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High Lactic Acid Production from Molasses and Hydrolysed Sugarcane Bagasse

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In this work is presented a process with high lactic acid production as well as high sugar conversion and low by-products formation. The microorganism *Lactobacillus plantarum* uses preferably 6-carbon sugars. However, the xylose consumption occurs since the hexose concentration is not high. *Lb. plantarum* was shown to have a capacity for lactic acid production from cellulosic and hemicellulosic hydrolysates without detoxification. The results were similar to that using glucose, which is the source of carbon currently used by the lactic acid industry.
Lactic acid is an organic acid with a hydroxyl and an acid functional group. It has an asymmetric carbon and it naturally occurs as two optical isomers, D and L lactic acid. Crystallinity and many other important physical properties such as rate of degradation, melting point and boiling point are controlled by the ratio of enantiomers used. Lactic acid production by fermentation has several advantages when compared to chemical synthesis, such as low temperatures, low energy consumption, better environmental concerns and high purity. Furthermore, by fermentation it is possible to obtain the optically pure lactic acid, while by chemical synthesis only the racemic mixture can be produced. Despite the wide variety of feedstock tested for the lactic acid production most of them have problems with price, seasonality, continuous availability for large-scale production, fermentation rate, the amount of contaminants present, yields of lactic acid, formation of by-products, location of lactic acid production plant due to both availability as logistics of transportation and use of it. Trying to find substrates that overcome some of these barriers, in this work is proposed the use of molasses from sugar industry, pentose and hexose from enzymatic hydrolysis of sugarcane bagasse aiming to produce lactic acid.

1. Introduction

Lactic acid is an organic acid with a hydroxyl group and asymmetric carbon. It is produced by humans, plants, animals and microorganisms (Vijayakumar, 2008). As monomer for poly-lactic acid (PLA) production it has gained prominence for a wide versatility of use and its favorable characteristics, such as biodegradability, biocompatibility, elasticity and a well-controlled profile of drugs release (Djukić-Vuković et al, 2013). PLA is considered one of the most promising biodegradable plastics, mainly due to its high chemical resistance, which is advantageous for the manufacture of fibers and films, while the heat resistance is favorable to the production of many utensils (Tanaka et al, 2006).
There is a commercial interest in the lactic acid that motivates the research towards developing a product with the desired qualities and economically feasible. Research efforts have been increasingly focused on finding new and effective nutritional sources always associated with new fermentation techniques. It reflects the attempt to implement these processes in industrial terms with a high substrate conversion and high lactic acid production (Bulut et al, 2004).
The demand for lactic acid is growing every year. However, the industrial production of lactic acid does not follow the market demand. That happens for several reasons, including the price of raw materials. The substrates used industrially are glucose and starch, both already processed, which ends up endear the

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process (Bomrungnok et al, 2012). In this sense, Brazil has conditions to strongly step into this market by using renewable raw materials from sugar cane, including molasses, pentose and hexose from hydrolyzed bagasse.

Although many microorganisms produce lactic acid, the most commonly used by the industry are *Lactobacillus sp.* (Zhao et al, 2013). The advantage of this use is related to its high tolerance to acid environments and for being easily genetically modified for selective production of lactic acid isomers (Kyla-Nikkila et al, 2000).

Bearing all these in mind the objective of this work is to assess whether different carbon sources from sugarcane can affect the yield and the productivity on the lactic acid fermentation using *Lactobacillus plantarum*.

2. Methodology

The methodology used in this work comprises the way to obtain molasses as well as the C5 and C6 liquors and the fermentation procedures adopted

2.1 Obtainment of C5 liquor, C6 liquor and molasses

The fermentations performed in this study were carried out using the hydrolyzed sugarcane bagasse; in this case, sugars came from the hemicellulose (C5) and cellulose (C6) molecules from the hydrolyzed liquor (Figure 1).

Initially the sugarcane bagasse was collected in the milling and then dried at room temperature. The material was pre-treated in full form without undergoing any washing process for removal of residual sugars or ash. To release of sugars from hemicellulose (≈ 70 % of xylose) it was made a hydrothermal pre-treatment with a diluted sulfuric acid in order to obtain a xylose's rich liquor with low concentration of inhibiting products for fermentation. This liquor is the C5 liquor.

The solids from the previous step were submitted to an enzymatic hydrolysis process, in order to break the cellulose molecules and, thus, to release the glucose molecules. This fluid phase is denominated C6 liquor.

The remaining solids are basically composed of lignin and ashes.

Molasses used on this study was obtained from the centrifugation step, during the sugar manufacturing process. It consisted mainly of reducing sugars (glucose and fructose) and not crystallized sucrose.

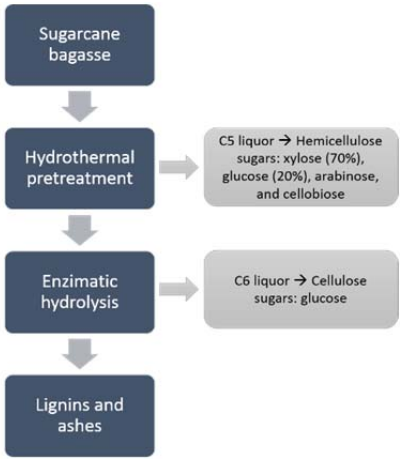

Figure 1: Process of obtaining C5 and C6 liqueurs from hydrolyzed sugarcane bagasse.

2.2 Fermentation process

Firstly, the microorganism *Lb. plantarum* was reactivated and stored in MRS (de Man, Rogosa and Sharpe) broth at 37 °C for 24 hours. In the next step, the microorganisms were transferred to a new MRS broth and it was placed in an orbital shaker at 37 °C for 16 hours at 120 rpm. At the end it was centrifuged, the supernatant was discharged and the cells were suspended in sterile water forming an inoculum suspension to be used in the tests.

The fermentations were conducted in 250 mL Erlenmeyer flasks with 200 mL of broth sterilized at 121 °C for 15 minutes. The broth composition is given in the Table 1.

Table 1: Final broth composition. Note that "Total sugar" corresponds to sugars of five or six carbons from each carbon source tested.

	Final broth composition		
Total sugar	50 g/L	Sodium acetate	5 g/L
Yeast extract	20 g/L	Calcium carbonate	30 g/L

The carbon sources tested were:

- C5 liquor (mainly xylose)
- C6 liquor (mainly glucose)
- Molasses
- C5 liquor + molasses - 50% of sugars from each one
- C6 liquor + molasses - 50% of sugars from each one
- Commercial glucose (used as control)

As a neutralizing agent it was used calcium carbonate in order to avoid the inhibition of the microorganism by the product, and the inoculum represented 10% of the final broth volume.
Post inoculation, flasks were placed in an orbital shaker at 37 °C for 24 hours at 150 rpm. After homogenization stage, a sample of 3 mL was used to check sugar and lactic acid content at the initial time of fermentation. This process was repeated after 24 hours of fermentation.

2.3 Analysis of sugars and lactic acid

To analyze sugar concentrations and lactic acid content it was used a High Performance Liquid Chromatography (HPLC) as shown in the Table 2.

Table 2: Parameters used to analyze sugar and lactic acid concentration on HPLC.

Parameter	Analyte	
	Sugar	Lactic acid
Column	Aminex ®HPX-87P 300 x 7,8 mm x 9 µm (Bio rad)	Aminex ®HPX-87H 300 x 7,8 mm x 9 µm (Bio rad)
Pre-column	Micro Guard De Ashing /Carb P	Micro Guard De Ashing /Cation H+ Refill
Detector	Refractive index detector	Refractive index detector
Mobile phase	Milli-Q water	Sulfuric acid 5mM
Flow	0,5 mL/min	0,6 mL/min
Injector	Automatic	Automatic
Column temperature	55°C	35°C
Detector temperature	50°C	35°C
Analysis' time	30 min	30 min

3. Results and Discussion

It was used the microorganism *Lb. plantarum* to produce lactic acid from different carbon sources from sugarcane, to know molasses, as fractions of sugar cane bagasse after pretreatment and hydrolysis (C5 and C6). The results show that it is possible to use the both molasses and bagasse from sugar cane to produce lactic acid. The use of hydrolyzed bagasse in this process can be an alternative to the viability of second-generation biotechnological products, since the utilization of derived sugars from hemicellulose, especially xylose, still represents a barrier in these processes, as far as production of ethanol is regarded.
On the studied condtions it was observed only homolactic fermentation, so that there was no presence of by-products such as alcohols, CO_2 or other organic acids (data not shown). The conversion rate was 1 mol $_{glucose}$: 2 moles $_{lactic\ acid}$ and 1 mol $_{xylose}$: 1.67 moles $_{lactic\ acid}$ (Holzapfel et al, 2014).
Using *molasses* as a carbon source it was achieved the highest lactic acid yield considering among the alternative sources of carbon, to know 88 %. The productivity using molasses was the highest, even considering the glucose control: 3.17 g L^{-1} h^{-1} (Figure 2). Molasses is a rich nutritional source, including a significant amount of mineral salts and vitamins. Thus, the association of molasses and yeast extract is able to provide to the microorganism all the needed compounds for a fast growth and a high lactic acid production, including the high availability of easy assimilation sugars by glycolysis pathway (Embden–Meyerhof–Parnas pathway). Although the yield of molasses was lower than that achieved with pure glucose (100 %), its

productivity was considerably higher, allowing to infer that molasses components exert a positive influence on the conversion rate of sugars into lactic acid.

When molasses and liqueurs from the hydrolyzed bagasse were mixed, it was possible to realize a decrease in the productivity as well as in the yield of lactic acid production process. The average productivity for *C5 liquor + molasses* and *C6 liquor + molasses* was 2.07 ± 0.1 g L^{-1} h^{-1} (Figure 2). The productivity reduction was 1.20 g L^{-1} h^{-1} and 1.00 g L^{-1} h^{-1} for C5 and C6 liqueurs associated with molasses, respectively. The average yield was 77 ± 2 %, which is 11 % lower than using pure molasses. This decrease in the yield and in the productivity can be strongly associated with inhibiting factors present in hydrolyzed bagasse liquors. This suggests a negative impact of the components of the hydrolysates (Gutierrez-Rivera et al, 2015), especially furfural and hydroxymethylfurfural. As it is known, many of these compounds are difficult to remove from the hydrolysates, including the phenolic compounds. The major phenolic components of the hydrolysates are 4-hydroxybenzoic acid, vanillin and catechol (Palmqvist et al, 2000). They also represent a great barrier to be overcome to the complete and efficient use of hydrolyzed liquors in the biotechnological processes as well as to the second generation products.

These compounds have been reported as causes for the reduction of the specific growth rate, reduction in the yield of biomass / ATP and reduced ethanol productivity in alcoholic fermentations (Palmqvist et al, 2000). Similarly, the effects of interaction among these compounds are most responsible for retardation of cell growth than the inhibition by the product. It indicates that other compounds, probably from lignin degradation during the hydrolysis, strongly contribute to the inhibition when using lignocellulosic hydrolysates in the fermentation processes (Palmqvist et al, 2000).

This fact is still reinforced by the average yield and productivity of *C6 liquor* and *C6 liquor + molasses*: 77 ± 2% and 2.31 ± 0.1 g L^{-1} h^{-1}, respectively. In fact, the use of *C6* either as pure or mixed with molasses showed no great differences in both yield and productivity. It is probably because the *C6 liquor* is basically composed by glucose. Therefore, the metabolic pathway for use of all the sugars from both *C6 liquor* and molasses is the glycolysis. Therefore, the only real difference between these two tests and the use of pure molasses is the presence of inhibitors from the hydrolyzed bagasse.

Unlike yeasts fermentations, acetic acid present in the hydrolyzates is not a major problem for the production of lactic acid. During the production of ethanol, the presence of weak acids involves a reduction of cytosolic pH (Axe et al, 1995; Gutierrez-Rivera et al, 2015), and this hampers the use of hydrolyzed. However, lactic acid bacteria are adapted to environments with low pH values, since its own metabolism is responsible for this feature producing lactic acid.

All this clearly depicts that the source of 6-carbon sugars to be fermented to lactic acid is an indifferent factor when compared to the inhibition exerted by some compounds present in the hydrolysates.

On the other hand, lactic acid production from xylose (*C5 liquor*) has a productivity of 0.9 g L^{-1} h^{-1} and achieved yield of 53 % (Figure 2). They are both considered low compared to the commercial glucose control and molasses. Even so, they are very promising results once they prove it is possible to produce lactic acid from C5 liquor without removing inhibitors. Xylose was consumed even in the presence of furfural, hydroxymethylfurfural and acetic acid, which are C5 liquor compounds that hinder in other biotechnological processes. The lower productivity is because the pentose metabolism is apparently slower than hexose metabolism, once it is suggested ATP production from xylose is lower than from glucose (Gutierrez-Rivera et al 2015). Besides, the low productivity is probably related with catabolite repression on xylose assimilation caused by remaining glucose from the hydrolysis process (Gutierrez-Rivera et al 2015).

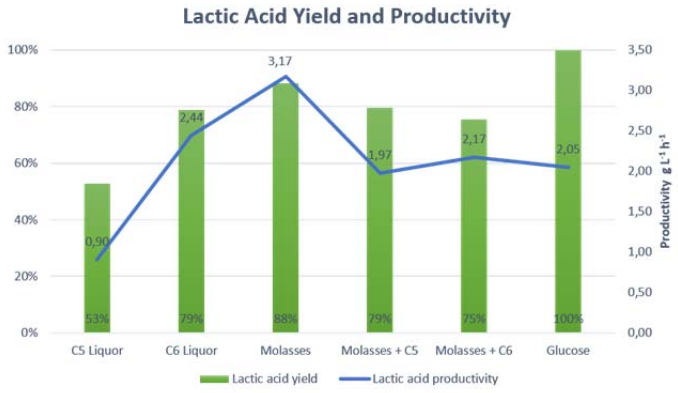

Figure 2: Lactic acid yield and a productivity in 24 hours of fermentation using six different carbon sources.

Besides, since it was not detected the presence of by-products and considering that the microorganism uses a homolactic fermentation to produce its own energy, the metabolic pathway used to consume xylose is the pentose-phosphate pathway (Holzapfel et al, 2014).
By looking to the sugar consumption (Figure 3), it is noted that glucose and fructose (6-carbons sugars) are always first consumed. It is associated with the fast and efficient breakdown of glucose and fructose by the glycolysis pathway. This process is very efficient in capturing energy for the microorganisms, being their principal route of survival.
Fermentations containing xylose (*C5 liquor* and *C5 liquor + molasses*) showed a distinctive pattern of consumption of this sugar. In the first case, when the xylose ratio is higher than that of 6-carbon sugars, almost 30% of the xylose present was consumed (Figure 3). In the second case, when xylose ratio is lower than 6-carbons sugar, the preferential consumption of glucose and fructose prevents the consumption of xylose, which does not reach 2%. It can be associated with a carbon catabolite repression (CCR), wherein the mixture of sugars derived from the lignocellulose is consumed sequentially, reducing the efficacy of the overall process (Kim et al 2010). In general, bacteria use the carbon sources through a specific hierarchical control to consumption of sugars. For it they use global transcriptional control and inducer exclusion, which results in the CCR (Titgemeyer et al, 2002). As in this case, many lactic acid bacteria use this sequential process of sugar consumption. In general, it makes the fermentation a very complex process, reducing the processes parameters as yield and productivity (Abdel-Rahman et al, 2011). A possible way to circumvent this process is the use of microorganisms capable of consuming pentose and hexose from lignocellulose at the same time (Abdel-Rahman et al, 2011; Guo et al, 2010; Kim et al 2010).
Still looking this fact, it was verified that the xylose concentration is inversely proportional to the lactic acid productivity. This is probably because the consumption of 6-carbon sugars is faster than the consumption of xylose, the first is always over before xylose. Therefore, 6-carbons sugar has the higher productivity in the process (Figure 3). However, these findings do not allow to infer that there is an interference relationship between proportion of xylose/glucose and productivity.
The commercial glucose test was used as control. It was intended to give the most favorable environmental for the lactic acid production by the *Lb. platarum* as in this case there were no inhibiting compounds.

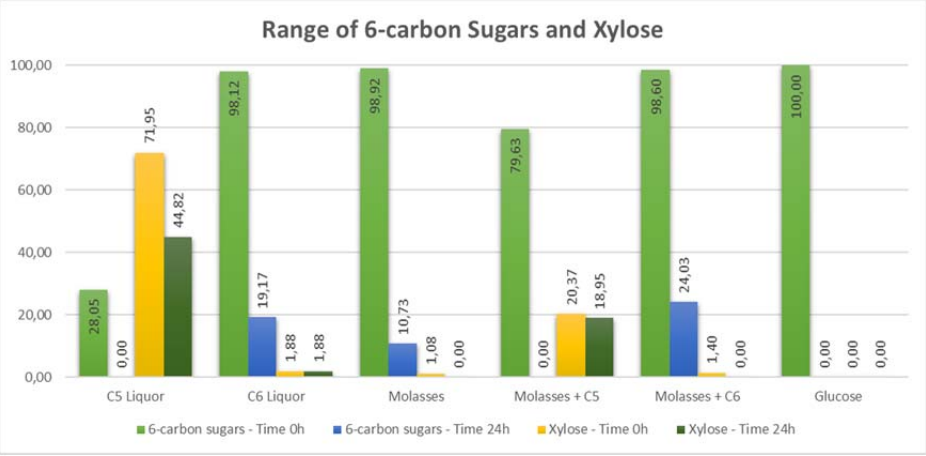

Figure 3: 6-carbons sugar and xylose composition in each fermentation test at 0 and 24 hours. Note that the chart values correspond to the values in percentage (%) of each component and not the real values of concentration.

4. Conclusions

In conclusion, it is emphasized that the microorganism *Lb. plantarum* uses preferably 6-carbon sugars, however the xylose consumption occurs since the hexose concentration is not high.
Lb. plantarum had shown to have a capacity for lactic acid production from cellulosic and hemicellulosic hydrolysate without detoxification which is very attractive in terms of robustness for an industrial process.
Xylose from hydrolyzed bagasse and without detoxification is consumed, although the hydrolyzed bagasse inhibitors (especially aromatic inhibitors) affect productivity and yield of lactic acid. In fact, what is limiting the use of hydrolysates, as much of C5 and C6 liqueurs is the presence of inhibitors.
The use of sugars and the lack of need for detoxification of the C5 liquor from sugarcane bagasse hydrolyzed is a crucial factor in the economic viability of second generation products. In this sense, the production of lactic

acid may be an alternative solution in allowing the second generation products, since the use of liquor with 5-carbon sugars has been very limited, for instance for ethanol production.

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