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2,3-Butanediol Production from Biowastes with *Bacillus Licheniformis*: a Preliminary Study

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2,3-Butanediol (2,3-BDO) is a promising platform compound which could be used to produce valuable derivatives such as methyl ethyl ketone and 1,3-butadiene. The biotechnological production of 2,3-BDO has been mainly studied with *Klebsiella* sp. under microaerophilic conditions. However the pathogenicity of these strains makes this process not industrially desirable.
The aim of this work was to study the possibility of producing 2,3-BDO with the non pathogenic microorganism *Bacillus licheniformis* ATCC-9789 using different sugars and biowastes as substrates. Shaken flask experiments were carried out using different monosaccharides commonly occurring in plant hydrolyzates (hexoses and pentoses), agroindustrial biowastes (sugar beet molasses and cheese whey) and the di- and monosaccharides occurring in them.
Flask fermentation of glucose produced 8.2±0,1 g/L 2,3-BDO after 18 hours, corresponding to a 40.4±1.0 g2,3-BDO/100g glucose yield. Under the same conditions, mannose was converted to 7.8±0.2 g/L 2,3-BDO with a yield of 39.4±1.8 g2,3-BDO/100g mannose. Pentoses (xylose and arabinose) were little or not consumed with no production of 2,3-BDO. Biowastes used at 20 g/L provided the corresponding sugars at approximately 10 g/L initial concentrations. While lactose in cheese whey was not used and converted into 2,3-BDO, 2.6±0.3 g/L 2,3-BDO were produced after 14.5 hours from sucrose occurring in molasses, corresponding to a yield of 26.6±3.4 g2,3-BDO/100g sucrose. Molasses appear therefore the most interesting feedstock for the production of 2,3-BDO with *Bacillus licheniformis* ATCC 9789.

1. Introduction

2,3-Butanediol is an organic molecule with two hydroxy substituents which is used in pharmaceutical, food, cosmetic and energy industrial fields. 2,3-Butanediol may be used directly as a liquid fuel, plasticizer or antibiotic, and is also the starting material for the production of 1,3-butadiene, diacetyl, methyl ethyl ketone and polyurethane, which are the monomers or initial reagents for the synthesis of artificial gums, flavorings, and cosmetic products (Koutinas et al., 2014).
2,3-BDO may be produced via fermentation from simple hexose and pentose sugars or from glycerol, through the partial oxidation to pyruvic acid. Given the high cost of pure sugars, potentially cheaper raw materials, such as hydrolyzed plant biomass, wood and corn, food industry waste or the wastes of biodiesel production have recently been investigated for 2,3-BDO production (Ji et al., 2011).
The microorganisms of the genus *Klebsiella*, in particular those belonging to the species *K. pneumoniae* and *K. oxytoca,* are considered the major 2,3-BDO producers. They are able to accumulate high amounts of 2,3-butanediol than the other microorganisms and to convert most of the sugars present in hydrolysates of cellulose and hemicellulose into 2,3-butanediol (Wang et al., 2014, Cheng et al., 2010). However, the pathogenicity of these species is generally seen as an obstacle to their use on a large scale. The microorganisms of the species *Bacillus licheniformis* are potentially the most promising for industrial production of 2,3-BDO, both due to their production capacity from glucose comparable or slightly lower than that of the principal strains belonging to the genus *Klebsiella*, and for the non-pathogenicity of the strain, that belongs to the class of risk 1 (Jurchescu et al., 2013; Perego et al., 2003; Nilegaonkar et al., 1992).

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The objective of this work was to evaluate the possibility of producing 2,3-BDO from sugars present in lignocellulosic biomass or waste from the food industry. For this purpose, a strain of the specie *Bacillus licheniformis* available in microbial culture collection was assayed on different hexoses and pentose monosaccharides, typically found in enzymatic hydrolysates of second generation lignocellulosic biomass. Moreover two agro-industrial wastes were assayed, namely cheese whey, i.e., the dairy industry waste rich in the disaccharide lactose, and molasses, the waste of the sugar production process rich in the disaccharide sucrose.

2. Materials and methods

2.1 Microorganism and chemicals

The microorganism used is *Bacillus licheniformis* ATCC 9789. The lyophilized microorganism was rehydrated and grown in rich soil Nutrient Broth (Beef Extract 3 g/L, peptone 5 g/L, recommended by ATCC) at 30 °C at 150 rpm shaking to prepare glycerol (20% v/v) stocks stored at -80 °C.
Pure sugars and microbial media components employed in this study were purchased by Sigma-Aldrich (Milan, Italy).Cheese whey powder was kindly provided by Lactogal (Portugal), contained about 50% sucrose. Molasse was kindly provided by Coprobi (Italy).

2.2 Experimental

The seed culture was prepared by inoculating 0.5 mL of -80°C stored culture into 50 mL of Nutrient Broth and incubated at 30°C and 150 rpm. After 21 hours incubation a volume of the grown culture was used as inoculum for the 2,3-BDO production experiments (initial optical density 600nm 0.4) in 50 mL of fermentation medium (Beef extract 10 g/L, Peptone 10 g/L, NaCl 5 g/L; Nilegaonkar et al., 1992). Sugars or agroindustrial wastes were added to the flasks just before the inoculation with the seed culture.
First, 2 hexoses (glucose and mannose) and 2 pentoses (xylose and arabinose) were tested individually. Furthermore, the strain was grown in absence of sugar, in order to verify microbial growth and 2,3-BDO production only in presence of the proteins and amino acids of the culture medium (Beef Extract and Peptone).
Then, cheese whey and molasses were tested individually as substrate. In addition the pure disaccharides present in the wastes were tested, in order to verify the possible presence of inhibitory effects on the process by other substances of the waste. Furthermore, the two monosaccharides that constitute the disaccharides were assayed singularly as substrate, in order to verify the ability of the strain to convert both and the possibility of using the enzymatic hydrolysate of the disaccharide, if the latter is not efficiently used or hydrolysed by the microorganism. Table 1 summarizes the tests carried out. Each carbon sources was added to the flask at the final concentration of 20 g/L. After inoculation, the flasks were incubated at 30°C and 100 rpm. Each experiment was carried out in duplicate.

Table 1: Carbon sources used in the flasks experiment

Monosaccharides	Biowastes	
Glucose	Cheese whey	Molasses
Mannose	Lactose	Sucrose
Xylose	Galactose	Fructose
Arabinose	Glucose	Glucose
No sugar		

2.3 Analytical methods

The main process parameters monitored were cell growth, substrate consumption, 2,3-BDO and by-products accumulation. Cell growth was assessed by measuring the optical density (OD) of the culture at 600 nm with a spectrophotometer (Prixma, Fulltech Instruments). The qualitative and quantitative analysis of substrates, 2,3-BDO and the main by-products (ethanol, acetate, succinate, lactic acid, acetoin) in the fermentation broth was performed with an high pressure liquid chromatography (HPLC) system (1260 Infinity Isocratic LC System, Agilent) coupled with refractive index detector (RID) using a Hi-Plex H column (8 pm, 300x7.7mm, Agilent) and a mobile phase consisting of 5 mM H_2SO_4 (flow 0.6 mL/min; injection volume 0.5 µL; temperature 65°C).

3. Results and discussion

3.1 Conversion of hexoses and pentoses in 2,3-BDO

Both hexoses (glucose and mannose) were converted in 2,3-BDO. In particular, as shown in Figure 1, the fermentation profiles of the two hexoses were comparable in terms of biomass, glucose consumption, 2,3-BDO production and byproducts accumulation, as well as in terms of substrate conversion, product concentration and yield (Table 2).

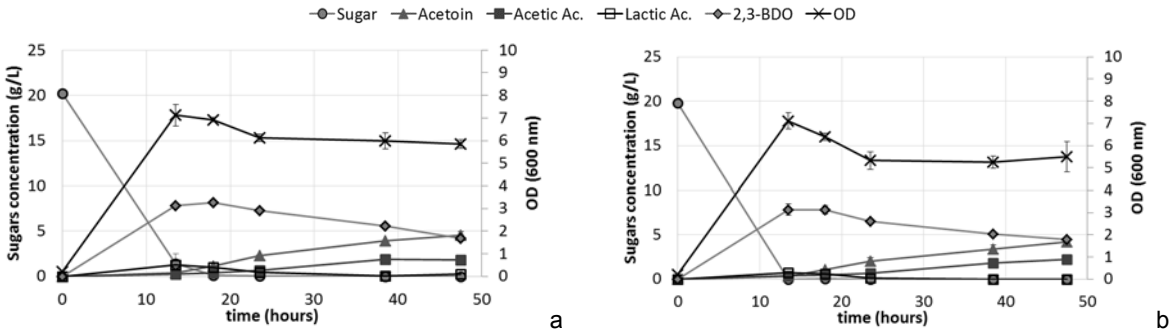

Figure 1: Microbial growth (OD600nm) and concentration of analytes (substrate, product and by-products) over time, during the fermentation of the sugars: glucose (a) and mannose (b).

After 13.5 hours fermentation the microorganism reaches the maximum growth (optical densities of 7.1±0.5 and 7.1±0.6 on glucose and mannose, respectively). The maximum concentration of 2,3-BDO is reached after 18 hours both from glucose (8.2 ± 0.1 g/L, yield equal to 40.4 ± 1.0 g/100g of glucose) and mannose (7.8±0.2 g/L, yield of 39.4±1.8 g/100g). Acetoin and acetic acid are produced after the complete consumption of the monosaccharide in concomitance with the degradation of 2,3-BDO.

Table 2: Main parameters related to the consumption of substrate and the formation of product in the fermentations conducted with several monosaccharides (hexoses and pentoses) and in the absence of sugars.

	Glucose	Mannose	Xylose	Arabinose	No sugar
Initial monosaccaride (g/L)	20.2±0.3	19.8±0.5	18.5±0.5	19.1±0.7	-
Residual monosaccaride (g/L)*	0	0	16±0.5	16.6±0.6	-
Conversion (g/100g)*	100	100	10.6±0.5	15.5	-
2,3-BDO max (g/L)	8.2±0.1	7.8±0.4	0	0	0
Yield max(g/100g)	40.4±1.0	39.4±0.8	0	0	-

*after 18 hours for glucose and mannose (maximum 2,3-BDO concentration); after 48 hours (end of the fermentation) for xylose and arabinose.

The results also indicate clearly that the strain does not produce 2,3-BDO from pentoses, which are little consumed during 48 hours of fermentation, as showed in Table 2. In the presence of pentoses the growth of the strain is markedly slowed down, leading to the achievement of an optical density (600nm) of about 6 after 38 hours of fermentation for xylose and about 4 after 18 hours for arabinose. The microorganism grows slowly even when grown only on the proteins and amino acids of Beef extract and Peptone without any sugars; in particular after 48 hours the highest optical density of 6.5 is reached. This growth is in line with that observed in the presence of the pentoses, indicating that the strain grows more slowly on the amino acidic of the media, and that the production of 2,3-BDO occurs from sugars, in particular hexoses. Microorganisms of *Klebsiella* species are able to metabolize a broader spectrum of sugars (Ji et al., 2011) than the *Bacillus licheniformis* strain used in this work. However, the yield of 2,3-BDO obtained from glucose by *Klebsiella spp*. usually ranges from 43 to 50 g 2,3-BDO/100 g glucose, which is comparable with that obtained in this work with *B. licheniformis* ATCC 9789. Higher 2,3-BDO titers, up to 150 g/L, have been also reported for *Klebsiella pneumoniae* from glucose under fed-batch conditions (Ma et al., 2009), suggesting that similar 2,3-BDO concentrations might be obtained after process optimization with *B. licheniformis* ATCC 9789.

3.2 Conversion of biowastes in 2,3-BDO

When cheese whey was used as substrate the initial concentration of lactose in the fermentation broth was about 10 g/L (instead of 20 g/L), because the of the lactose content of about 50% w/w. The organism slightly consumes the lactose present in the cheese whey but it does not ferment it to 2,3-BDO. Likewise, pure lactose and pure galactose are not converted in 2,3-BDO by the strain (Figure 2). This evidence indicates that cheese whey is not an agro-industrial waste suitable for the production of 2,3-BDO with this microorganism. Furthermore when cheese whey, lactose and galactose are used as substrate the microorganism grows slowly on the amino acidic fraction of the medium as described above. Lactose is apparently not converted to 2,3-BDO also by *Klebsiella oxytoca* (Champluvier et al. 1989), while can be fermented to 2,3-BDO by *Klebsiella pneumoniae* with yields comparable to those obtained from glucose (46 g2,3-BDO/100g of lactose) (Lee et al.1984).

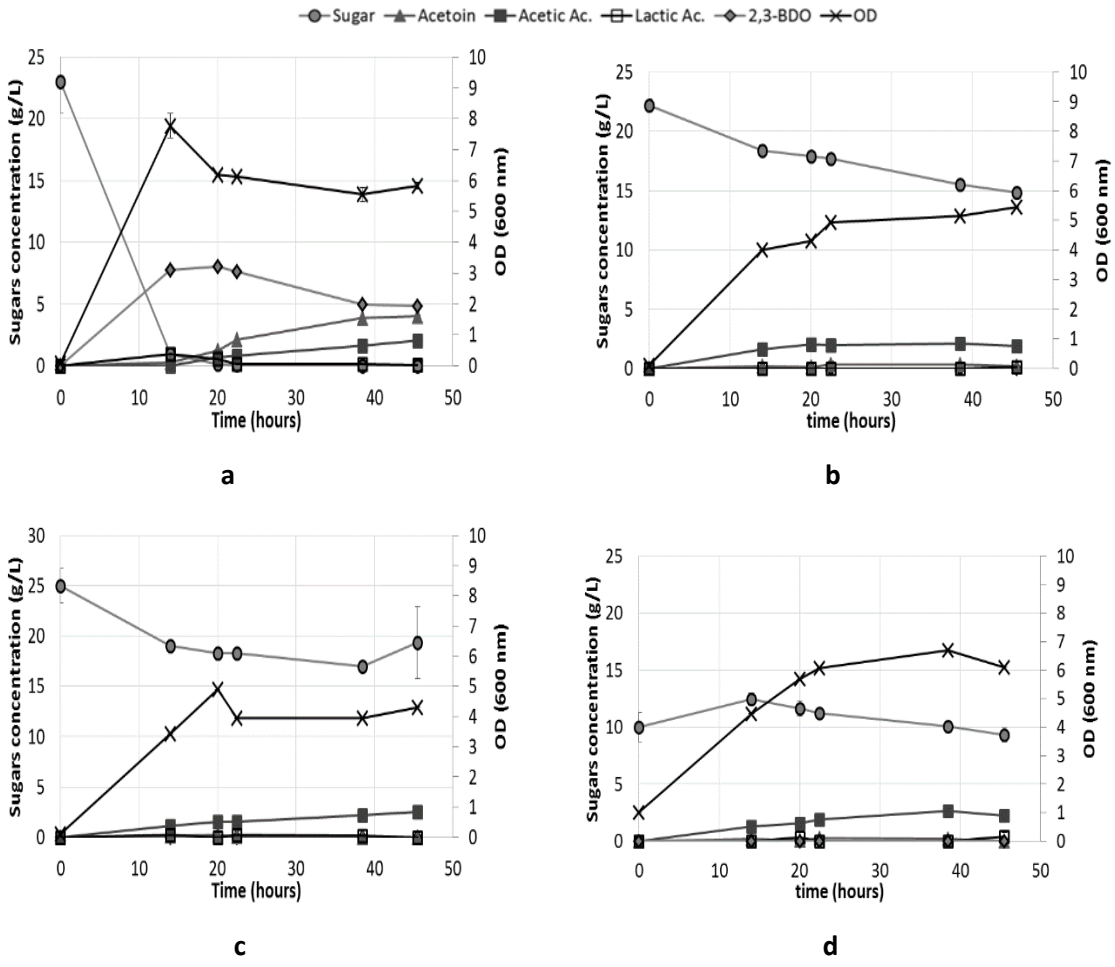

Figure 2: Microbial growth (OD600) and concentration of analytes (substrate, product and by-products) over time, during the fermentation of glucose (a), galactose (b), lactose(c), cheese whey (lactose)(d).

Figure 3 shows the Optical density (600nm) and the concentration of the substrate, product and by-products versus fermentation time when sucrose occurring in molasses, pure sucrose and the monosaccharides occurring in sucrose (glucose and fructose) are used as substrate. As regards glucose and fructose, the optical density reaches the maximum of 6.8±0.3 and 5.9±0.0, respectively, after 14.5 hours. The maximum 2,3-BDO concentration and yield are reached after almost 19 hours for both monosaccharides, being those from fructose about 70% of those obtained with glucose (Table 3). When pure sucrose is used, the same 2,3-BDO concentration is reached with a yield of approximately 85% of that obtained from glucose, as Table 3 shows. The lower yield obtained with sucrose with respect to glucose probably is not due to a partial hydrolysis of the disaccharide in the two monosaccharides, but to the lower conversion efficiency of fructose, which represents 50% by weight of sucrose.

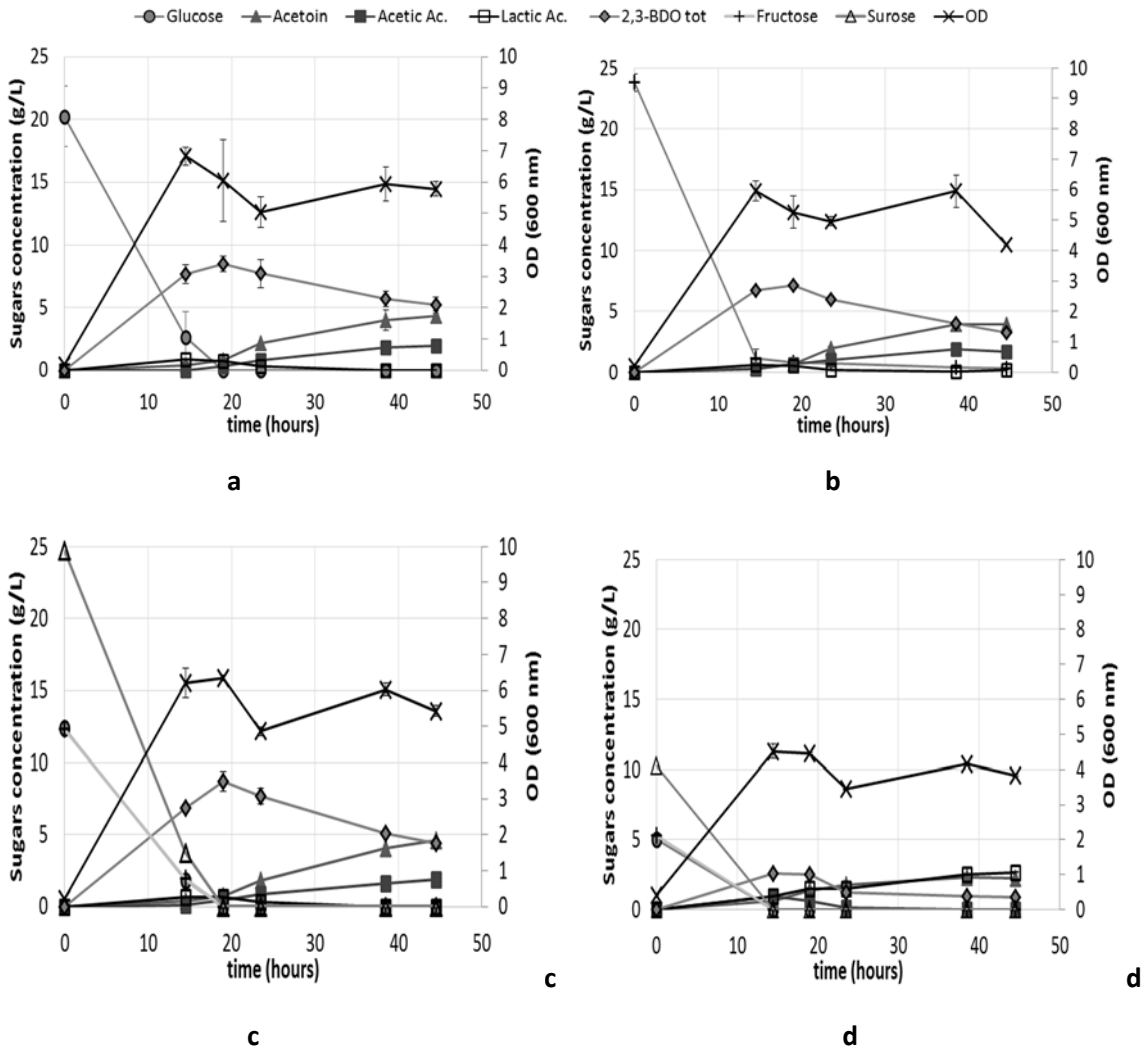

Figure 3: Microbial growth (OD600) and concentration of analytes (substrates, product and by-products) over time, during the fermentation of glucose (a), fructose (b), sucrose(c), molasses (sucrose)(d).

Table 2: Main parameters related to the consumption of substrate and the formation of product in the fermentations conducted with molasses and the sugars contained in it.

	Glucose	Fructose	Sucrose	Molasses (Sucrose)
Initial Monosaccaride (g/L)	20.2±2.4	23.8.1±0.7	24.7±1.1	10.3±0.7
2,3-BDO max (g/L)	8.5±0.6	7.1±0.5	8.7±0.1	2.6±0.3
Fermentaiton time (hours)*	19	19	19	14.5
Residual monosaccaride (g/L)	0	0.8±0.1	0	0
Yield (g/100g)	42.1±2.0	29.9±1.3	36.3±0.4	26.6±3.4
Conversion (g/100g)	100	96.7	100	100

* time after which the maximum concentration of 2,3-BDO was reached.

As regards molasses, the initial concentration of sucrose in the fermentation is about 10 g/L (instead of 20 g/L), since the content of sucrose in molasses is about 50% by weight. Sucrose is completely consumed after 14.5 hours, although complete sucrose consumption might have been reached earlier. The corresponding maximum 2,3-BDO concentration of 2.6 ± 0.3 g/L and yield (26.6±3.4 g/100g sucrose) are recorded after that time (14.5 h). This yield is significantly lower than that obtained with pure sucrose and lower than that reported from cane molasses for *Klebsiella oxytoca* (42 g 2,3-BDO/100 g; Afschar et al., 1991). It should be

recognized, however, that the initial concentration of sucrose in this fermentation was about the half of that used for pure sucrose, and that sucrose may have been completely consumed before the first sampling carried out after 14.5 hours of incubation. Thus, it is possible to speculate that a higher concentration and yield of 2,3-BDO from molasses could be obtained during the first 14.5 hours of fermentation, at the moment of complete sucrose consumption. Similarly, a higher concentration of the product and a higher yield could be obtained after 14-18 hours of fermentation using a double concentration of molasses, corresponding to an initial concentration of sucrose of 20 g/L. The data obtained indicate that it is therefore possible to produce 2,3-BDO from molasses, and suggest that concentrations and yields comparable to those obtained from pure sucrose might be obtained after process optimization.

4. Conclusions

In conclusion this work had the aim to evaluate the possibility to produce 2,3-BDO with the strain *Bacillus licheniformis* ATCC 9789 from sugars occurring in lignocellulosic biomass or biowaste from the food industry. The results show that the microorganism is able grows and produces 2,3-BDO starting from hexoses monosaccharides such as glucose, mannose, fructose, from the disaccharide sucrose, and from molasses. It is not able to use and produce 2,3-BDO from pentoses xylose and arabinose and the hexoses galactose, from the disaccharide lactose and the cheese whey. The strain is therefore promising for the production of 2,3-BDO from molasses.

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