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ETHANOLIC FERMENTATION OF SUCROSE, SUGARCANE JUICE AND MOLASSES BY *ESCHERICHIA COLI* STRAIN KO11 AND *KLEBSIELLA OXYTOCA* STRAIN P2

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ABSTRACT

Escherichia coli KO11 and *Klebsiella oxytoca* P2 recombinants fermented sucrose to ethanol. In minimal medium with 2% or 12% added sucrose KO11 produced 75% and 41%, respectively, of the maximum theoretical yield (0.54g ethanol/g sucrose). In Luria-Bertani (LB) broth with up to 8% sucrose, KO11 presented a 94-96% yield and with 12% sucrose, KO11 presented about 69% yield (44.5g ethanol/L). P2 presented 55% of the theoretical maximum yield in minimal medium supplemented with 2% sucrose and 47% of the maximum in 12% sucrose. In LB broth with 2 or 4% sucrose, P2 presented 94-95% of the theoretical maximum yield, which fell to 73% with 8% added sucrose (31.4g ethanol/L) and 58% with 12% sucrose (37.5 g/L). Volumetric productivity in LB broth containing 2% sucrose was 0.41 g/L/h for KO11 and 1.1 g/L/h for P2, while in LB broth with 12% added sucrose, productivity was 0.96 g/L/h (KO11) and 1.4 g/L/h (P2). During fermentation of sugar cane juice by *E. coli* KO11 and *K. oxytoca* P2 produced 39.4 g/L and 42.1 g/L ethanol when supplemented with 0.5% yeast extract, micronutrients and thiamine. In sugar cane juice supplemented with LB broth ingredients, KO11 ethanol fermentation was inhibited with production of only 23.0 g ethanol/L, while P2 produced 44.2 g/L. Ethanol production by KO11 and P2, respectively, in sugarcane juice was a) 25.3 and 30.2 g/L with 0.2% ammonium sulfate, b) 24.9 and 31.6 g/L with ammonium sulfate and micronutrients, c) 25.6 and 37.5 g/L with ammonium sulfate, micronutrients and thiamine. During molasses fermentation *E. coli* KO11 presented low ethanol production and high lactic acid production. *K. oxytoca* P2 produced 25 g ethanol/L in molasses diluted 10-fold in water, with or without addition of 0.5% yeast extract, and 27.8 g/L with addition of LB broth ingredients after 96h. P2 produced 24.5, 26.9, and 28.0 g ethanol/L in molasses diluted 10-fold in vinasse, vinasse with 0.5% added yeast extract and with LB broth ingredients, respectively.

Key words: *Escherichia coli* KO11, *Klebsiella oxytoca* P2, *Zymomonas mobilis*, sucrose fermentation, ethanol fermentation

INTRODUCTION

Petroleum is the most widely used fuel source on the planet. Petroleum will undoubtedly be depleted since it is a non-renewable fossil fuel source. It is estimated that crude oil production will decline worldwide by 2010, and become drastically reduced by 2050 (8). Brazil was compelled to search for new energy sources to substitute gasoline after the 1973 “petroleum crisis”. The Brazilian “National Alcohol Program” (Proálcool) was created in 1975, with its main objective being to promote substitution of gasoline by ethanol as automotive fuel (6). Through Proálcool, Brazilian ethanol production grew from 556 million liters in 1975/76 to 11.4 billion liters in 1985/86, turning the country into one of the world´s biggest ethanol producers and consumers.

Brazil developed a technology for motor combustion with ethanol and was the pioneer in large-scale use of alcohol as automotive fuel. In the 1980’s, about 95% of the vehicles produced in the Brazil were equipped with alcohol combustion

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motors. Currently, the majority of ethanol produced in Brazil is used as a gasoline additive, at a percentage of 24-26% (31). However, use of hydrated alcohol as automotive fuel is increasing currently because of the rising popularity of vehicles that can use alcohol as well as gasoline or their mixture in differing proportions (Flex fuel technology). Flex-fuel vehicles represented 58.9%, gasoline engines 35.6% and alcohol engines 1% of all car sales in Brazil in July of 2005 (10).

Alcohol fuel presents several advantages over gasoline: alcohol combustion does not cause an increase in atmospheric CO_2 concentration, and is less polluting than gasoline because alcohol produces less toxic substances and less gaseous emissions (26). Ethanol is a pure substance of known composition, whereas gasoline is a mixture of different compounds. Alcohol is also a more secure energy source because it is renewable (11,23) and can be produced anywhere in the world from biomass.

In the United States, ethanol is produced by fermentation of cornstarch (23), which competes for the limited agricultural land needed for food and feed production (28), representing an obstacle to increasing ethanol utilization. In Brazil, ethanol is produced primarily from sugarcane juice, utilizing the yeast *Saccharomyces cerevisiae* for fermentation. Molasses, a residue of the sugar industry, is also used in Brazil for ethanol fermentation (31). Other sugars substrates, such as lactose from cheese whey (1,9,14,21) and pentoses and hexoses from lignocellulosic biomass, can be converted to ethanol (1,2,3,12, 17,23,28,32). The yeast *S. cerevisiae* and the bacterium *Zymomonas mobilis* are highly efficient in alcohol fermentation but they can not to use many source of sugars substrates (7). Therefore, there is great interest in construction of recombinant microorganisms which combine the efficient alcoholic fermentation observed in *S. cerevisiae* and *Z. mobilis* with the capacity to utilize a broad variety of sugar substrates (17). To this end, *Escherichia coli* KO11 (25) and *Klebsiella oxytoca* P2 (32) recombinants were constructed by integrating *Z. mobilis* genes *pyruvate decarboxylase* (*pdc*) and *alcohol dehydrogenase* II (*adh*B) into their chromosomes.

The *E. coli* KO11 (KO11) and *K. oxytoca* P2 (P2) recombinants ferment sugar substrates that are not fermented by *S. cerevisiae* and *Z. mobilis*. The efficiency of KO11 and P2 in conversion of various substrates to ethanol, including simple sugars (5,16,21,22,29), starch (15), and hydrolyzed lignocellulose (2,3,11,12,13) has been determined. In this study we examined growth and ethanol fermentation of sucrose, sugarcane juice and molasses by these recombinants.

MATERIALS AND METHODS

Bacterial strains and media

The recombinants *E. coli* KO11 (25) and *K. oxytoca* P2 (32), both containing *Z. mobilis pyruvate decarboxylase* (*pdc*) and *alcohol dehydrogenase* II (*adh*B) genes were used. Stock cultures were maintained in LB broth with 40% glycerol. Chloramphenicol (40 µg/mL) was added to all stock cultures, inoculum preparations and fermentation experiments.

Fermentation of sucrose was conducted in LB broth and minimal medium (MM) containing per liter: 2g ammonium sulfate, 0.2g magnesium sulfate, 0.7g dibasic potassium phosphate, 0.3g monosodium phosphate, 5 mL of a micronutrient solution (per liter: 5g disodium EDTA, 0.22g zinc sulfate. $7H_2O$, 0.5g calcium chloride, 0.5g ferrous sulfate.$7H_2O$, 0.1g ammonium molybdate. $4H_2O$, 0.16g cupric chloride, 0.16g cobalt chloride and 0.5g manganese sulfate) and 1 mL thiamine solution (14). Thiamine stock solution (0.1% w/v) was filter sterilized.

Half-liter flasks containing minimal medium were autoclaved at 121ºC for 20min. Sucrose (Merck) stock solution (40% w/v) was sterilized at 121ºC for 20min and added to the media at final concentrations of 2%, 4%, 8% and 12% in a final volume of 200 mL. One mL micronutrient solution, 200 µL thiamine solution, 200 µL chloramphenicol (40 µg/mL) and sucrose solutions were added to the media after cooling.

Fermentation of sugarcane juice

Sugarcane juice was centrifuged at 8.000*g* for 8min. and 200 mL aliquots were autoclaved at 121ºC for 15min in 500-mL fermentation flasks. Sugarcane juice was fermented under the following conditions: a) without supplements, b) with 0.5% yeast extract, c) with 0.5% yeast extract, micronutrients and thiamine, d) with LB broth compounds, e) with 0.2% ammonium sulfate, f) with 0.2% ammonium sulfate and micronutrients and g) with 0.2% ammonium sulfate, micronutrients and thiamine. Micronutrients, thiamine and chloramphenicol were added as previously described.

Fermentation of sugarcane molasses

Molasses was diluted 10-fold in distilled water or in vinasse. Two hundred mL were autoclaved at 121ºC for 15min in 500-mL fermentation flasks and fermented under the following conditions: a) without supplementation, b) with 0.5% yeast extract and c) with LB broth reagents.

Fermentation inoculum preparation

Recombinant bacteria stored in LB broth/glycerol at -20ºC were transferred to LB agar containing 2% sucrose and incubated at 30ºC for 24h. After growth, one well isolated colony was transferred from a plate to 10 mL LB broth containing 2% sucrose, incubated overnight at 30ºC. This culture was added to 300 mL of LB broth containing 2% sucrose and once again incubated overnight at 30ºC under agitation (100 rpm). After growth, optical density at 550nm (OD) of the cell culture was measured (Milton Roy model Spectronic 20 D spectrophotometer) to determine the volume needed to produce a fermentation medium OD of 1.0, equivalent to approximately

0.33 g dry weight of cells per liter (25,32). This volume was centrifuged at 7000*g* for 8 min and 4ºC and the pellet transferred directly to the fermentation medium (4).

Fermentation experiments, sampling and analytical procedures

Fermentation was carried out in pH-controlled reactors, as previously described (4). A pH/ORP controller (Cole-Parmer model 5656-00) was used to automatically maintain the minimal pH at 6.0 through addition of 2M KOH. Batch fermentation was carried out in duplicate and under continuous stirring at 100 rpm using a star-shaped magnetic stirrer (Variomag® Electronicrührer Multipoint HP stirrer). Fermentations were run at 30ºC in a circulating water bath (Cole-Parmer Polystat Imersion Circulator).

Samples were removed for OD and ethanol determination throughout the 96h incubation period. Samples for ethanol determination were centrifuged in microtubes at 11750g and supernatants were stored at -20ºC for ethanol determination. The pH and amount of 2M KOH used were recorded several times during the incubation for correction of the dilution that occurred during fermentation. Ethanol concentration was determined by gas chromatography using a Hewlett Packard Chromatograph (5890 series II plus), equipped with a HPWAX (Hewlett Packard) polyethylene glycol capillary column (25 m x 0.2 mm x 0.2 mm), flame ionization detector (FID) and electronic integrator (HP 3395 Integrator), using nitrogen as carrier gas. Ethanol concentration was determined by comparison with a calibration curve using propanol as internal standard. The following chromatograph conditions were used: 1 µL sample injection volume; column temperature: 50 to 80ºC (8ºC/min.); injector temperature: 200ºC; detector temperature: 250ºC. All tests were run in duplicate with two or more repetitions, and results are expressed as the average of all repetitions.

Fermentation parameters

The following definitions apply to this study: a) ethanol production: concentration of ethanol produced (g/L); b) volumetric productivity: ethanol produced per volume of medium per unit of time (g/L/h) and c) percent of maximum theoretical yield (%): ethanol produced in relation to the maximum theoretical yield. This maximum theoretical yield of ethanol from sucrose was assumed to be 0.54 g ethanol/g sugar (the other 0.46 g being converted to CO_2). Maximum theoretical yields were calculated in relation to the amount of sugar present at the beginning of the fermentations, with no corrections made for unconsumed sugar.

RESULTS

Fermentation of sucrose in minimal medium

Escherichia coli KO11 presented maximum growth in MM after about 36h, reaching 0.8 g cells/L (OD 2.4) at 2% sucrose and 1.1 g/L (OD 3.2) in MM at a sucrose concentration of 4% or more (Fig. 1A). *Klebsiella oxytoca* P2 presented maximum growth after about 24h, reaching an OD of 2.0-2.5, at 2% to 4% sucrose, and OD of 4.0 at 8% to 12% sucrose (Fig. 1C). Flocculation of *K. oxytoca* P2 in medium containing sucrose interfered with OD determination and OD values reported in Fig. 1C therefore do not accurately represent the cell mass produced by P2.

Ethanol production by *E. coli* KO11 and *K. oxytoca* P2 in MM using sucrose as carbon source is presented in Figs. 1B and 1D. Ethanol production, percent of maximum theoretical yield and volumetric productivity by both recombinants are listed in Table 1. Strain *E. coli* KO11 produced more ethanol than *K. oxytoca* P2 in MM, except at 12% sucrose. Ethanol production in medium with 2% sucrose was 75% of the maximum theoretical yield for KO11 and 55% for P2. Recombinant KO11 presented 41% (26.6 g ethanol/L) of the maximum theoretical yield while P2 presented 47% (30.9 g ethanol/L) at a 12% sucrose concentration (Figs. 1B and D and Table 1). The carbon source was probably the limiting factor for ethanol production at 2% to 4% sucrose while other nutrients were limiting at 8% to 12% sucrose. Ethanol production could probably increase at these higher sugar concentrations if the incubation time were increased, resulting in greater ethanol production efficiency. Recombinant P2 presented a lower yield but a higher volumetric productivity than KO11 (Table 1), converting sugar to ethanol more rapidly at all sugar concentrations tested.

The pH of growth medium with 2% - 4% sucrose increased after 60-72h incubation of KO11 while an increase in growth medium pH occurred after 36h at 2% - 4% sucrose, and after 48h at 8% sucrose for strain P2.

Table 1. Ethanol production (g/L), Maximum theoretical yield [a] (%), and Volumetric productivity (g/L/h) [b] by *Escherichia coli* strain KO11 and *Klebsiella oxytoca* strain P2 in minimal medium added of sucrose.

	Sucrose added to minimal medium							
	2%		4%		8%		12%	
	KO11	P2	KO11	P2	KO11	P2	KO11	P2
Ethanol production	7.7	5.9	15.8	11.3	25.9	21.3	26.6	30.9
Maximum theoretical yield	75	55	70	52	60	49	41	47
Volumetric productivity	0.23	0.3	0.41	0.47	0.43	0.64	0.37	0.66

[a] Maximum theoretical yield of ethanol from sucrose is considered 0.54 g ethanol/g sucrose (more 0.46 g carbon dioxide/g sucrose);
[b] Calculated from the early stages of fermentation.

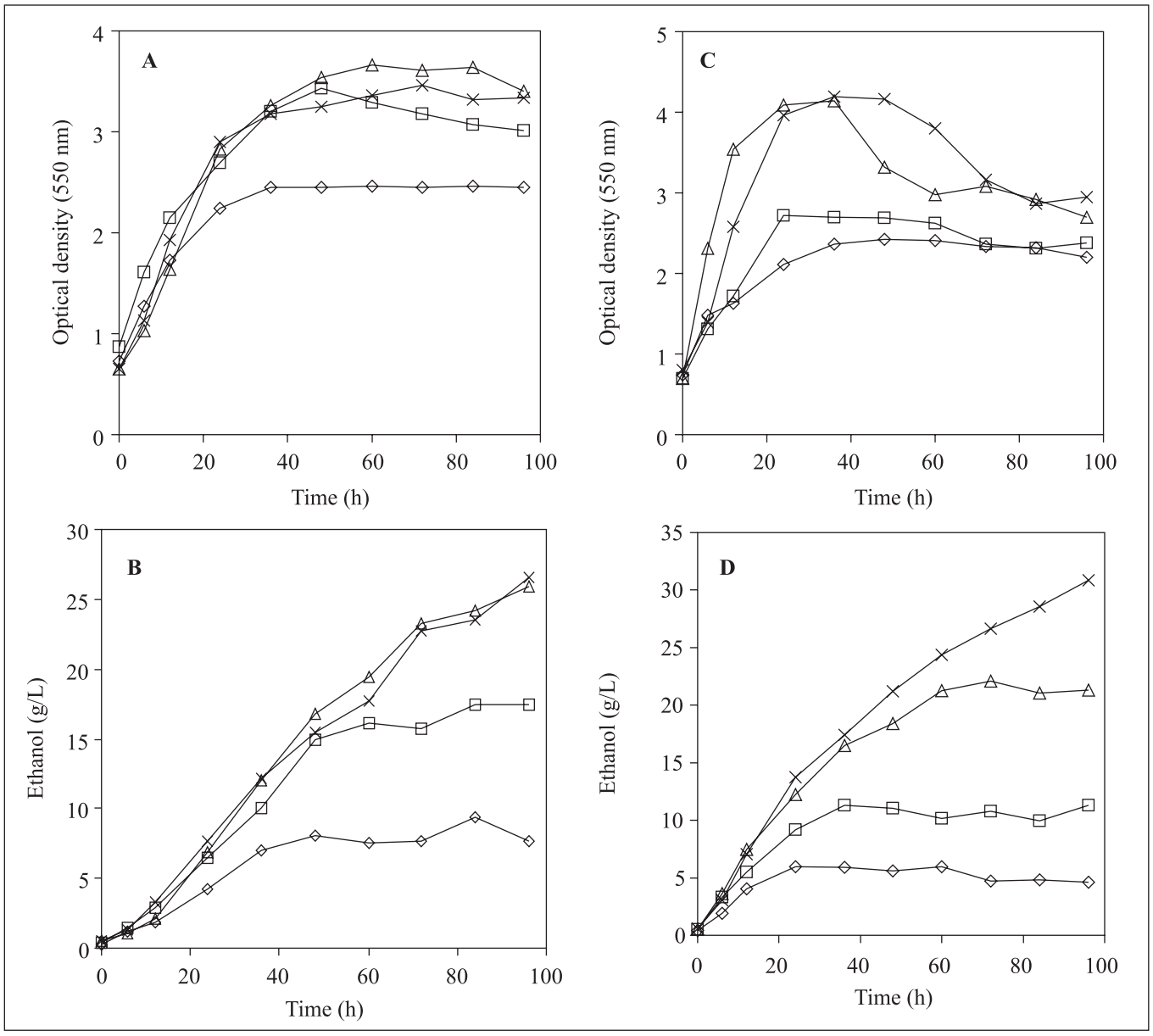

Figure 1. Growth and ethanol production by *Escherichia coli* KO11 (A and B) and *Klebsiella oxytoca* P2 (C and D) in minimal medium containing sucrose in the concentrations (w/v) of 2% (◇), 4% (□), 8% (△) and 12% (×).

Fermentation of sucrose in LB broth

Growth and Ethanol production is presented in Fig. 2, while Table 2 summarizes ethanol production, percent of maximum theoretical yield, and volumetric productivity for KO11 and P2. Recombinant KO11 (Fig. 2A) grew slightly more slowly than *K. oxytoca* P2 (Fig. 2C). KO11 cell mass reached about 1.7 g/L (OD 5.0) at a 2% sucrose concentration and about 2.0 g/L (OD 6.0) at higher sucrose concentrations after 24h. *K. oxytoca* P2 presented an OD of 5.0 at 2% sucrose and 7.0 at 12% sucrose after 24h incubation (Fig 2C).

Ethanol production was similar for both recombinants at 2% to 4% sucrose, corresponding to about 94-95% of the maximum theoretical yield. Both recombinants produced 20.3 g ethanol/L at 4% sucrose. Nevertheless, KO11 presented higher ethanol production at 8% to 12% sucrose. While KO11 presented about 96% efficiency (41.3g ethanol/L) in LB containing 8% sucrose, P2 presented only about 73% efficiency (31.4g ethanol/L). At a 12% sucrose concentration, KO11 presented 68.7% efficiency (44.5 g ethanol/L) and P2 presented 57.9% efficiency (37.5g ethanol/L). *K. oxytoca* P2 converted

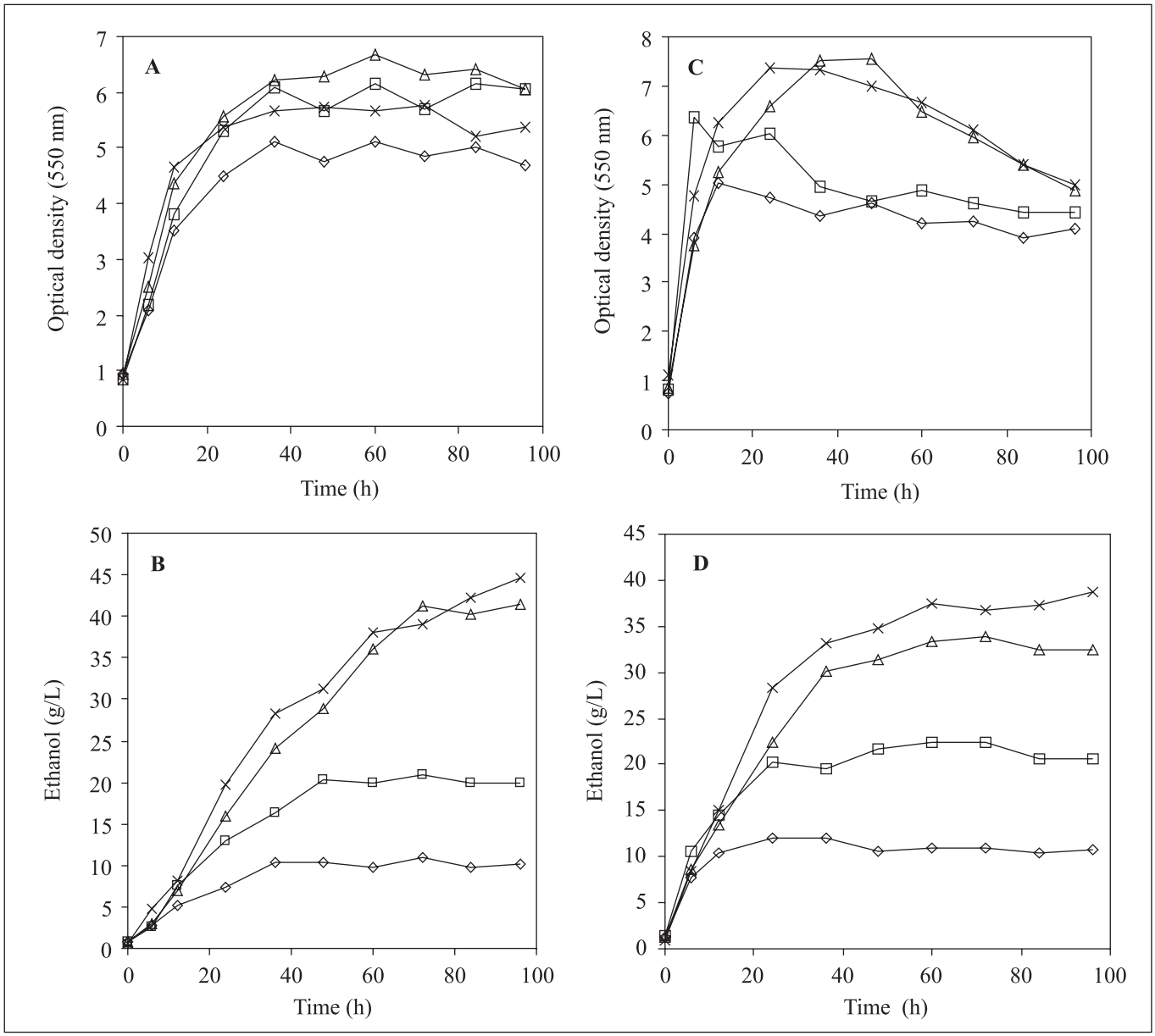

Figure 2. Growth and ethanol production by *Escherichia coli* KO11 (A and B) and *Klebsiella oxytoca* P2 (C and D) in LB broth containing sucrose in the concentrations (w/v) of 2% (◊), 4% (□), 8% (△) and 12% (×).

sucrose to ethanol faster than *E. c*oli KO11, and presented higher volumetric productivity. Increase in medium pH occurred after 48-60h of fermentation by KO11 and after only 12-36h fermentation by strain P2.

Fermentation of sugarcane juice without supplementation, supplemented with yeast extract and with yeast extract, thiamine and micronutrients

Fig. 3 presents growth and ethanol production by *E. coli* KO11 and *K. oxytoca* P2 under these conditions. Although KO11 and P2 did not grow in sugarcane juice without supplements (Fig. 3A), ethanol yield was approximately 11 g/L after 96h (Fig. 3B). Both bacteria presented rapid growth and ethanol production when supplements were added. In sugarcane juice with 0.5% yeast extract, ethanol production was approximately 34 g/L for both bacteria after 96h. Upon addition of yeast extract, thiamine and micronutrients, KO11 ethanol production rose to 39.4 g ethanol/L and P2 production to 42.1 g/L after 96h (Fig. 3B). Ethanol production in this case was slightly higher than in sugarcane juice with only yeast extract added.

Table 2. Ethanol production (g/L), Maximum theoretical yield [a] (%), and Volumetric productivity (g/L/h) [b] by *Escherichia coli* strain KO11 and *Klebsiella oxytoca* strain P2 in LB broth added of sucrose.

	Sucrose added to LB broth							
	2%		4%		8%		12%	
	KO11	P2	KO11	P2	KO11	P2	KO11	P2
Ethanol production	10.2	10.3	20.3	20.3	41.3	31.4	44.5	37.5
Maximum theoretical yield	94.4	95.4	94	94	95.6	72.7	68.7	57.9
Volumetric productivity	0.41	1.08	0.81	1.52	0.75	1.21	0.96	1.4

[a] Maximum theoretical yield of ethanol from sucrose is considered 0.54 g ethanol/g sucrose (more 0.46 g carbon dioxide/g sucrose); [b] Calculated from the early stages of fermentation.

Fermentation of sugarcane juice supplemented with LB broth reagents

Fig. 4 shows growth and ethanol production by KO11 and by P2 in sugarcane juice supplemented with LB broth components. *E. coli* KO11 produced 18.5 g of ethanol/L after 24h, with strong inhibition of fermentation after this time, reaching only 23 g ethanol/L after 96h (Fig. 4B), a much lower value than obtained from sugarcane juice with 0.5% yeast extract (34 g/L, Fig. 3B). To test the effect of NaCl on growth and ethanol production, KO11 was grown in sugarcane juice supplemented with LB broth reagents without NaCl. Growth and ethanol production were slightly higher, with ethanol production reaching about 30 g of ethanol/L within 96h, 7 g/L higher than when grown in medium with all LB components added. In this medium, P2 presented higher alcoholic production (Fig. 4B), reaching 44.2 g ethanol/L after 96h. This value is approximately twice that of the ethanol produced by KO11 under the same conditions.

Fermentation of sugarcane juice supplemented with ammonium sulfate, with ammonium sulfate and micronutrients and with ammonium sulfate, micronutrients and thiamine

E. coli KO11 and *K. oxytoca* P2 presented little growth in sugarcane juice with ammonium sulfate (Fig. 5A). Ethanol production (Fig. 5B) was greater than the 11 g/L produced in sugarcane juice alone (Fig. 3B). Likewise, addition of micronutrients and thiamine did not result in increased ethanol production by KO11. In all cases KO11 produced only about 25 g ethanol/L after 96h (Fig. 5B).

K. oxytoca P2 presented better fermentation than KO11 in sugarcane juice supplemented with ammonium sulfate. P2 ethanol production after 96h was similar when fermenting sugarcane juice supplemented with ammonium sulfate (30.2 g/L) or with ammonium sulfate and micronutrients (31.6 g/L). However, when thiamine was also added, ethanol production by P2 increased to 37.5 g ethanol/L. This value is higher than

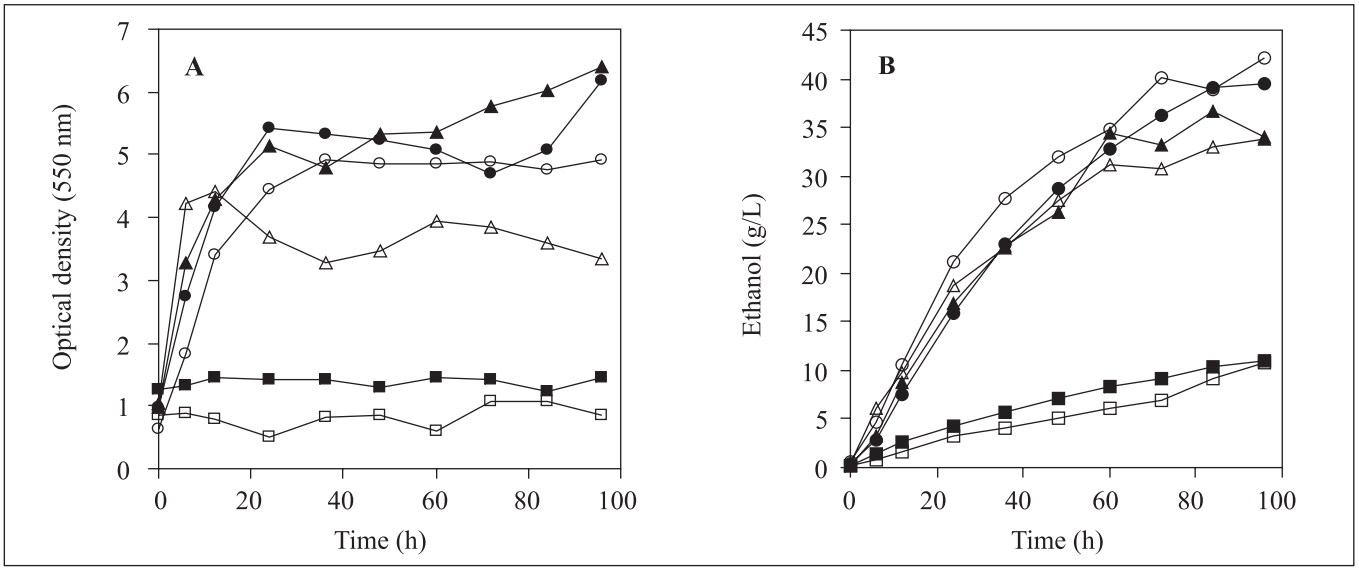

Figure 3. Growth (A) and ethanol production (B) by *Escherichia coli* strain KO11 (closed symbols) and *Klebsiella oxytoca* strain P2 (opened symbols) during sugarcane juice fermentation. [■ □] Without supplementation; [▲ △] supplemented with 0.5% yeast extract; [● ○] supplemented with 0.5% yeast extract, thiamine and micronutrients.

that obtained in sugarcane juice supplemented with yeast extract (34 g/L, Fig. 3B), but is lower than obtained with addition of yeast extract, micronutrients and thiamine (42.1 g/L, Fig. 3B) or LB components (44.2 g/L, Fig. 4B). These results indicate that thiamine addition improves alcoholic yield by P2, an effect not observed for KO11.

Fermentation of sugarcane molasses

Results of KO11 and P2 fermentation of molasses diluted 10-fold in distilled water and in vinasse are presented in Fig. 6A and 6B, respectively. *E. coli* KO11 molasses fermentation diluted in water or in vinasse resulted in low ethanol yields. Suplementation of molasses did not result in an increase in

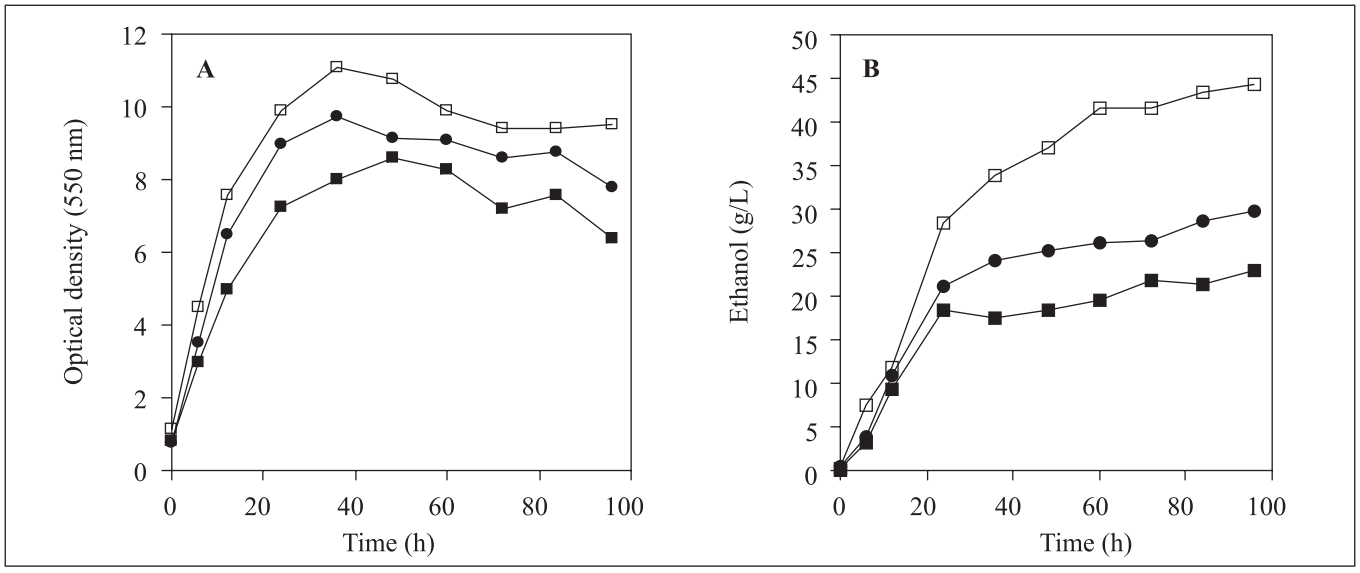

Figure 4. Growth (A) and ethanol production (B) by *Escherichia coli* strain KO11 (closed symbols) and *Klebsiella oxytoca* strain P2 (opened symbols) during sugarcane juice fermentation. [■ □] Supplemented with LB broth reagents; [●] supplemented with LB broth reagents without NaCl.

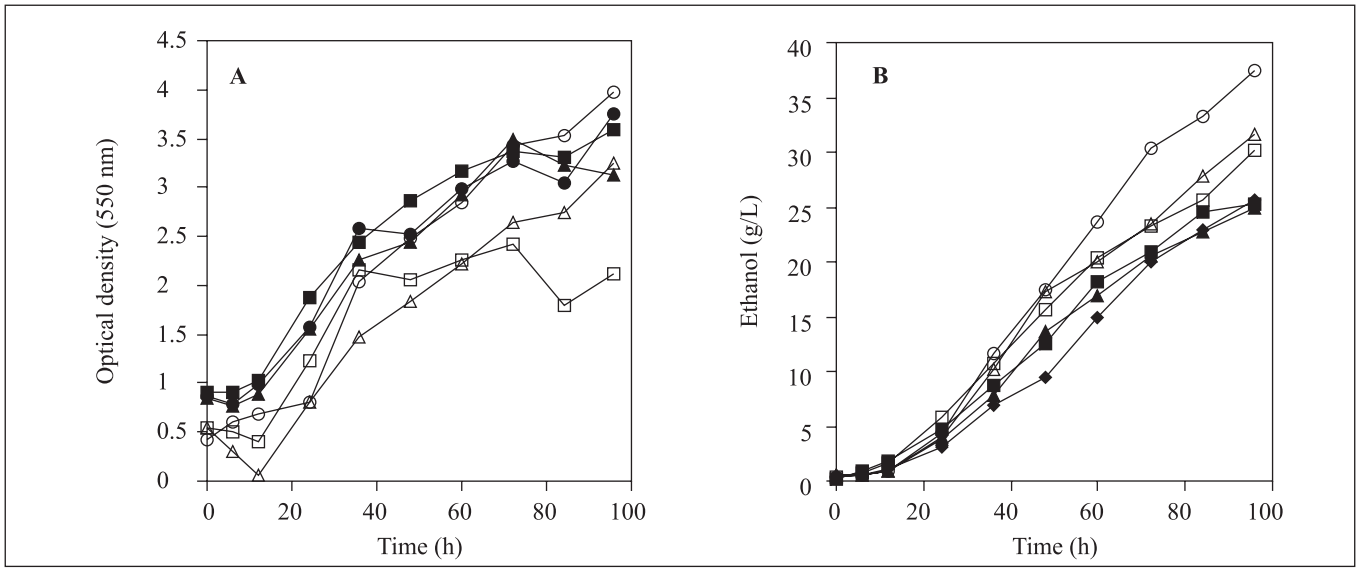

Figure 5. Growth (A) and ethanol production (B) by *Escherichia coli* strain KO11 (closed symbols) and *Klebsiella oxytoca* strain P2 (opened symbols) during sugarcane juice fermentation. [■ □] Supplemented with 0.2% ammonium sulfate; [▲ △] supplemented with 0.2% ammonium sulfate and micronutrients; [● ○] supplemented with 0.2% ammonium sulfate, micronutrients and thiamine.

ethanol production by KO11, that produced only 18.1 g ethanol/L whithout suplementation and about 15.5 g/L with yeast extract or LB reagents. In molasses diluted in vinasse, KO11 produced 14.4 g ethanol/L without supplements and about 16.6 g/L with yeast extract or with LB components. There was a relatively KOH consumption during fermentation to maintain pH. Acid analysis by HPLC indicated high lactic acid concentration in the fermentation medium (results not shown), indicating a possible loss of ethanologenicity by KO11.

In contrast to KO11, *K. oxytoca* P2 fermented molasses efficiently. In molasses diluted in water, ethanol production after 96h was 25.6 g/L (no supplements), 24.6 g/L (with yeast extract) and 27.8 g/L (with LB). In molasses diluted in vinasse, ethanol production was 24.4 g/L (no supplements), 24.6 g/L (with yeast extract) and 25.1 g/L (with LB). Addition of yeast extract or LB promoted rapid P2 fermentation that reached a maximum ethanol yield within 24h and stabilized after this time probably because of sugar depletion in the medium.

DISCUSSION

For sucrose fermentation, *Escherichia coli* KO11 generally presented better fermentation results if only ethanol produced is considered. However, if one considers volumetric productivity, P2 presented better results than KO11. Rapid ethanol production by P2 was also reported by others authors (12), who concluded that P2 presents dominance in the earlier part of the fermentation in co-culture with other organisms. A probable explanation for lower ethanol yield from P2 in relation to KO11 when sugar is a limiting factor, is its higher cell mass production and diversion of part of the sugar to capsule polysaccharides production. This can be observed by comparing the 8% sucrose curves in Figs. 2B and 2D. *K. oxytoca* P2 growth stabilized in ethanol after 36h, while *E. coli* KO11 presented the same result after only 72h. In both cases, the curves indicate sugar depletion in the medium, since KO11 produced nearly the maximum theoretical yield (96%) and P2 presented 73%, with the remaining sugar probably having been utilized for capsule production.

The *K. oxytoca* P2 culture flocculated in medium containing sucrose. Flocculation presents some advantages for alcohol production, facilitating separation of cells and eliminating the need for centrifugation for cell recovery in an industrial setting (27). Flocculation is also a way to maintain high cell density in a continuous-flow bioreactor. It was observed that flocculation in *Z. mobilis* is influenced by the ethanol concentration in the medium (27). The influence of ethanol concentration on *K. oxytoca* P2 flocculation was not investigated. This flocculation might explain the progressive decline in OD observed.

According to Beall *et al.* (4), the rise in medium pH provides a convenient marker for completion of fermentation and appears to coincide with maximum ethanol concentration. Increase in medium pH occurs due to catabolism of complex nutrients, which releases ammonia after sugar depletion (4). The rapid increase in medium pH observed for recombinant P2 confirms the greater ethanol production efficiency of this strain as compared to KO11. Complex nutrients are absent in MM and the rise in pH observed in this medium may be explained by cell lysis and release organic macromolecules that can be utilized by growing cells.

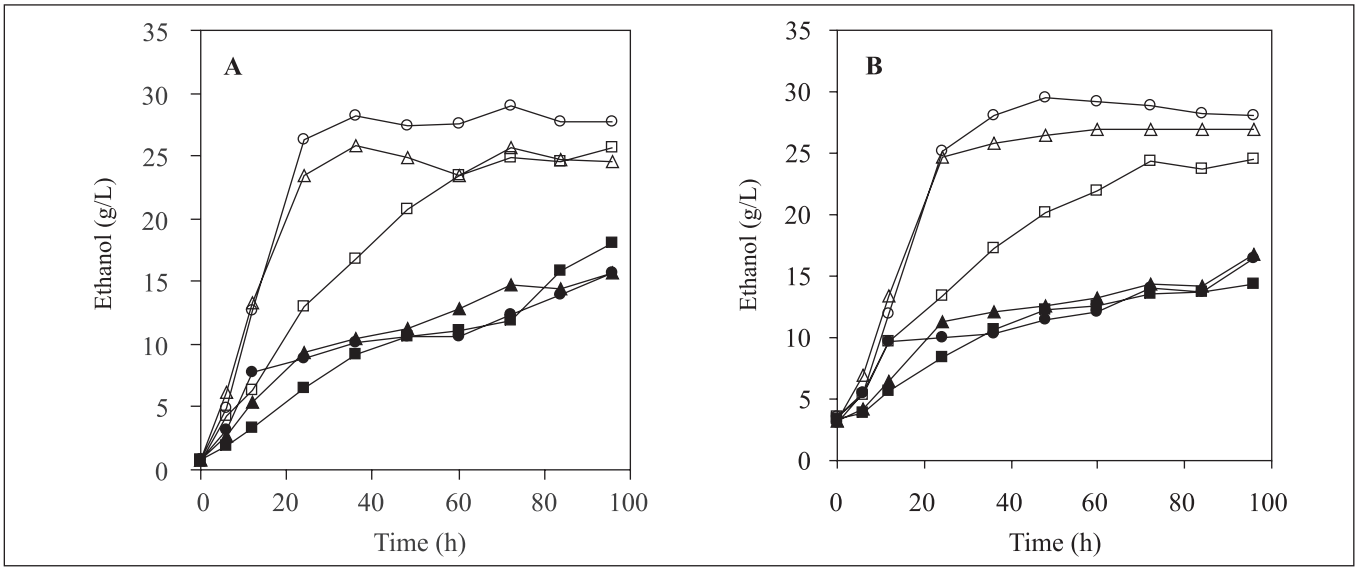

Figure 6. Ethanol production by *Escherichia coli* strain KO11 (closed symbols) and *Klebsiella oxytoca* strain P2 (opened symbols) during sugarcane molasses fermentation diluted 10X in distillated water (A) or diluted 10X in vinasse (B). [■ □] Without supplementation; [▲ △] supplemented with 0.5% yeast extract; [● ○] supplemented with LB broth reagents.

The results obtained in this work for KO11 are similar to those observed in a previous study (24), in which greater than 90% of the maximum theoretical yield was reached upon fermentation of 9% sucrose. However, P2 presented poorer results than in that study, producing 73% of the maximum theoretical yield with 8% sucrose and 58% with 12% sucrose.

Growth and fermentation of sugarcane juice to ethanol by KO11 and P2 required addition of nutrients. For instance, 0.5% yeast extract was sufficient for rapid growth of both recombinants, but addition of yeast extract, micronutrients and thiamine promoted better ethanol production. The low KO11 ethanol yield in sugarcane juice containing LB broth (23 g/L), as compared to the yield in sugarcane juice with 0.5% yeast extract (34 g/L) was unexpected. When NaCl was omitted from the medium, KO11 ethanol production increased to 30 g of ethanol/L after 96h. Previous studies reported reduction in the fermentation rate of *E. coli* pLOI297, the recombinant strain that gave rise to KO11, when more than 200 mM of NaCl was added to the medium (4). Similarly, a decrease in sugarcane juice fermentation by *Z. mobilis* was described at NaCl concentrations greater than 7 g/L, and a reduction in biomass production from 2.8 to 0.9 g/L was observed when the salt concentration increased from 3 to 9 g NaCl/L (18). These results suggest that high osmolarity of the medium could be responsible for the reduction in alcoholic fermentation of the sugarcane juice supplemented with LB components. These results are in agreement with those of Underwood *et al*. (30), who also observed that high growth medium osmolarity reduced KO11 growth and volumetric production. In contrast to KO11, P2 presented the best fermentation result in sugarcane juice supplemented with LB (44.2 g ethanol/L after 96h), producing more than twice the concentration KO11 under the same conditions. Thus, P2 was not affected by the high osmolarity of the medium.

Addition of 0.2% ammonium sulfate to sugarcane juice resulted in growth and ethanolic fermentation by both recombinants. Nevertheless, micronutrient and thiamine supplements did not improve KO11 ethanol production, with only 25 g ethanol/L produced, only higher than in fermentation of sugarcane juice supplemented with LB. P2 also presented better ethanol production in sugarcane juice supplemented with inorganic nutrients. Although micronutrients and thiamine did not improve KO11 ethanol production, addition of these nutrients resulted in higher ethanol production by P2. Addition of thiamine alone increased P2 ethanol production, but had no effect on fermentation by KO11.

Molasses and vinasse are byproducts of the sugar-alcohol industry in Brazil. Molasses is used as additive in ethanol production due to its high sugar content while vinasse is used for irrigation due to its high micronutrient content. The ethanol concentration in vinasse is relatively high (about 3.5 g/L) since it is a residue of the alcohol industry and is obtained in the final distillation process. *E. coli* KO11 fermented molasses with low efficiency under all conditions of dilution and supplementation. The high amount of KOH used to maintain the pH during fermentation can be related to loss of ethanologenicity by KO11. Previous studies describe high KO11 instability during continuous fermentation, with rapid ethanologenicity loss (19,20). The poorer results obtained in this work with sugarcane juice containing LB and with molasses may be due to the instability of the Pet operon described. Unlike KO11, *K. oxytoca* P2 fermented molasses with high efficiency. The recombinant *K. oxytoca* P2 presented better results than *E. coli* KO11 in fermentation of sugarcane juice and molasses, producing more ethanol and presenting higher volumetric production and higher stability during fermentation.

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RESUMO

Fermentação etanólica de sacarose, caldo de cana-de-açúcar e de melaço por *Escherichia coli* KO11 e *Klebsiella oxytoca* P2

As bactérias recombinantes *Escherichia coli* KO11 e *Klebsiella oxytoca* P2 fermentaram sacarose a etanol. Em meio mínimo com 2% ou 12% de sacarose, KO11 apresentou, respectivamente, 75% e 41% do rendimento máximo teórico (0,54g de etanol/g de sacarose). No caldo Luria-Bertani (LB) com até 8% de sacarose, KO11 apresentou rendimento de aproximadamente 94-96% e com 12% de sacarose, KO11 apresentou cerca de 69% de rendimento (44,5g de etanol/L). A porcentagem do rendimento máximo teórico obtida com P2 em meio mínimo com 2% de sacarose foi de 55% e com 12% de sacarose foi de 47%. Em LB com 2 ou 4% de sacarose, P2 apresentou 94-95% do rendimento máximo teórico, porém somente cerca de 73% com 8% de sacarose (31,4g de etanol/L) e 58% com 12% de sacarose (37,5 g/L). A produtividade volumétrica em LB contendo 2% de sacarose foi de 0,41 g/L/h para KO11 e de 1,1 g/L/h para P2, enquanto que em LB com 12% de sacarose, a produtividade foi 0,96 g/L/h (KO11) e 1,4 g/L/h (P2). Durante a fermentação do caldo de cana, *E. coli* KO11 e *K. oxytoca* P2 produziram, respectivamente, 39,4 g de etanol/L e 42,1 g/L quando suplementado com 0,5% de extrato de levedura, micronutrientes e tiamina. No caldo de cana suplementado com os reagentes do meio LB, KO11 apresentou forte inibição da fermentação alcoólica, produzindo apenas 23,0 g de etanol/L, enquanto que P2 produziu 44,2 g/L. A produção de etanol por KO11 e P2, no caldo de cana suplementado com a) 0,2% de sulfato

de amônio foi, respectivamente: 25,3 e 30,2 g/L, b) com sulfato de amônio e micronutrientes: 24,9 e 31,6 g/L, c) com sulfato de amônio, micronutrientes e tiamina: 25,6 e 37,5 g/L. Durante a fermentação do melaço, *E. coli* KO11 apresentou baixa produção de etanol e alta produção de ácido láctico. *K. oxytoca* P2 produziu 25 g de etanol/L a partir de melaço diluído 10X em água, com ou sem adição de 0,5% de extrato de levedura e 27,8 g/L com reagentes do caldo LB após 96h. P2 produziu 24,5, 26,9, e 28,0 g de etanol/L em melaço diluído 10X em vinhoto, vinhoto com 0,5% de extrato de levedura e com os reagentes do caldo LB, respectivamente.

Palavras-chave: *Escherichia coli* KO11, *Klebsiella oxytoca* P2, *Zymomonas mobilis*, fermentação da sacarose, fermentação etanólica

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