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Ethanol production from corn cob hydrolysates by *Escherichia coli* KO11

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Corn cob hydrolysates, with xylose as the dominant sugar, were fermented to ethanol by recombinant *Escherichia coli* KO11. When inoculum was grown on LB medium containing glucose, fermentation of the hydrolysate was completed in 163 h and ethanol yield was 0.50 g ethanol/g sugar. When inoculum was grown on xylose, ethanol yield dropped, but fermentation was faster (113 h). Hydrolysate containing 72.0 g/l xylose and supplemented with 20.0 g/l rice bran was readily fermented, producing 36.0 g/l ethanol within 70 h. Maximum ethanol concentrations were not higher for fermentations using higher cellular concentration inocula. A simulation of an industrial process integrating pentose fermentation by *E. coli* and hexose fermentation by yeast was carried out. At the first step, *E. coli* fermented the hydrolysate containing 85.0 g/l xylose, producing 40.0 g/l ethanol in 94 h. Baker's yeast and sucrose (150.0 g/l) were then added to the spent fermentation broth. After 8 h of yeast fermentation, the ethanol concentration reached 104.0 g/l. This two-stage fermentation can render the bioconversion of lignocellulose to ethanol more attractive due to increased final alcohol concentration.
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Introduction

The Brazilian alcohol program represents an outstanding example of a renewable alternative energy source to fossil fuels. At its height in 1985, vehicles powered solely by hydrous alcohol shared 90% of the new car market [5]. At present, anhydrous alcohol is used as an additive to petrol in a blend of 22–24%. The fuel ethanol industry is struggling with many difficulties, but the future of the program remains promising and growth of ethanol production is still expected [4].

The most important agricultural feedstock for the production of ethanol in Brazil is sugarcane [17,19]. In addition, there is a great potential to utilize abundant and renewable lignocellulosic residues as sources of fermentable sugars for conversion into ethanol with pentose-utilizing microorganisms as biocatalysts, in a parallel process integrated with the current national production of alcohol from sugarcane by conventional yeast.

In lignocellulosic pretreatment processes, the hemicellulose component is readily hydrolysed to pentoses and hexoses. The complete bioconversion of all these sugars to ethanol is essential to increase the efficiency and reduce the costs of the process [8].

Genetic improvements of microorganisms have been made either to enlarge the range of substrate utilization or to channel metabolic intermediates specifically towards ethanol [1,10,11]. Many authors have shown that genetically engineered *Escherichia coli* K011 is capable of metabolizing pentoses and hexoses to ethanol with conversion efficiency near the theoretical maximum [2,6,7,15].

In Brazil, residues such as sugarcane bagasse and corn cob may be used in fermentation processes by using an efficient microorganism like *E. coli* K011, in order to expand the capacity of existing conventional ethanol plants and increase the national overall production. Such new biofuel projects may largely contribute to the modernization of the Brazilian distilleries and alcohol program [15].

While there is a possibility to apply this technology and thus create new alternative agroindustrial businesses, improvement of the fermentation process is also required. Many steps such as pretreatment, detoxification and addition of nutrients need to be optimized to generate a competitive lignocellulose ethanol plant [18].

The present study examined the ability of recombinant *E. coli* KO11 to produce ethanol from corn cob hemicellulosic hydrolysates. It investigated the possibility of improving ethanol production using a combination of hydrolysate detoxification with microorganism adaptation or high cellular concentration inocula. Rice bran was tested as an extra source of nutrients. The feasibility of implementing a two-stage sequential fermentation scheme for the production of ethanol was also demonstrated, in which pentose fermentation was performed by *E. coli* KO11 and hexose fermentation by baker's yeast.

Materials and methods

Microorganism

Recombinant *E. coli* KO11 was used [9]. It was grown on plates containing solid LB medium supplemented with 20.0 g/l xylose and 40.0 mg/l chloramphenicol, and incubated at 30°C for 24 h. Isolated colonies were transferred daily to new plates containing the medium indicated above.

Preparation of inocula and fermentation experiments

E. coli KO11 was cultured on solid LB medium supplemented with 20 g/l glucose or xylose, and incubated at 30°C for 24 h. Isolated

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colonies were transferred to 250-ml cultivation flasks containing 50 ml of liquid LB medium supplemented with 40 g/l glucose or xylose, and cultivated at 30°C for 6 h on a rotatory shaker. The resulting cultures were diluted 10-fold into volumetric flasks containing fermentation medium. In order to achieve higher cellular concentrations, 250-ml cultivation flasks with baffles and containing glass beads were also tested.

Corn cob hemicellulosic hydrolysate preparation

The hydrolysate was prepared by dilute acid treatment: 100 g of corn cob was immersed in 600 ml of 1% sulphuric acid (vol/vol) for 24 h. The excess acid was removed and the corn cob was hydrolysed in an autoclave at 120°C for 40 min. The liquid phase from the acid hydrolysis was recovered, filtered, heated to 80°C, overlimed by adding CaO up to pH 10.0 and supplemented with 1.0 g/l sodium sulphite. After cooling at room temperature, the hydrolysate was centrifuged and the pH was adjusted to 7.0 with sulphuric acid.

For the fermentation experiments, the hydrolysate was supplemented with tryptone (10 g/l) and yeast extract (5 g/l). Extra nutritional addition of rice bran (20 g/l) was also tested. Rice bran solution was prepared by adding 1000 ml of distilled water to 200 g of rice bran, autoclaved at 127°C for 15 min and then centrifuged to recover the supernatant.

Fermentation experiments

All experiments were conducted at 30°C, using 100-ml volumetric flasks with 45 ml of corn cob hydrolysate, in a rotatory shaker. The flasks were sealed with rubber stoppers drilled to allow the insertion of a needle through which carbon dioxide was vented and samples were taken. Consumption of sugars and production of ethanol were determined by HPLC, using a Bio-Rad HPX-87H, as described earlier [15].

Calculation of fermentation parameters

The maximum theoretical yield of ethanol from xylose is 0.51 g ethanol/g sugar. The volumetric productivity (Q_P) was calculated by dividing the maximum ethanol concentration by the time required to achieve such a concentration (g/l h). The ethanol yield ($Y_{P/S}$) was calculated as the maximum concentration of ethanol produced divided by the concentration of sugar initially present in the medium (g ethanol/g sugar).

Results and discussion

Important secondary phytobiomass residues such as corn cob and sugarcane bagasse could be readily used to expand the capacity of existing ethanol plants in Brazil. A biomass-to-ethanol facility using pentose-rich hemicellulosic hydrolysate as starting material and *E. coli* KO11 as biocatalyst could be integrated into an existing plant where alcohol is produced from hexoses by conventional yeasts.

The transformation of lignocellulose to ethanol is highly complex and many steps are required such as pretreatment, detoxification and addition of nutrients [16,18]. Acid pretreatment efficiently releases sugar monomers from hemicellulose, but generates inhibitory substances. A detoxification step is necessary and many strategies to inactivate or remove these toxic compounds have been investigated [3,12,13]. Together with detoxification, addition of nutrients comprises a significant fraction of the production costs [16].

In this study, hemicellulosic hydrolysates prepared from corn cob were used as feedstock for the production of ethanol by *E. coli* KO11. A combination of hydrolysate detoxification with microorganism adaptation or utilization of high cellular concentration inocula was investigated to improve ethanol production in this step. Overlimed and neutralized hydrolysates were prepared for fermentation by adding tryptone and yeast extract; *E. coli* KO11 appeared to grow well on these materials.

When the inoculum was grown on LB medium containing glucose (Figure 1), fermentation of the hydrolysate was complete within 163 h, reaching a maximum ethanol concentration of 39.7 g/l, which is equivalent to a yield of 0.50 g ethanol/g sugar. When *E. coli* KO11 was previously grown on plates containing solid LB medium supplemented with xylose as sole sugar source and the inoculum was grown on LB medium containing xylose (Figure 2), ethanol yield was lower, but complete utilization of xylose was faster, requiring 113 h. Previous adaptation of bacterial cells on medium containing xylose would have a positive influence on the subsequent fermentation of pentose-rich hydrolysates.

Hydrolysates prepared from corn cob were also investigated using rice bran solution to provide vitamins [14]. Fermentation media supplemented with rice bran were metabolized efficiently by *E. coli* KO11. Maximum ethanol concentration was obtained within 69 h and ethanol yield approached 0.50 g ethanol/g sugar. Without rice bran supplementation, the recombinant strain metabolized xylose more slowly. Furthermore, ethanol yield dropped to 0.45 g ethanol/g sugar (Table 1). Since the addition of nutrients comprises a considerable fraction of the production costs, besides the addition of rice bran solution, other inexpensive and easily available materials must be found in attempts to substitute yeast extract and tryptone, without affecting ethanol yield and productivity.

To examine the possibility of improving the fermentation using higher initial cell concentrations, inocula were prepared by transferring fresh colonies to cultivation flasks with or without

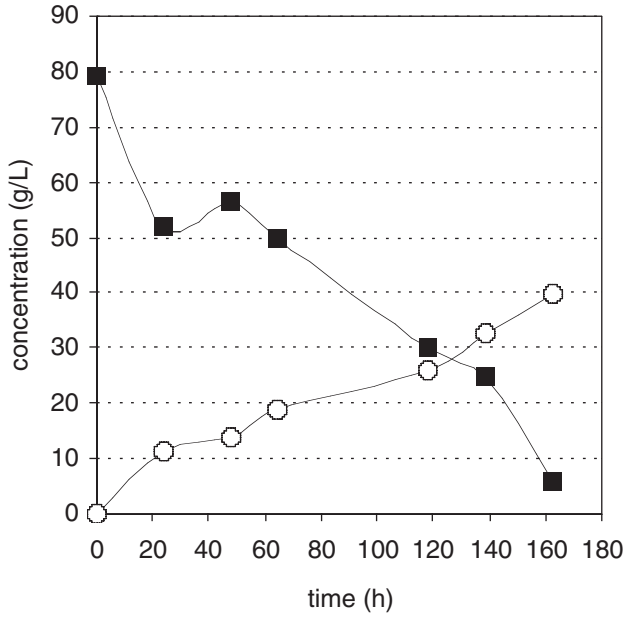

Figure 1 Fermentation of hemicellulose hydrolysate from corn cob by *E. coli* KO11. Inoculum grown on LB medium supplemented with glucose. (■) Sugar consumption; (○) ethanol production.

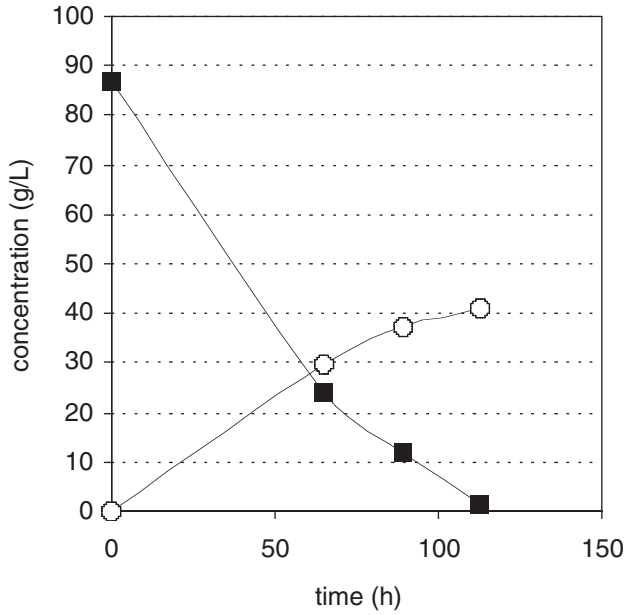

Figure 2 Fermentation of hemicellulose hydrolysate from corn cob by *E. coli* KO11. Cells were previously adapted on LB medium supplemented with xylose. (■) Sugar consumption; (○) ethanol production.

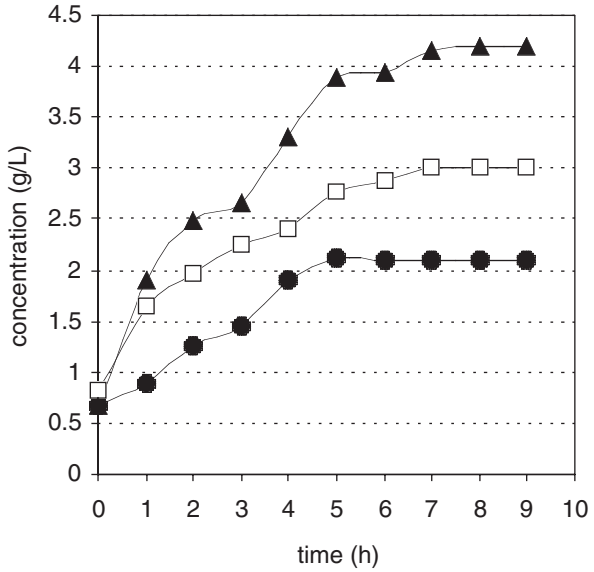

Figure 3 Growth of *E. coli* KO11 in LB medium, using cultivation flasks with or without baffles, and addition of glass beads. (●) Flasks without baffles; (□) flasks with baffles; (▲) flasks with baffles and addition of glass beads.

baffles, containing LB medium supplemented with xylose. Further, these flasks were used in combination with the addition of glass beads to provide greater turbulence and enhance homogenization of the medium. When flasks without baffles were used and glass beads were not added, the cellular concentration achieved 2.0 g/l within 5 h and no further growth was observed. The inoculum concentration increased significantly to 4.2 g/l when flasks with baffles and glass beads were used (Figure 3). Increase of cellular concentration was assumed to be related to an increase of aeration and homogenization provided by the baffles and glass beads.

Two LB media flasks supplemented with 50 g/l glucose were inoculated with different inocula concentrations: 4.2 g/l and 2.0 g/l. Maximum ethanol concentration of 17.8 g/l was reached in 43 h for fermentation medium inoculated with 2.0 g/l cells, approximately 40% higher compared to fermentation medium inoculated with 4.2 g/l cells.

LB medium supplemented with 42 g/l xylose and corn cob hydrolysates were also inoculated with the inocula concentrations mentioned above and similar results were observed. In LB medium containing xylose, maximum ethanol concentrations were 23.0 and 19.6 g/l, and ethanol yields were 0.53 and 0.46 g/g (g ethanol/g sugar consumed) for fermentations using lower and higher cellular concentration inocula, respectively. Fermentations of corn cob hydrolysates were essentially complete within 163 h in both cases, but final ethanol concentrations of 39.7 and 28.0 g/l were reached for lower and higher concentration inocula, respectively (Figure 4).

Fermentation cultures inoculated with an inoculum concentration of 2.0 g/l presented higher ethanol yield and productivity compared to cultures started with an inoculum concentration of

Table 1 Xylose consumption and ethanol production from corn cob hemicellulosic hydrolysate by *E. coli* KO11

Time (h)	Concentration (g/l)			
	Supplemented with TR+YE+RB		Supplemented with TR+YE	
	Xylose	Ethanol	Xylose	Ethanol
0	72.21	0.00	85.00	0.00
24	38.27	20.44	48.18	20.80
48	16.99	29.64	25.59	29.72
69	0.00	36.05	4.10	37.85
77	–	–	0.00	38.50

Rice bran solution was tested as an extra supplement. TR, tryptone; YE, yeast extract; RB, rice bran.

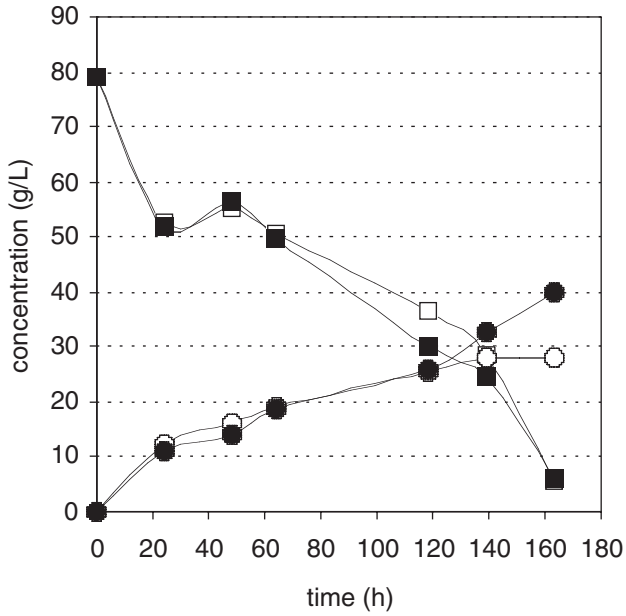

Figure 4 Influence of different inoculum on fermentation of corn cob hemicellulose hydrolysate by *E. coli* KO11. (I) Inoculum concentration of 4.2 g/l: (□) sugar consumption; (○) ethanol production. (II) Inoculum concentration of 2.0 g/l: (■) sugar consumption; (●) ethanol production.

Table 2 Fermentation of corn cob hydrolysate by recombinant *E. coli* KO11 and 150 g/l sucrose by baker's yeast (100 g/l) in a two-stage alcohol production process

Time (h)	Concentration (g/l)		
	Xylose	Sucrose	Ethanol
0	84.76	0.00	0.00
24	58.60	0.00	9.90
48	41.18	0.00	18.92
72	4.14	0.00	38.72
94	3.67	0.00	40.00
110	0.00	0.00	35.00
111	0.00	150.00	32.14
112	0.00	ND	36.00
113	0.00	ND	60.50
114	0.00	ND	72.50
115	0.00	ND	85.00
116	0.00	ND	93.00
117	0.00	ND	100.30
118	0.00	0.00	107.00

ND, not determined.

4.2 g/l. These are surprising results because generally higher cellular concentrations result in improvement of fermentation performance. It was verified that this difference in final ethanol concentrations was not due to higher ethanol evaporation from flasks containing fermentation medium inoculated with higher cellular concentration inocula. This difference may be related to the metabolism of the bacteria; the microorganism may be consuming ethanol or producing less ethanol in a condition of high cell density.

Finally, a two-stage fermentation strategy was studied, utilizing both recombinant *E. coli* KO11 and baker's yeast, to achieve a higher final alcohol concentration. In the first step, strain KO11 fermented the corn cob hydrolysate supplemented with tryptone and yeast extract, and a maximum ethanol concentration (40.0 g/l) was obtained within 94 h. Ethanol yield was 0.47 g ethanol/g sugar, which is 92% of the theoretical maximum.

In the second step, 100 g/l baker's yeast (wet weight) and 150 g/l sucrose were added to the spent fermentation broth immediately following the end of the first step. After 8 h, sucrose was successfully fermented by yeasts and the final ethanol concentration accumulated in the medium was 107 g/l (Table 2).

E coli KO11 produces acetic, lactic and succinic acids in small amounts under anaerobic conditions, but after the completion of the first step, the pH of the medium was 6.0 and it was not necessary to adjust it before adding sucrose and yeast. Apparently, the hemicellulosic hydrolysate presents a buffering capacity due to the presence of compounds that act as buffers at the pH of the fermentation, and the presence of acids produced in the previous step by *E. coli* did not affect the subsequent ethanol production by yeast. Furthermore, it is not necessary to remove bacterial cells prior to the addition of yeast cells. With an ethanol final concentration of 10%, bacterial cells die and there are no adverse impacts for the environment.

The two-stage fermentation scheme reported above could be utilized in Brazil, with the recombinant strain *E. coli* KO11 performing fermentation of pentose-rich media and conventional yeast metabolizing hexose-rich media. At present, the total ethanol production volume derives exclusively from the fermentation of sugarcane juice by yeasts. If the amount of fermentable sugars found in hemicellulose could be recovered and utilized, it would add a considerable bonus over the total amount of ethanol produced in the country. Besides corn cob, sugarcane bagasse must be viewed as an important potential raw material for ethanol production. Sucrose was chosen as starting substrate in the second step because it is abundant in Brazil and new possibilities for its utilization must be studied.

It would be possible to take advantage of the attributes of both *E. coli* KO11 and conventional yeasts in a single vessel. *E. coli* KO11 can efficiently convert pentoses into ethanol and conventional yeasts show high efficiency for fermenting hexoses, high ethanol tolerance and long residence time in fermentation vats. This two-step process demands a specific plant design, in which fermentation of hemicellulosic hydrolysates by *E. coli* occurs in an independent vessel. Sucrose and yeasts are added to this vessel after the completion of the first step. This process could be incorporated with the objective of maximizing productivity, making the best use of the available raw materials, resources and facilities of the installed sugar–alcohol units.

Conclusions

(1) *E. coli* KO11 can efficiently ferment xylose-rich hemicellulosic hydrolysates to ethanol. Previous physiological adaptation of the cells in medium containing xylose was important to improve the fermentation of these hydrolysates.

(2) Inexpensive material such as rice bran solution can be used effectively as a nutritional supplement to improve the fermentability of hemicellulosic hydrolysates. Ethanol yields near the theoretical maximum were achieved within 69 h.

(3) The production of higher concentration inocula (4 g/l) was obtained using cultivation flasks with baffles and addition of glass beads. However, ethanol yields were higher when synthetic media and hydrolysates were inoculated with lower concentration inocula (2 g/l).

(4) Baker's yeast was able to metabolize sucrose rapidly, even in the presence of compounds from the metabolism of recombinant strain *E. coli* KO11 produced in a previous fermentation step.

(5) Addition of baker's yeast and sucrose after the fermentation of corn cob hydrolysate by *E. coli* KO11 increased the ethanol concentration to 100 g/l, rendering the bioconversion of lignocellulose to ethanol more attractive.

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References

1 Aristidou A and M Pentillä. 2000. Metabolic engineering applications to renewable resource utilization. *Curr Opin Biotechnol* 11: 187–198.

2 Asghari A, RJ Bothast, JB Doran and LO Ingram. 1996. Ethanol production from hemicellulose hydrolysates of agricultural residues using genetically engineered *Escherichia coli* strain KO11. *J Ind Microbiol* 16: 42–47.

3 Barbosa MFS, MJ Beck, JE Fein, D Potts and LO Ingram. 1992. Efficient fermentation of *Pinus* sp. acid hydrolysates by an ethanologenic strain of *Escherichia coli*. *Appl Environ Microbiol* 58: 1382–1384.

4 Berg C. 1999. World Ethanol Production and Trade to 2000 and Beyond Published in site, www.distill.com/berg.
5 Carvalho LCC. 1996. The prospects for ethanol production in Brasil: future remains promising despite current difficulties. *Int Sugar J* 98: 289–294.
6 Dien BS, RB Hespell, LO Ingram and RJ Bothast. 1997. Conversion of corn milling fibrous co-products into ethanol by recombinant *Escherichia coli* strains KO11 and SL40. *World J Microbiol Biotechnol* 13: 619–625.
7 Dien BS, LB Iten and RJ Bothast. 1999. Conversion of corn fiber to ethanol by recombinant *E. coli* strain FBR3. *J Ind Microbiol Biotechnol* 22: 575–581.
8 Gong CS, NJ Cao, J Du and GT Tsao. 1999. Ethanol production from renewable resources. *Adv Biochem Eng Biotechnol* 65: 207–241.
9 Ingram LO, K Ohta and BE Wood. 1998. Recombinant cells that highly express chromosomally integrated heterologous genes. US Patent 5821093.
10 Ingram LO, PF Gomez, X Lai, M Moniruzzaman, BE Wood, LP Yomano and SW York. 1998. Metabolic engineering of bacteria for ethanol production. *Biotechnol Bioeng* 58: 204–214.
11 Ingram LO, HC Aldrich, ACC Borges, TB Causey, A Martinez, F Morales, A Saleh, SA Underwood, LP Yomano, SW York, J Zaldivar and S Zhou. 1999. Enteric bacterial catalysts for fuel ethanol production. *Biotechnol Prog* 15: 855–866.
12 Larsson S, A Reimann, NO Nilvebrant and LJ Jönsoon. 1999. Comparison of different methods for the detoxification of lignocellulose hydrolysates of spruce. *Appl Biochem Biotechnol* 77-79: 91–103.
13 Martinez A, ME Rodriguez, ML Wells, SW York, JF Preston and LO Ingram. 2001. Detoxification of dilute acid hydrolysates of lignocellulose with lime. *Biotechnol Prog* 17: 287–293.
14 Silva SS, JD Ribeiro, MG Felipe and M Vitolo. 1997. Maximizing the xylitol production from sugar cane bagasse hydrolysate by controlling the aeration rate. *Appl Biochem Biotechnol* 63–65: 557–564.
15 Takahashi CM, KG de C Lima, DF Takahashi and F Alterthum. 2000. Fermentation of sugar cane bagasse hemicellulosic hydrolysate and sugar mixtures to ethanol by recombinant *Escherichia coli* KO11. *World J Microbiol Biotechnol* 16: 829–834.
16 von Sivers M, G Zacchi, L Olsson and B Hahn-Hagerdahl. 1994. Cost analysis of ethanol production from willow using recombinant *Escherichia coli*. *Biotechnol Prog* 10: 555–560.
17 Wheals AE, LC Basso, DMG Alves and HV Amorim. 1999. Fuel ethanol after 25 years. *Trends Biotechnol* 17: 482–487.
18 Zaldivar J, J Nielsen and L Olsson. 2001. Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. *Appl Microbiol Biotechnol* 56: 17–34.
19 Zanin GM, CC Santana, EPS Bon, RCL Giodano, FF deMoraes, SR Andrietta, CCC Neto, IC Macedo, D LahrFo, LP Ramos and JD Fontana. 2000. Brazilian bioethanol program. *Appl Biochem Biotechnol* 84–86: 1147–1161.