molasses, always in shaker and in the same conditions listed above) there were obtained the seed cultures to the assays. To each assay it was used a fresh seed culture (always centrifuged and at 10 °BRIX), from the mother seed culture (described above) or from younger ones prepared later.

3.2 SELECTION OF THE BEST ETHANOL PRODUCER STRAIN

In a preliminary test, both strains (NRRL 806 e ATCC 35001, named from here “806” and “FAT”, respectively) were inoculated in soybean molasses at 10 and 15 °BRIX. It were used 125 ml erlenmeyers with utile volume of 100ml, and the rate of inoculation was 10% (90ml + 10ml). Fermentations were conduced at the stove (30 °C) and samples were collected at 3, 6, 9, 12, 14 and 16h of fermentation (besides the samples collected in the moment of the inoculation, “time 0”) for ethanol content determination.

To further tests, strain 806 was chosen, because it presented superior results concerning ethanol production.

3.3 ASSAYS WITH INITIAL AERATION OF THE FERMENTATION BROTH

A first try of increasing the ethanol production was the use of agitation (that provides either extra oxygen for the cells and better homogenization of the

broth) in the first hours of fermentation. There were employed soybean molasses solutions of 5 and 10 °BRIX. At the start of fermentation the flasks were submitted to agitation of 120 rpm, at 30 °C. After a period of time, the flasks were stored at a stove (30 °C too). In this assay there were tested two levels of time for the initial agitation: 3h and 6h. Samples were collected at 0, 6, 18 and 24h of fermentation. The inoculation rate was of 10% and the incubation was conducted at a temperature of 30 °C. Erlenmeyers of 125ml (utile volume of 100ml) were helpful on these tests.

After some tests with pH and molasses concentration, it was decided to retry some assays with initial aeration (due to not conclusive results coming from the previous one). The new test (conducted in duplicate) was made with a higher soybean molasses concentration (18 °BRIX) and pH adjusted to 6,0 at the start of the fermentation. There were analysed four levels of initial agitation: 1h, 3h and 4h, besides the control point (no agitation at the start). Samples were collected 21h after the start of fermentation.

3.4 EFFECT OF SEED CULTURE AGE

In this assay it was evaluated the influence of seed culture age (15 and 21h of aerobic cultivation for seed culture obtainment) and the soybean molasses concentration (10 °BRIX and 20 °BRIX). The inoculation rate was fixed in 10%, the stove incubation was carried out at 30 °C and the pH was 5,5 (natural soybean molasses pH, approximately). Amber flasks were used (30ml of total volume and 25ml of utile volume), covered with cotton and Kraft paper.

3.5 EFFECT OF pH AND SOYBEAN MOLASSES CONCENTRATION

In this assay there were evaluated the pH (5,0 and 6,0) and, again, the soybean molasses concentration, but now in another levels (20 and 30 °BRIX). The samples were taken 16h after the fermentation. The inoculation rate was of 10%, the temperature of 30 °C, and a seed culture age of 21h. Once more, amber flasks were used, in the same conditions described above for the previous experiment. The samples were collected after 16h of fermentation.

In another assay (carried out in duplicate) the only variable tested was the pH. The chosen levels were: 4,0; 5,0; 6,0; 7,0 and 8,0. The soybean molasses concentration used was of 18 °BRIX (since it was not known exactly the concentration of molasses that start to inhibit the growth, it was adopted a conservative strategy: to use a lower concentration than a previously tested one that did not cause any problems to the strain to growth). The inoculation rate, temperature and seed culture age were the same that described on the last paragraph. Since the manipulation of the amber flasks were causing some troubles (the main one: difficulties of homogenization to collect the samples) erlenmeyers flasks were used on this assay (125ml of total volume, with an utile volume of 100ml. Samples were collected at time 0h and at 20h of fermentation. As in other assays, the concentration of ethanol at 20h was deduced from the concentration of time 0 (ethanol coming from the seed culture).

To analyze the soybean molasses separately, the chosen levels were: 17,5; 20,0; 22,5; 25,0 and 27,5 °BRIX (tests conduced in duplicate). The pH was fixed at 6,0 (function of preview results). The same conditions of temperature, rate of inoculation and seed culture age were adopted in relation to the previous assays. Erlenmeyer flasks were used (same volumes described

above). Samples were collected at time 0h and at 20h of fermentation. The concentration of ethanol at 20h was deduced from the concentration of time 0 (ethanol coming from the seed culture).

3.6 EFFECT OF INOCULATION RATE

Five different levels of inoculation rate were employed (in duplicate): 10%, 20%, 30%, 40% and 50%. The samples were collected 19h after the inoculation, and the incubation was carried out at 30 °C, and the flasks contained a final concentration of soybean molasses of 18 °BRIX. It was chosen the pH of 5,5 instead of the pH 6,0. The explanation for this choice: since there were great difference in the volumes of seed culture, it was better to use the natural pH of soybean molasses (approximately 5,5) instead of another one (it would be harder to establish homogeneous conditions in all the flasks otherwise). The seed culture was 21h aged and erlenmeyers of 125ml with 100ml of utile volume were used.

3.7 KINETICS OF PRODUCTION OF ETHANOL AND CONSUMPTION OF SUGARS

3.7.1 Assays in erlenmeyer flasks

It were realized two kinetics tests (in duplicate) for the determination of ethanol and sugars profile during the process. Some of the conditions adopted in these experiments were chosen at the light of results observed in previous ones. To say: soybean molasses concentration (15 °BRIX and 20 °BRIX), pH (6,0 for both assays) and seed culture age (21h for both assays). The inoculation rate, however, was maintained at 10% (for both assays) to simplify

the procedures and not to change so much other conditions (like concentration and pH) and also to facilitate eventual comparisons with other assays. The tests were conducted at 30 °C and there were used erlenmeyers of 125ml with 100ml of utile volume.

3.7.2 Assay in bench scale batch reactor

Similar conditions that those cited above were fixed to ferment a 15 °BRIX broth of soybean molasses in a batch reactor (BE Marubishi, MDL) of 6l total volume (5l of utile volume). To say: pH (6,0), seed culture age (21h), inoculation rate (10%) and temperature (30 °C). The temperature was controlled automatically and the agitation rate was of 65 to 70 rpm, that is enough to homogenize the medium but not enough to provide extra oxygen (what is not desirable in this step, the production of ethanol).

3.7.3 Fermentation by an yeast in batch reactor

In order to compare the performance of *Zymomonas mobilis* with a well known ethanol producer, a similar test of that related above was carried out with *Saccharomyces cerevisiae* (a commercial product, named "LEVASAF"). The concentration of the soybean molasses was 15 °BRIX, the pH was adjusted to 5,0 (best condition for the yeast), the temperature was automatically controlled at 30 °C and the agitation rate was of 70 rpm. The utile volume of the reactor was of 5l. It was used a mass of cells that corresponded to a start concentration for the process of $1,8 \times 10^7$ CFU/ml (about one log higher than the number of viable cells of bacteria at time 0 on the last described experiment).

3.8 ACID AND ENZIMATIC HYDROLYSIS

Preliminary tests (in duplicate) were performed to determine the better concentration of each one of the tested acids (chloridric acid, sulfuric acid and phosphoric acid) to perform the hydrolysis. The criterion adopted was: the smallest concentration capable of degrade the content of complex sugars for a 15 °BRIX soybean molasses solution. The tested levels of phosphoric acid tested were: 0,1N and 1,0N. To chloridric acid: 0,25N; 0,5N; 1,0N and 2,0N. To sulfuric acid: 0,1N; 0,2N; 0,5N and 1,0N. After the selection (HCl: 0,5N; H₂SO₄: 0,2N; H₃PO₄: 1,0N), the hydrolysis were performed with the selected levels of each acid (in duplicate) in a medium containing soybean molasses at 18 °BRIX. The time was of 15 minutes and the temperature of 121 °C (since in all the other assays it was used the sterilization process with autoclave, the temperature of that step was exploited to the hydrolysis step). After that, the media had its pH adjusted to 6,0 with the add of sodium hydroxide, and the inoculation was performed. The temperature of the fermentation was of 30 °C, the seed culture age was of 21h and the rate of inoculation of 10%. Erlenmeyers of 125ml were used (100ml of utile volume).

Besides the acid hydrolysis, it was performed an enzymatic one too. The conditions used were the same that optimized by SIQUEIRA, P. F. (2006). The cited conditions are: pH 5,0; concentration of enzyme: 500(μ l of enzyme) / (l of medium); 24h of incubation; temperature of 55 to 60 °C. The conditions of the assay (soybean molasses concentration, pH at the moment of inoculation, seed culture age, inoculation rate, flasks used) were the same that described for the hydrolysis assay. The fermentations were carried out until 20h, when samples were collected. Samples were collected at time 0 too (both for acid and

enzymatic proofs). The concentration of produced ethanol was obtained by subtracting the read at 20h from that of 0h.

3.9 EXPERIMENTAL PLAN WITH 3 VARIABLES: SOYBEAN MOLASSES CONCENTRATION, pH AND RATE OF INOCULATION

The fixed conditions of this test were the seed culture age (21h) and temperature (30 °C). The test was conducted in duplicate. Each variable assumed two levels, and the choice was based on results of previous experiments: soybean molasses concentration (17,5 and 27,5 °BRIX), pH (5,0 and 7,5) and inoculation rate (10 and 20%). To soybean molasses concentration, there were chosen equidistant values from the point that gave the better ethanol production (22,5 °BRIX). To pH, there were chosen values centered on pH 6,25, because the test with pH as the sole variable both pH 6,0 and 7,0 showed good results, a little bit better for 6,0 than 7,0. So, the centered point was chosen to be nearer to 6,0 than 7,0. The inoculation rate levels were chosen at lower levels. It was considered not only the results of previous tests, but also economical and practical points. A central point was considered too: 22,5 °BRIX; pH 6,25; rate of inoculation of 15%. At the same conditions of the central point, it was performed an assay with enzymatic hydrolysis, the same way as described in topic 3.8. The flasks used were erlenmeyers of 125 ml with 100ml of utile volume (80 + 20 for the tests with 20% of rate of inoculation; 85 + 15 for those one with 15% and 90 + 10 for those with 10%). The medium was prepared in such a way that the final concentration (after inoculation) reached the values listed above. Samples were collected at the time 0, 18h and 24h. Ethanol levels were determined by subtracting the final ones per the initial ones.

3.9.1 Calculation of the main effects

The main effect is calculated as the media of individual effects and allows to define what is the median effect of the examined variable over the conditions of the another ones, using the table of coefficients in contrast (- or +). Mathematically, the main effect can be represented by:

$$\text{Main effect} = 2(\sum y^+ - \sum y^-) / b^a \quad (\text{Equation 1})$$

Where:

- y corresponds to the media of the measured individual effects;
- + and – correspond to level “high” or “low” of the variable;
- b^a corresponds to the number of total experiments of the plan

3.9.2 Secondary interactions effect (or effects of 2nd order)

Considering, for example, the variables concentration (C) and pH (P), it can be written that the interaction effect of these two variables (CP), will be gived for:

$$(TC) = 2[(\sum y^{++} + \sum y^{-}) - (\sum y^{-+} + \sum y^{+-})] / b^a \quad (\text{Equation 2})$$

3.9.3 Trifatorial effect (or effect of 3rd order)

In this case, the trifatorial interaction Concentration (C) X pH (P) X Inoculation rate (T) can be defined as:

$$(CPT) = \frac{2[(y^{+--} + y^{-+-} + y^{--+} + y^{+++}) - (y^{---} + y^{++-} + y^{+-+} + y^{--+})]}{b^a} \quad (\text{Equation 3})$$

3.9.4 Standard deviation calculation for effects

It can be demonstrated that, to a factorial plan type 2^a , the estimative of effects variance is given by:

$$S_e^2 = \frac{1}{n2^{a-2}} S^2 \quad (\text{Equation 4})$$

Where:

- n: corresponds to the number of replicates of each conjunct,
- a: is the number of factors
- S^2 : is the estimative of population variance

Considering that exist “n” replicates to each one of the 2^a experiments of the plan (in this case 2^3), and if $y_{i1}, y_{i2}, y_{i3}, \dots, y_{in}$ are observations of the experiment “i”, so it can be stated that:

$$S_i^2 = \frac{1}{n-1} \sum_{j=1}^n (y_{ij} - \bar{y}_i)^2 \quad (\text{Equation 5})$$

Equation 5 is an estimative of variance for the experiment “i”, where $i = 1, 2, 3, \dots, 2^a$ and \bar{y}_i the respective media. Combining the estimatives of the 2^a experiments, it is obtained the estimative for total variance:

$$S^2 = \frac{1}{2^a(n-1)} \sum_{i=1}^{2^a} \sum_{j=1}^n (y_{ij} - \bar{y}_i)^2 \quad (\text{Equation 6})$$

So, considering that S^2 is a good estimative for populational variance

(σ^2), it can be written that:
$$S_e = \pm \sqrt{\frac{1}{n-2}} S^2 \quad (\text{Equation 7})$$

3.10 PREPARATION OF SAMPLES FOR ETHANOL AND SUGARS DETERMINATION

In all the assays the samples were collected in ependorfs of 2ml (usually 1ml of utile volume) and after that centrifuged at 10000 rpm. The supernatant was collected and diluted from 5 to 10 times in distilled water. The diluted fluid was filtered with the aid of a syringe coupled with Millipore filters (22 μm). The filtered fluid was analyzed on *Varian* HPLC and column *Shodex* KS 801, with refraction index detector. Pattern samples of each one of the involved compounds (ethanol, stachyose, raffinose, sucrose, fructose, glucose) were prepared, and filtered to injection. The concentration of the injected pattern samples were of 1ml/l and 2ml/l (except for ethanol, for which the pattern samples were of 1ml/l; 5ml/l and 10ml/l). This way were obtained the calibration curves for each compound. The resulting areas in the chromatograms obtained by the injection of samples from the assays were compared with the calibration curves and multiplied by the respective factors (5 to 10).

3.11 BIOMASS DETERMINATION

Due to difficulties concerning the determination of biomass by dry weight (soybean molasses contains a series of compounds that are partially soluble. These compounds sometimes appear on the supernatant and sometimes on the precipitate. The precipitate is far from being a homogenous paste) there

was chosen the technique of counting of growth colonies. The method used was the *drop plate* (drops of 30 μ l). There were performed serial dilutions of the original sample for this purpose. Drops from these dilutions were let down on Petri dishes containing ZM solid medium (same composition of ZM medium plus 15g/l of bacteriological agar). After the drops, the plates were incubated at the stove, at 30 °C. Two days of incubation were enough for colonies counting (visual perception). The results obtained were given in CFU/ml (colony formation units per milliliter), being considered on the calculations both the volume of the drop and the used dilution.

4 RESULTS AND DISCUSSION

4.1 STRAINS REACTIVATION

Both strains of *Zymomonas mobilis* were reactivated successfully and were well adapted to the soybean molasses medium. Seed cultures cultivated in aerobic conditions (120 rpm on shaker) presented countings ranging from 10^7 to 10^8 CFU/ml.

4.2 SELECTION OF THE BEST ETHANOL PRODUCER STRAIN

Both strains were able to produce ethanol on diluted soybean molasses. However, the liquid production of ethanol by the strain 806 was higher than the production for the strain that was named as "FAT". This fact was observed for both the concentrations of soybean molasses tested: 10 °BRIX and 15 °BRIX.

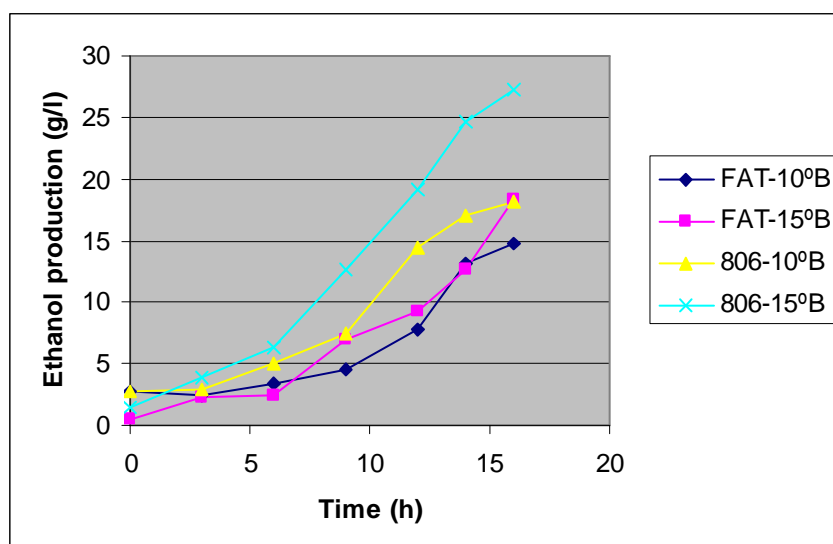
The figure 1 shows the profile of ethanol production from time 0h to time 16h of fermentation. The table 1 shows the calculated liquid ethanol production ([ethanol] at 16h minus [ethanol] at time 0h).

For the test with soybean molasses at 10 °BRIX the performance of the strain 806 was 27,3% better than the other strain. And for the test with soybean molasses at 15 °BRIX the difference was even larger: 44,9%. Therefore, the strain 806 was selected for further tests.

TABLE 1. LIQUID PRODUCTION OF ETHANOL BY THE STRAINS 806 AND FAT AT 16H OF FERMENTATION.

Etanol – produção líquida em 16h (g/l)			
FAT – 10 °BRIX	FAT – 15 °BRIX	806 – 10 °BRIX	806 – 15 °BRIX
12,1	17,8	15,4	25,8

FIGURE 1. COMPARISON BETWEEN THE STRAINS 806 AND FAT CONCERNING ETHANOL PRODUCTION.



4.3 ASSAYS WITH INITIAL AERATION OF THE FERMENTATION BROTH

When cultivated in aerobic conditions *Z. mobilis* uses expressive part of the carbon and nitrogen sources for growth (biomass). When in anaerobic conditions, the carbon sources are used almost entirely to acquisition of energy via Entner-Doudoroff pathway. When this way is activated, the bacteria convert the carbon sources into carbonic gas and ethanol in equimolar proportion (SPRENGER, 1996).

The idea of initial aeration is to make possible that the biomass growth takes place faster, and so the ethanol production could be enhanced.

The results, however, show no evidence of significative difference in ethanol production when oxygen is provided (by agitation) in the starting hours of the fermentation. Both assays in 5 °BRIX and in 10 °BRIX, the flasks maintained 6h under agitation showed results slightly worse (6,5g/l and 13,4g/l at time 18h, respectively for 5 and 10 °BRIX) than that maintained only 3h under agitation (7,9 g/l and 15,0 g/l at time 18h, respectively for 5 and 10 °BRIX). And both results at 10 °BRIX (3h and 6h of agitation) are worse than that presented in figure 1, yellow line (15,4g/l; previous topic). Therefore, these tests suggest that initial aeration is not a decisive factor for ethanol production by this strain on this conditions. However, in most concentrated soybean molasses broths, the effect could be different.

A possible explanation for this results: the yield of ethanol from sugars might have been reduced in the assays with initial agitation. The carbon sources that could have been converted in ethanol might have been used to biomass growth. Samples collected from the flasks of 5 °BRIX were collected at the time 6h. At this time, one of the flasks (5 °BRIX, shaker 6h) was been

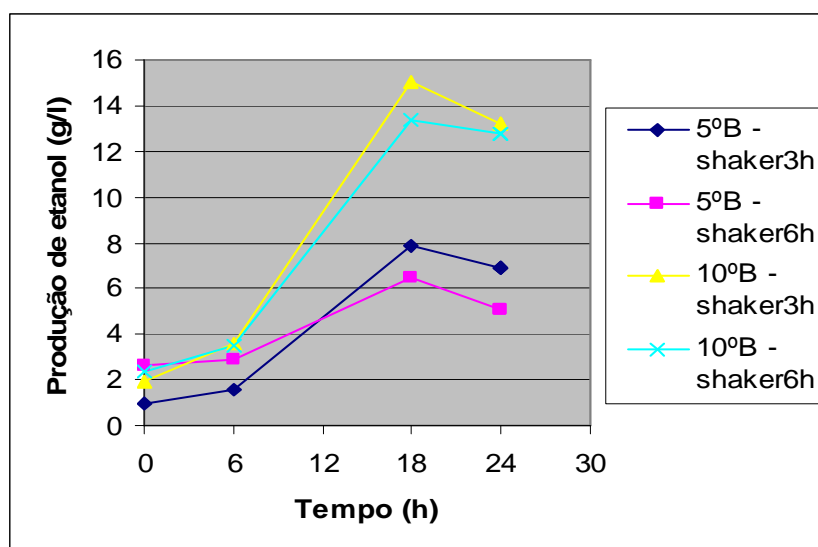
transferred from the shake the stove and another one (5 °BRIX, shaker 3h) was already been transferred to the stove (and was there since the third hour). The results of colony counting are:

* 5 °BRIX, shaker 6h: $4,8 \times 10^7$ CFU/ml;

* 5 °BRIX, shaker 3h: $8,3 \times 10^6$ CFU/ml.

In other words, the agitation really seemed to improve cell growth, but, this way reduced the ethanol production by not allowing the carbon sources to be converted in product.

FIGURE 2. EFFECT OF THE INITIAL AERATION ON ETHANOL PRODUCTION FOR BROTHS AT 5 AND 10 °BRIX.

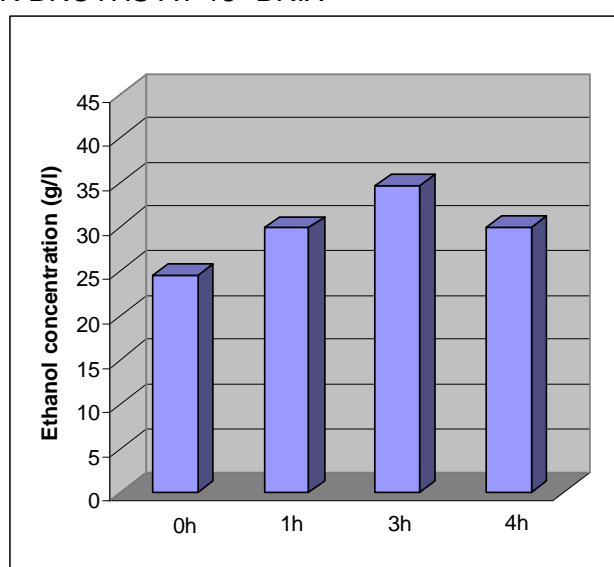


Since the results showed that initial aeration did not work on fermentation at lower soybean molasses concentrations, another assay was performed at a higher concentration. At this point, data from pH and molasses concentration were at disposal. So, a concentration of 18 °BRIX was used, and the pH adjusted to 6,0. The results are in favor of the aeration now. The flasks fermented with 3h of agitation showed the best results (34,67g/l of ethanol),

followed by those ones fermented at 4h and 1h (29,85 and 29,83g/l respectively) and the worst result was observed for the flasks that were stored on the stove since the beginning of the fermentation (24,48g/l).

The possible explanation: agitation favored cell growth by providing oxygen to the media on the starting hours, and then the ethanol production has taken place rapidly. Besides this, the agitation provides a better homogenization of the medium components, and the carbon and nitrogen sources of the molasses are accessed easily by the cells, as well as the cells distribution are closer to uniformity on the medium. When the flask is in a static position, the cells tend to sediment, and cells near the bottom probably will have no access to nutriment, and will die easily. However, too much agitation can contribute to an excessive cell growth, reducing the yield of ethanol from carbon sources. It seemed to have happened with the flasks maintained for 4h on shaker.

FIGURE 3. EFFECT OF THE TIME OF INITIAL AERATION ON ETHANOL PRODUCTION FOR BROTHS AT 18 °BRIX



4.4 EFFECT OF SEED CULTURE AGE

The seed culture age had shown to be an important factor for ethanol production. Assays were carried out with two different seed culture ages: 15 and 21 hours of aerobic fermentation. These seed cultures were tested in broths at 10 and 20 °BRIX. In both assays the older one presented better results with 16 hours of fermentation. Table 2 summarizes the results:

TABLE 2. PRODUCTION OF ETHANOL IN 16H OF FERMENTATION, IN G/L, FOR DIFFERENT SEED CULTURES AGES IN TWO SOYBEAN MOLASSES CONCENTRATIONS

Age (h)	15	21
Concentration (°B)		
10	14,8 g/l	17,2 g/l
20	21,3 g/l	29,4 g/l

From table 2 it is observed that the older seed culture enhanced the production in a shorter fermentation (16h). At 10 °BRIX the difference between the ethanol concentrations were of 16,2% while at 20 °BRIX the difference was even larger: 38,0%. A hypothesis to explain this: the cells of the older culture were probably at the late exponential phase, while the younger was not yet. A fact that can help to explain the results: the cellular contents of the seed cultures (listed below):

* Seed culture of 15h: $3,3 \times 10^7$ CFU/ml

* Seed culture of 21h: $1,6 \times 10^8$ CFU/ml

Thinking not only in the “quality” of the cells, but also in its “quantity” the explanations may become clearer.

Another point to be observed: about the soybean molasses concentration effect, it can be hypothesized that at 20 °BRIX some level of inhibition may have started to occur, because the yields are not proportional (the concentration was 2 times higher in the assays with 20 °BRIX, but the ethanol production was not).

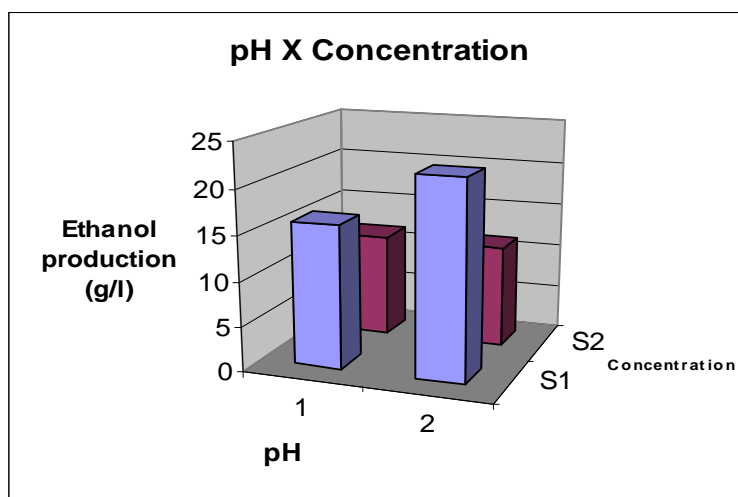
Experiments carried out after this one were conducted with seed cultures fermented for approximately 21h.

4.5 EFFECT OF pH AND SOYBEAN MOLASSES CONCENTRATION

It was carried out an experiment to evaluate the influence of soybean molasses concentration and the medium pH. On the last experiments related, some important information was obtained about the soybean molasses concentration effect. It was already known at this point that some level of inhibition could take place for concentrations above 20 °BRIX. To confirm this, the levels chosen were 20 and 30 °BRIX, while the pH levels were chosen centered on soybean natural pH: 5,0 and 6,0.

Results are shown in figure 4.

FIGURE 4. INFLUENCE OF PH AND SOYBEAN MOLASSES CONCENTRATION ON ETHANOL PRODUCTION. PH LEVELS: 5,0 AND 6,0. CONCENTRATION LEVELS: 20 °BRIX AND 30 °BRIX.



The higher level of production was observed at 20 °BRIX and pH 6,0 (22,1 g/l).

The test showed that at 30 °BRIX the bacterial strain produces quantities significantly lower of ethanol (11,7 g/l and 11,6g/l), probably due to its metabolism inhibition by compounds present in excessive amounts (salts and sugars). Another argument is the reduced water activity at this concentration of molasses.

Besides this, the results with pH 6,0 were better than with pH 5,0 for the concentration of 20 °BRIX (22,1g/l and 16,1g/l respectively).

The assay with pH as the unique variable (figure 5) confirmed that the pH 6,0 is close to be the ideal pH for ethanol production. The fermentation with pH 7,0 got also good results (what suggests that the ideal pH is between 6,0 and 7,0, closer to 6,0 than 7,0)

The assay with soybean molasses (figure 6) concentration confirmed the inhibition by substrate and predicted the zone that could give the better results: around 22,5 °BRIX (at least on the conditions tested, and in erlenmeyer flasks).

FIGURE 5. INFLUENCE OF PH ON ETHANOL PRODUCTION.

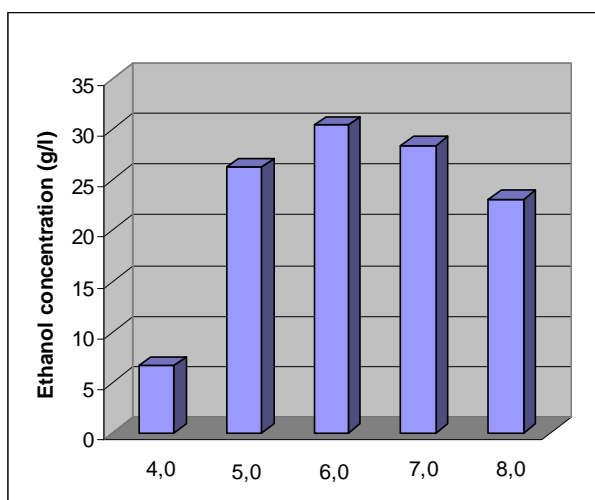
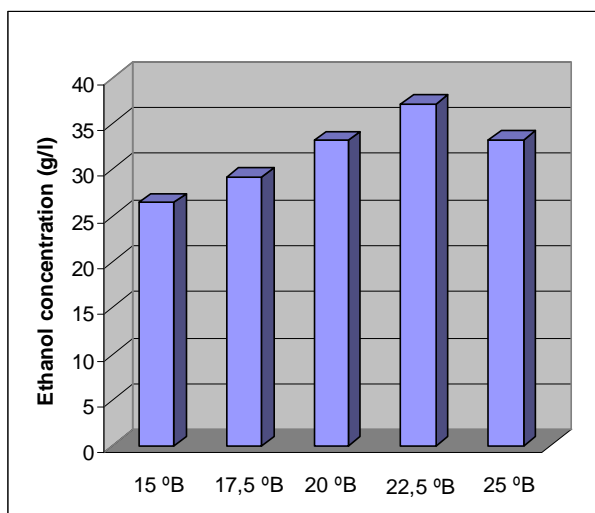


FIGURE 6. INFLUENCE OF SOYBEAN MOLASSES CONCENTRATION ON ETHANOL PRODUCTION.



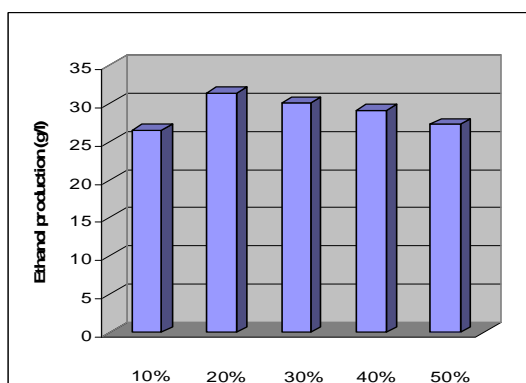
4.6 EFFECT OF INOCULATION RATE

An attempt to accelerate the process is to increase the number of initial cells at the starting of the fermentation. With this aim, the rate of inoculation was ranged from 10 to 50%.

The best results was achieved for the 20% inoculated flasks (31,4 g/l), followed by the assays with 30% (30,0 g/l), 40% (29,1g/l) and 50% (27,2g/l)

The experiment confirms the hypothesis previously assumed, but other tests are necessary to warrant an economical justification in the choice of higher levels of inoculation.

FIGURE 7. EFFECT OF INOCULATION RATE ON ETHANOL PRODUCTION



4.7 KINETICS OF PRODUCTION OF ETHANOL AND CONSUMPTION OF SUGARS

Table 3 summarizes the results for sugar consumption and ethanol productivity and yields:

TABLE 3. TOTAL SUGAR CONTENT, ETHANOL PRODUCTION, PRODUCTIVITY AND YIELDS FOR FERMENTATION IN ERLLENMEYERS AT 15 AND 20 °BRIX AND IN REACTOR AT 15 °BRIX (*Z. MOBILIS*) AND IN REACTOR AT 15 °BRIX (*S.CEREVISAE*).

	Fermentation at 20 °BRIX in flasks	Fermentation at 15 °BRIX in flasks	15 °BRIX in reactor (<i>Z. mobilis</i>)	15 °BRIX in reactor (<i>S.cerevisae.</i>)
Initial total sugar content (g/l)	147,5	112,4	116,8	115,1
Ethanol liquid production (g/l)	26,0	24,2	29,3	27,7
Ethanol productivity (g/l.h)	1,53	1,42	1,83	1,73
Yield of ethanol over total sugars (g ethanol)/ (g sugar)	0,18	0,22	0,25	0,24
Yield of ethanol over consummated sugars (g ethanol)/ (g sugar)	0,35	0,40	0,49	0,46
Maximum theoretical amount of ethanol (g/l)	38,5	30,9	30,5	31,0
Ratio between the ethanol liquid production and the maximum theoretical amount (%)	67,6	78,1	96,0	89,2

* The data correspond for lectures at 17h in the case of flask experiments, and at 16h for the reactor experiments.

4.7.1 Assays in erlenmeyer flasks

Ethanol production was monitored since the instant of inoculation until 24h of fermentation, to broths at 15 and 20 °BRIX, always with pH 6,0 and 21

hours aged seed culture. Also, it was monitored the profile of sugars consumption.

The results show that the bacteria had greater facility to growth and to produce ethanol at 15 °BRIX. Until the 15th hour of fermentation, the rate of production of ethanol in the flasks with 15 °BRIX soybean molasses are slightly higher than those one fermented at 20°BRIX. However, the greater production was observed for the assay at 20 °BRIX, with 21 hours of fermentation (32,2g/l).

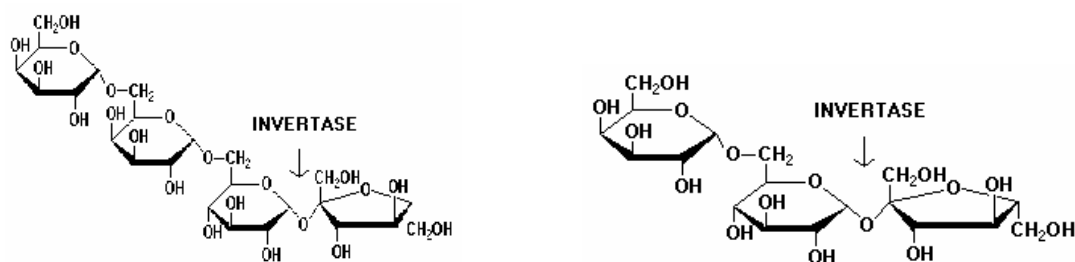
The results suggest that the exponential growth phase is achieved earlier to the assay with the medium more diluted, but the overall production stills higher for the 20 °BRIX broth. At the light of this results and of those one for the soybean molasses concentration as the unique variable, there is perspective of future studies with intermediate concentrations of molasses, or even slightly higher than 20 °BRIX..

The number of cells in the seed culture was determined: $1,3 \times 10^7$ CFU/ml. The fermented broth at 15 °BRIX presented $9,7 \times 10^6$ CFU/ml after 24h and the 20 °BRIX, $4,0 \times 10^6$ CFU/ml at the same instant. One more evidence of the starting of cell growth inhibition at this level of molasses concentration (at least in the tested conditions, in flasks).

With relation to the sugars consumption, it is clear that the strain is able to cleave stachyose (a tetrasaccharide). This result is very interesting and motivates future studies, as the range of substrates used as sole carbon sources by *Zymomonas mobilis* is, knowingly, restricted (SPRENGER, 1996). Raffinose, a trisaccharide, was not consummated. The apparent increasing in its concentrations an evidence that the bacteria is able to cleave only one of the bindings of stachyose, freeing a trisaccharide (that is detected at the same time

of raffinose, but is not raffinose in fact) and also a monosaccharide, possibly fructose, since there is an O-glycosidic binding in stachyose, like shown in figure 8:

FIGURE 8. STRUCTURAL FORMULAS OF STACHYOSE AND RAFFINOSE, RESPECTIVELY.



Also, it is possible to verify that the bacteria easily hydrolyze a disaccharide (possibly sucrose). In the assay at 15 °BRIX, at 6h of fermentation, the levels of disaccharides abruptly fall from 46,6g/l to 16,4g/l. From the 8th hour on, the level of disaccharides stays almost constant (about 13g/l). To the assay with 20 °BRIX, the behavior is analogous, but a little less accelerated: in 10 hours of fermentation, the disaccharides level falls from 62,4g/l to 18,2g/l, and after that stays almost constant at a level of approximately 16g/l. This noticeable residual level of disaccharide is an evidence that *Z. mobilis* is not able to hydrolyse melibiose (another disaccharide) that is detected at the same time of sucrose.

The starting levels of fructose and glucose increases at the first hours of fermentation due, mainly, to stachyose and sucrose hydrolysis and after are consumed almost entirely until 14 hours (glucose) and 17 hours (fructose) for the assay with 15 °BRIX and consumed almost entirely until 17 hours (glucose)

and 20 hours (fructose) for the assay with 20 °BRIX. In fact, the levels of glucose near the end of the process are not enough to be detected by the HPLC (and so, appear as zero). The peaks of fructose has the same detection time as galactose. Basal levels almost constant to fructose + galactose peaks at the end of fermentation (around 5g/l for 15 °BRIX and 6g/l for 20 °BRIX) suggest that this strain does not metabolizes galactose, what is not a surprise.

Interpreting the results from table 3 for flask experiments: the ethanol absolute production at 17h is higher at 20 °BRIX than 15 °BRIX, but the necessary time to the carbon sources exhaustion is higher too (probably, again, due to the inhibition of growth by substrate concentration or excessive salt concentrations). Besides this, the yields of ethanol, even if calculated at 24h are lower for the 20 °BRIX fermentation. So, in flask, static fermentations, the limitations of substrate concentration for soybean molasses medium are possibly close to this range (20 °BRIX). The results of figure 6 (already commented) support this hypothesis too.

About the yields of ethanol over sugars: the yields are something near to the their theoretical maximum (78,1% for 15 °BRIX fermentation and 67,6% for 20 °BRIX fermentation), but the yields of total sugars are very low yet (0,22 (g ethanol/ g sugar) and 0,18 (g ethanol/ g sugar) respectively for the most and the less diluted concentrations tested). It is a reflex of the narrow ability of *Zymomonas mobilis* to deal with the complex sugars. So, it is justifiable a try to hydrolyse the medium, in order to increases the amounts of assimilable sugars.

4.7.2 Assay in bench scale batch reactor

In general meanings, the same phenomena were observed for the test in reactor, when compared to those one in flasks, in terms of sugars consumption. There was, again, evidenced the bacterial ability to break stachyose, the apparent increase in raffinose concentration, the initial increase of glucose and sucrose (due to cleavage of most complex sugars) followed by rapid consumption (first all the glucose is exhausted, and a short time later, the fructose). Once more, residual levels of a disaccharide (probably melibiose) were observed and of galactose also (that is detected together with fructose).

Comparing the reactor test (15 °BRIX) with that one carried out in erlenmeyers (15 °BRIX too), it is possible to conclude that the rate of consumption of sugars and consequent ethanol production is pretty higher. While the necessary time to the erlenmeyer assay reach the plato is about 20h, it takes only about 10h in the reactor assay. Besides this point, the yield of ethanol in the reactor is pretty much closer to the maximum theoretical yield (96,6% at 20h and 96,0% at 16h) than that for static ones (82,8% at 20h and 78,1% at 17h).

These results can be explained, again, by the fact that the medium is well homogenized in the reactor (it is submitted to agitation of 65 to 70 rpm) when compared to the flask assays. This way, the cells have an easier access to nutriments, and hence, produce ethanol rapidly.

4.7.3 Fermentation by an yeast in batch reactor

Even when compared to the yeast, *Zymomonas mobilis* presented good results (reactor proof) in respect of the ethanol production profile, as can be

observed on figure 9. At the 8th hour of fermentation, the bacterium has almost reached the plato of ethanol, while the yeast took about 10h to achieve such stage. Table 3 shows that the production, the productivity and the yields for ethanol are slightly higher for *Zymomonas mobilis* at 16h of fermentation. Remark for the yield of ethanol / consummated sugars compared to the theoretical maximum^{OBS1}. For the bacterium, this yield is 96,0% of the maximum at 16h, and for the yeast, 89,2%. The yeast is able to consume a fraction a little bit larger of the soybean molasses sugars when compared to the bacterium (60,1g/l against 59,0g/l at 16h) but even with this drawback for *Zymomonas mobilis* it obtained a little bit higher ethanol production (29,3g/l against 27,7g/l at 16h). Combining these last two informations, it becomes clear why *Zymomonas mobilis* had a better relative yield (96,0%). This data is an evidence that the yeast uses a larger fraction of carbon sources to produce biomass than the bacterium.

The profile of viable cells during the fermentation for the flasks experiment is presented in figure 15, while the profile for batch fermentation is presented in figure 16. Figure 15 is another evidence that at 20 °BRIX a considerable level of inhibition starts to take place. Figure 16, curiously, suggests that the biomass growth rate is increased only after the reaching of plato for ethanol production (after 12 or 14 hours of fermentation). It does not mean that there is no growth during the phase of production, but means that the dead and replication rates are almost equal at this period.

OBS1: the maximum theoretical yield of ethanol from consummated sugars is calculated by considering that all carbon sources converge to ethanol and carbonic gas. The ratio between the molecular mass of ethanol and the

sum of the molecular masses of ethanol and carbonic gas gives, approximately, 0,51. So, the mass of ethanol produced will be no higher than about 51% of the mass of sugars consummated. This calculation gives the maximum theoretical production, and is listed in table 3 for each of the cases analyzed.

FIGURE 9. KINETICS OF ETHANOL PRODUCTION

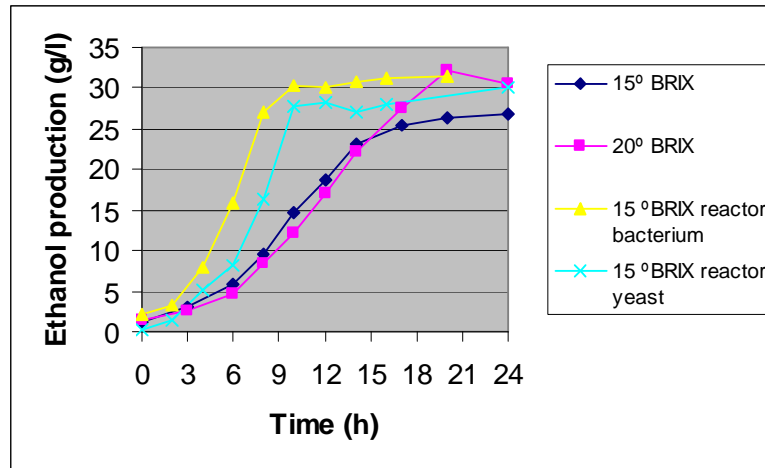


FIGURE 10. KINETICS OF STACHYOSE CONSUMPTION

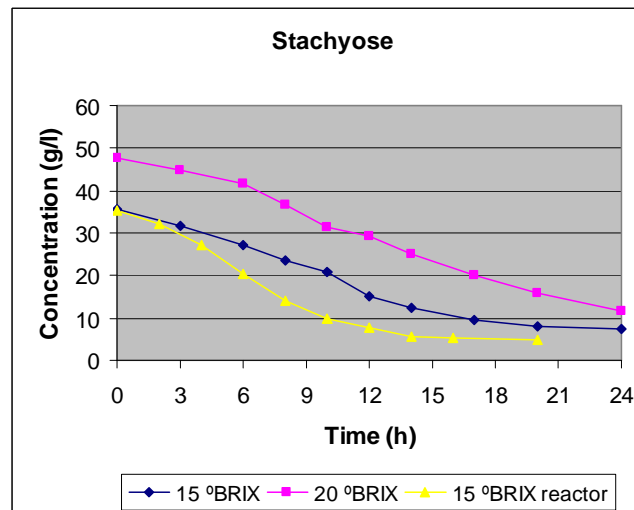


FIGURE 11. (APPARENT) PROFILE OF RAFFINOSE CONCENTRATION DURING THE FERMENTATIONS

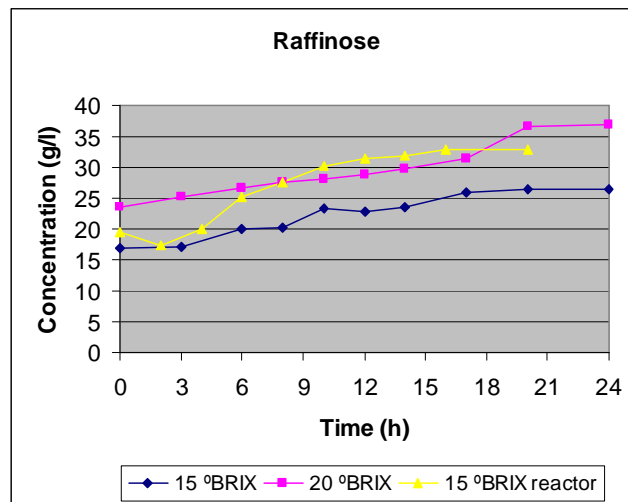


FIGURE 12. KINETICS OF DISACCHARIDES CONSUMPTION

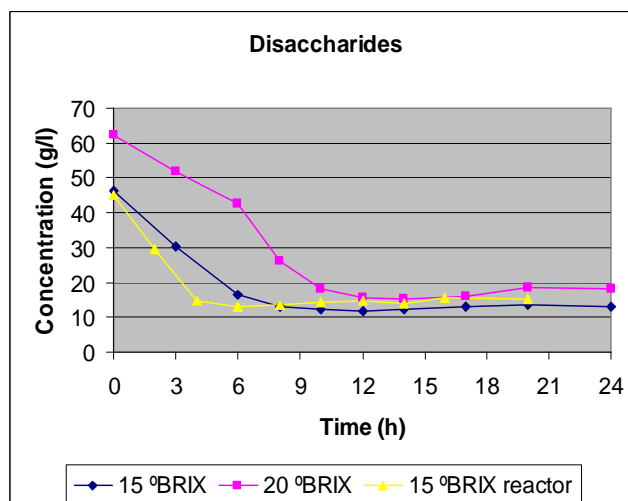


FIGURE 13. KINETICS OF GLUCOSE CONSUMPTION

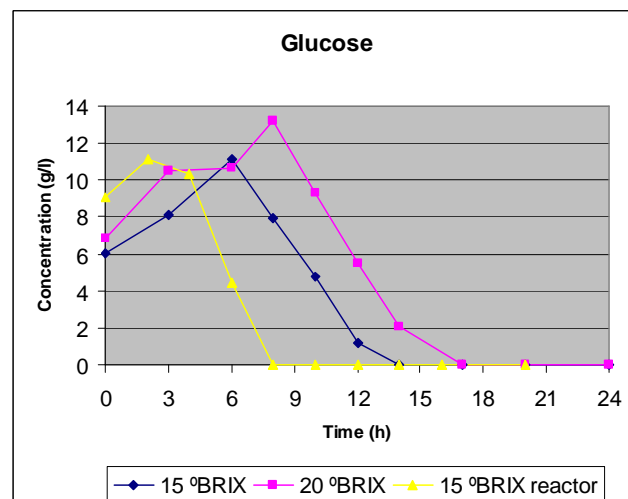


FIGURE 14. PROFILE OF FRUCTOSE PLUS GALACTOSE CONCENTRATIONS DURING THE FERMENTATIONS

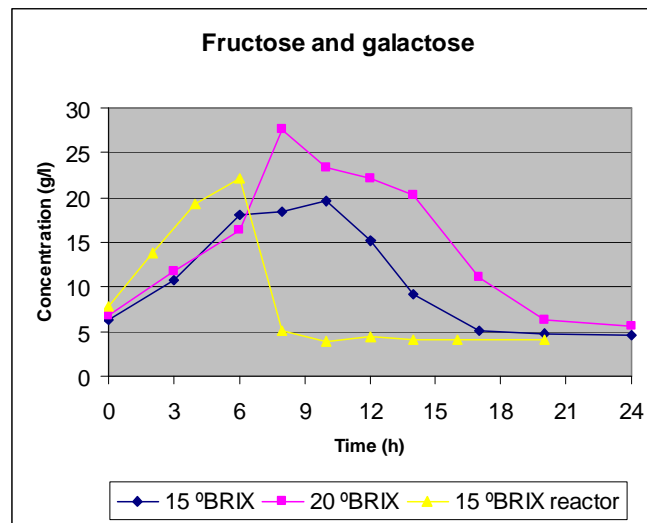
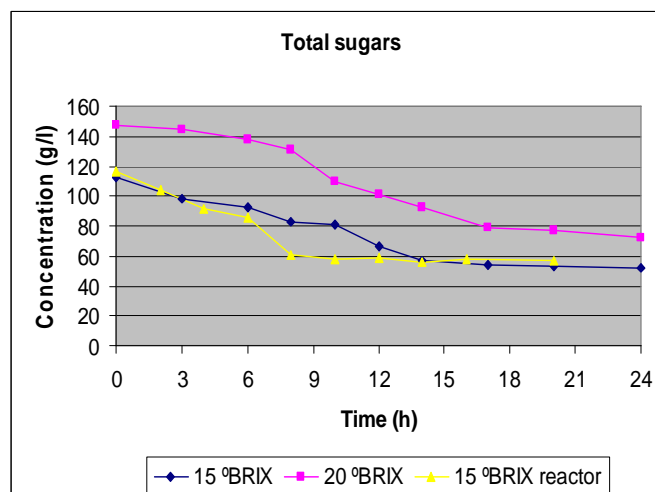


FIGURE 15. KINETICS OF TOTAL SUGARS CONSUMPTION



4.8 ACID AND ENZYMATIC HYDROLYSIS

TABLE 4. PROFILE OF SUGARS CONCENTRATIONS FOR TESTS OF HYDROLYSIS PERFORMED AT 121 °C BY 15 MIN.

		Sta	Raf	Disac	Glu	Fru+Gal	T. S.
Control		38,8	13,6	53,6	3,0	4,0	113,0
HCl	0,25N	0,3	23,8	17,0	27,9	41,3	110,3
	0,5N	0,2	0,1	4,3	33,4	44,6	82,5
	1,0N	0	0	3,3	26,8	19,9	50,1
	2,0N	0	0	2,6	20,8	15,0	38,4
H ₂ SO ₄	0,1N	8,5	21,0	16,4	19,9	30,0	95,9
	0,2N	0,2	2,0	8,5	32,8	46,1	89,5
	0,5N	0	0	3,2	33,6	43,7	80,4
	1,0N	0	0	1,1	24,1	24,6	49,8
H ₃ PO ₄	0,1N	36,3	14,1	54,3	4,1	5,3	114,0
	1,0N	0	24,8	11,1	24,2	35,3	95,4

* The results are shown in g/l of the corresponding sugars

** Sta: stachyose

** Raf: raffinose

** Disac: disaccharides

** Glu: glucose

** Fru+Gal: fructose plus galactose

** T.S.: total sugars

Table 4 contains the results that allowed the selection of the acids levels.

There were chosen the levels that produced higher amounts of simple sugars, like glucose and fructose. It is interesting to note that higher acid concentrations spoil the sugar content (the concentration of total sugars is reduced with increasing acid concentration).

Figure 17 show the results of the assays carried out with the selected acid concentrations for hydrolysis. It is also shown the results for the fermentation with enzyme.

It is clear that the all the acid hydrolysis were able only to improve very slightly the ethanol productions at 20h of fermentation (2,0%; 2,4% and 2,8% for cloridric, phosphoric and sulfuric acid, respectively).

The explanation can be given at the light of results shown in table 4 and the kinetics of sugars presented last topic: without hydrolysis, *Zymomonas mobilis* is not able to degrade raffinose, only stacchyose and sucrose, to finally metabolize fructose and glucose. The acid hydrolysis broken the complex sugar raffinose, increasing the amount of simple sugars in the medium, but at the same time spoiled significant part of them (chemical reactions). The liquid result was a slightly improvement of the process, wich will probably be not economically justifiable.

The enzyme was able to increases the amount of simple sugars without spoiling them. The ethanol production was increased for 33,2% in relation to the control. It is clear that the use of commercial enzyme will not justify economically the process, but the results motivate further studies. An interesting idea is to co-cultivate *Zymomonas mobilis* and another bacterial strain able to free the enzyme α -1,6 galactosidase into the medium. The conditions should be adapted, certainly.

FIGURE 16. EFFECT OF ACID AND ENZYMATIC HYDROLYSIS ON ETHANOL PRODUCTION

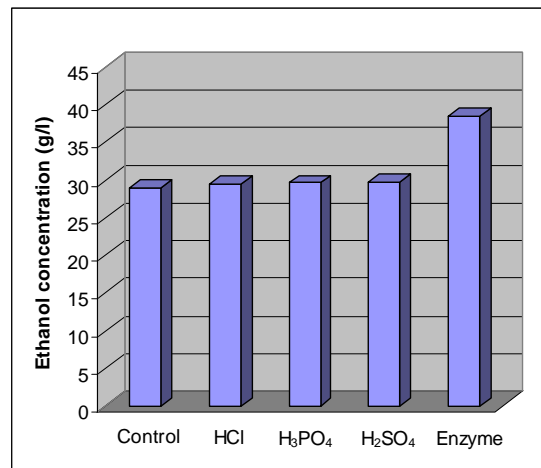


FIGURE 17. VIABLE BIOMASS FOR THE ERLLENMEYERS ASSAYS

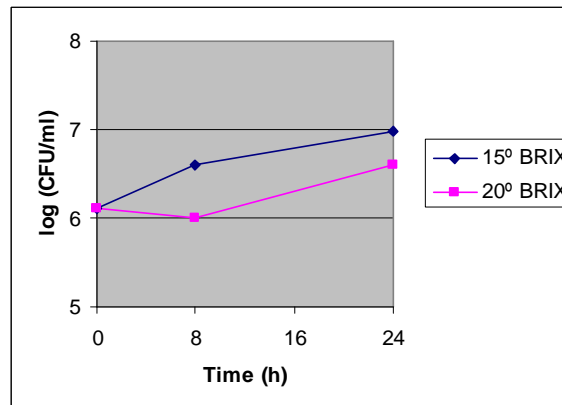
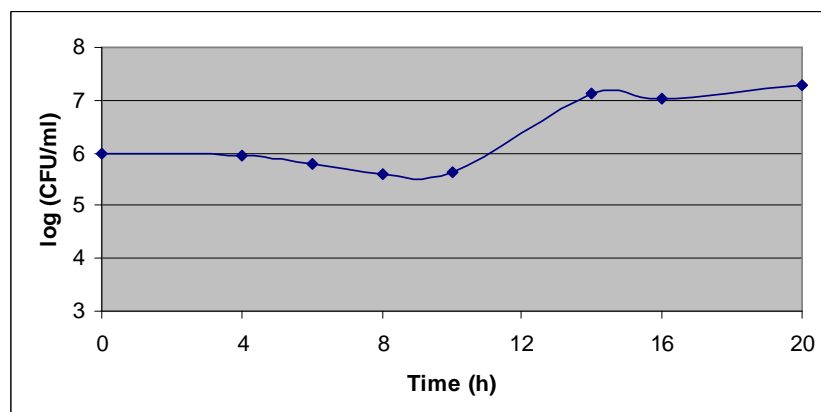


FIGURE 18. VIABLE BIOMASS FOR THE REACTOR ASSAY



4.9 EXPERIMENTAL PLAN WITH 3 VARIABLES: SOYBEAN MOLASSES CONCENTRATION, pH AND RATE OF INOCULATION

The statistical analysis was applied for both the data from 18h of fermentation and for 24h of fermentation.

TABLE 5. SIMBOLOGY AND RESULTS FOR THE TEST: SAMPLES COLLECTED WITH 18H OF FERMENTATION.

Concentration (C), %								17,5 (-)	27,5 (+)	
pH (P)								5 (-)	7,5 (+)	
Rate (T) %								10 (-)	20 (+)	
exp., y	C	P	T	Interactions				Replicates		
				CP	CT	PT	CPT	First	Second	Media
1	-	-	-	+	+	+	-	25,8	25,3	25,6
2	+	-	-	-	-	+	+	5,4	11,3	8,4
3	-	+	-	-	+	-	+	28,2	30,6	29,4
4	+	+	-	+	-	-	-	25,5	30,8	28,1
5	-	-	+	+	-	-	+	28,3	27,9	28,1
6	+	-	+	-	+	-	-	6,9	-	6,9
7	-	+	+	-	-	+	-	27,9	25,9	26,9
8	+	+	+	+	+	+	+	24,6	31,1	27,8

TABLE 6. EFFECTS FOR THE VARIABLES OF THE EXPERIMENT AT 18H.

	Concentration	=	-9,69	±	1,35
Main effect	pH	=	10,84	±	1,35
	Rate	=	-0,43	±	1,35
	CP	=	9,86	±	1,35
Secondary effect	CT	=	-0,45	±	1,35
	PT	=	-0,97	±	1,35
Terciary effect	CPT	=	1,55	±	1,35

TABLE 7. SIMBOLOGY AND RESULTS FOR THE TEST: SAMPLES COLLECTED WITH 24H OF FERMENTATION.

Concentration (C), %						17,5(-)	27,5(+)		
pH (P)						5(-)	7,5(+)		
Rate (T) %						10(-)	20(+)		
				Interactions				Results	
exp., y	C	P	T	CP	CT	PT	CPT	0, 0, 0	34,4
1	-	-	-	+	+	+	-		28,8
2	+	-	-	-	-	+	+		17,8
3	-	+	-	-	+	-	+		29,0
4	+	+	-	+	-	-	-		29,8
5	-	-	+	+	-	-	+		28,9
6	+	-	+	-	+	-	-		18,0
7	-	+	+	-	-	+	-		27,4
8	+	+	+	+	+	+	+		29,0

TABLE 8. EFFECTS FOR THE VARIABLES OF THE EXPERIMENT AT 24H.

	Concentration	=	-0,54
Main effect	pH	=	5,41
	Rate	=	-4,90
	CP	=	1,96
Secondary effect	CT	=	0,24
	PT	=	6,05
Tertiary effect	CPT	=	0,19

The central point (concentration of 22,5 °BRIX; pH 6,25 and rate of 15%) did not enter the statistical analysis. The result for 18h of the central point is: 26,5 g/l. And for 24h: 34,4 g/l.

The assay with enzymatic hydrolysis had the following results: 39,8g/l and 46,6g/l for 18h and 24h, respectively. These results are impressive.

Analyzing the values of the effects (main and interaction) and considering the standard deviation of these effects, it can be concluded that:

* The pH has a positive effect for both the times of fermentation (+10,84 and +5,41) and the concentration had a sensible oppose effect for the time 18h (-9,69), but had little influence at time 24h (-0,54). It means that for shorter

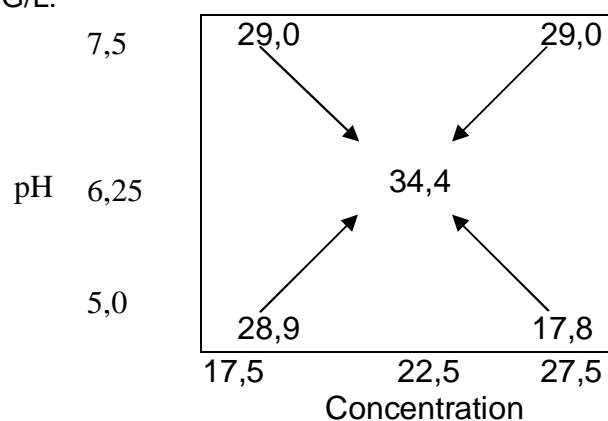
fermentations (18h), higher soybean molasses concentrations do not present good ethanol productions, but at 24h, the results are almost equivalent. The rate of inoculation did not show significant effect on this assay. A possible explanation: maybe its importance could be noted only in shorter fermentations.

* The interaction between pH and concentration is the only significative interaction. It means that one variable cannot be analyzed separately from the other. It was interesting to note that at higher soybean molasses concentrations, good results were obtained with higher pHs. It is an important data for further researches. Attempts to increase the concentration should be linked to pH increases. Another interesting result: the pHs were read after the fermentation (at 24h), and the average pH of the broths fermented at a starting pH of 7,5 was 5,4. The average pH of those fermented at 5,0 was 4,7. This decrease in pH can be explained by the production of CO₂. Future attempts to control the pH during the fermentation may result in better ethanol yields.

About the central point, it gave slightly lower ethanol productions at 18h, but the higher production at time 24h (34,4g/l).

It means that, at 24h of fermentation, the optimum point is limited by the tested values, as the figure 19 suggests:

FIGURE 19. STATISTICAL TECHNIQUE OF STATIONARY EVOLUTION FOR PH AND CONCENTRATION AT 24H. THE VALUES IN THE BOX ARE THE ETHANOL PRODUCTIONS, IN G/L.



The assays with enzymatic hydrolysis had a production 50,3% higher than the central point at 18h and 35,3% higher than the central point at 24h. Once more, future researches with co-cultivations have a prerogative.

FIGURE 20. BENCH SCALE BATCH REACTOR USED FOR FERMENTATIONS (BE MARUBISHI, MDL)



5 CONCLUSIONS

This work showed that *Zymomonas mobilis* strains NRRL 806 and ATCC 35001 were able to grow and produce ethanol in diluted soybean molasses, without addition of any salts and any extra carbon or nitrogen sources. Of the two tested strains, the NRRL 806 was a better ethanol producer.

Some environmental conditions of fermentation, aiming to increase and accelerate ethanol production were tested. The initial aeration helped in most concentrated broths; the optimal pH is between 6,0 and 7,0; the concentration that provides better production was between 20 and 25 °BRIX, but from 20 °BRIX up was observed levels of growth inhibition. Also, it was shown that older seed cultures (21h) gave better results than younger ones (15h). The increasing inoculation rate seemed to accelerate the production of ethanol (it is probable that the optimum point is between 10 and 30%).

The kinetics tests revealed that *Zymomonas mobilis* was able to degrade stachyose and sucrose, but not raffinose, melibiose and galactose. It can uptake glucose and fructose. In the assays on agitated bench scale batch reactor, the ethanol production, its rate and productivity and its yields were far superior from static assays. Also, *Zymomonas mobilis* presented better yields when compared to *Saccharomyces cerevisiae* on the reactor experiment (96,0% of the theoretical maximum against 89,2% for the yeast).

The acid hydrolysis helped only slightly on ethanol production, but the assays with enzyme provided excellent results, even though larger process scales would not be economical by using commercial enzyme.

6. PERSPECTIVES

Some results of this work will certainly encourage future researches, and the implementation of a scale pilot process may be possible. The optimum conditions must be refined yet, but some ranges are already defined. The assays on batch reactor showed that *Zymomonas mobilis* has a good potential for substitute yeasts on soybean molasses fermentation, due to its higher yields and productivity. Allied with this fact, the enzyme assays provided excellent results. Efforts can be made to implement a larger scale process using *Zymomonas mobilis* in co-culture with another strain that is able to produce α -1,6 galactosidase. Also, if pH may be controlled during the process, the ethanol yields for higher soybean molasses concentrations could be increased. Implementation of continuous process could be the following step. Finally, this work is a real proof that this byproduct from soybean industry, the soybean molasses, is a potential option for obtaining biofuel. This theme is growing importance day by day, due to non renewable energy resources exhaustion, and certainly researches on this area will appear more frequently.

7. REFERENCES

MACHADO, R. **Produção de etanol a partir de melaço de soja**. 1999. Porto Alegre – RS – Brazil;

MONTENECOURT, B. S. **Biology of Industrial Organisms**. Benjamin-Cummings Publishing Co. California, 1985;

ROUSSOS, S; SOCCOL, C. R.; PANDEY, A.; AUGUR, C. **New Horizons in Biotechnology**. ISBN 4020-1718-9. Kluwer Academic Publishers. Dordrecht, Netherlands, 2003.

SWINGS, J.; DE LEY, J. *Bacteriol. Rev.* Vol. 41, 1977. pg. 1-46;

SWINGS, J.; DE LEY, J. **Bergies Manual of Systematic Bacteriology**. Vol. 2. William & Wilkins. Baltimore, 1984;

SIQUEIRA, P. F., **Production of Bio-Ethanol from Soybean Molasse by *Saccharomyces cerevisiae***. 2006, Curitiba – PR – Brazil;

DAVIS, L.; ROGERS, P.; PEARCE J.; PEIRIS, P. **Evaluation of *Zymomonas mobilis*-based ethanol production from a hydrolysed waste starch stream**. *Biomass and Bioenergy* 30 (2006); 809 – 814;

TANAKA, K.; HILARY, Z. D.; ISHIZAKI, A. **Investigation of the Utility of Pineapple Juice and Pineapple Waste Material as Low-Cost Substrate for**

Ethanol Fermentation by *Zymomonas mobilis*. Journal of Bioscience and Bioengineering, vol 87; 5: 642-646, 1999;

RUANGLEK. V.; MANEEWATTHANA, D.; TRIPETCHKUL, S. **Evaluation of Thai Agro-industrial Wastes for Bioethanol Production by *Zymomonas mobilis*.** Process Biochemistry 41 (2006) 1432-1437;

SPRENGER, G. A. **Carbohydrate Metabolism in *Zymomonas mobilis*: a Catabolic Highway with some Scenic Routes.** Microbiology Letters 145 (1996) 301-307;

KALNENIEKS, U.; GALININA, N.; TOMA, M. M.; MARJUTINA, U. **Ethanol Cycle in an Ethanologenic Bacterium.** FEBS Letters 522 (2002) 6-8.