

# Sugar Cane Juice Fermentation by *Zymomonas mobilis* CP4 Subjected to Inhibition by Ethanol and High Initial Concentration of Substrate

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## ABSTRACT

Sugar cane juice fermentation by *Z. mobilis* CP4 subject to stress by ethanol and high concentration of sucrose was investigated. Supplementation with saponifiable portion of soybean oil deodourising distillate (SOD) at 5; 10 and 15 mL/L. The ethanol production resulted values of 15.91; 16.99 and 15.63 g/L respectively. Values of  $Y_p/s$  0.35; 0.36 and 0.37 were achieved, which represented 20.69; 24.14 and 27.59% respectively higher when unsupplemented medium fermentation was carried out.

**Key words:** *Zymomonas mobilis*, fermentation, lipids, ethanol

## INTRODUCTION

For the fuel ethanol industry to expand, there is a requirement for search of more competitive ethanologenic microorganisms. *Zymomonas mobilis* has attracted the attention as a promising bacterium regarding improving ethanol production (Daugulis *et al.*, 1997). Microorganisms are usually subjected to stressing conditions in ethanol distilleries. High sucrose concentrations and ethanol are a case in point (Falcão de Moraes *et al.*, 1993). Growth in high osmolar sugar solutions promotes by-products formation and the substrate is not fully metabolised. Ethanol has been established as the primary end product responsible for inhibiting fermentations (Moreau *et al.*, 1997; Hallsworth, 1998). Tano (1999) has found that concentration of ethanol added in the fermentation medium as low as 25 mL/L has an inhibitory effect. The fermentation showed a long lag phase and it was incomplete as sugars were not fully metabolised.

Cells of microorganisms grown in the presence of ethanol show changes in the lipid composition of the cell plasma membrane (Hermans *et al.*, 1991; Weir & Chase Jr., 1995; Mizoguchi & Hara, 1997). In *Z. mobilis* there are two possible mechanisms involving lipids to explain the ethanol tolerance. In the first mechanism, it is postulated

that the high levels of cyclic lipids in the cell membranes protect the bacterium from the toxic effects of ethanol (Hermans *et al.*, 1991). In the second mechanism, it is postulated that the high levels of cis-vaccenic acid in the phospholipids of the bacterial membrane protect the bacterium from ethanol toxicity (Ohta *et al.*, 1981; Moreau *et al.*, 1997).

There has been several publications dealing with the inhibitory effect of both ethanol and high initial concentration of substrate. These publications have used enrichment of fermentation medium such as salts, vitamin, proteins and lipids aiming fermentation improvement (Weir & Chase Jr., 1995; Ohta *et al.*, 1981; Mizoguchi & Hara, 1997; Lawford & Rousseau, 1997; Duarte *et al.*, 1996). Minimization of cost are also associated with nutritional supplement for large scale production of ethanol (Lawford & Rousseau, 1997).

Commercial dispersants and detergents are utilised when lipids are used as supplement in the culture medium (Ohta & Hayashida, 1983; Duarte *et al.*, 1996; Bruheim *et al.*, 1997).

Deodourising distillate is a by-product of the soybean oil refining process. It is a lipid-rich material and its composition presents aldehydes, ketones, peroxides, hydrocarbons, tryglicerides and fatty acids. Its highly complex composition

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depends on soy bean variety and soy bean oil processing utilised. Free fatty acids and triglycerides constitute its saponifiable portion. (Almeida *et al.*, 1994; Kao *et al.*, 1998).

In the currently study we have used the saponifiable portion of SOD as a nutritional supplement for ethanol production by *Z. mobilis* CP4, subjected to inhibitory fermentation conditions. Such conditions were achieved by adding ethanol into the culture medium and a high initial sucrose concentration.

## MATERIAL AND METHODS

**Microorganism:** Bacterial strain used in this study was *Zymomonas mobilis* var. *recifensis*. It was maintained by monthly transfer to the following medium (g/L): sucrose 50, yeast extract 5,  $\text{KH}_2\text{PO}_4$  1,  $(\text{NH}_4)_2\text{SO}_4$  1,  $\text{MgSO}_4$  0.5.

Fermentation medium contained (g/L) sugar cane juice with total reducing sugars adjusted to 150, yeast extract 5,  $\text{KH}_2\text{PO}_4$  1,  $\text{MgSO}_4$  0.5,  $(\text{NH}_4)_2\text{SO}_4$  1. The media in duplicates were supplemented with SOD at the following concentrations (mL/L): 5; 10 and 15. These media concentrations were carried out in the three repetitions according statistical methodology by Barros Neto *et al.* (1996). The media were sterilised by autoclaving and after cooling, 25 mL/L the ethanol GR was added. The saponifiable portion of SOD was extracted according to Rabassi (1997). A sample of 20g of SOD was mixed with 40mL of ethanol and then let it boil. After adding 20 g of KOH at 50% (W/V), it was refluxed. After cooling HCl was added for neutralization

The total reducing sugar concentration of 150 g/L in the cane juice and lipid supplementation were utilised according to statistical method (Barros Neto *et al.*, 1996). The determination of biomass was by measuring the absorption at 605 nm and the corresponding dry weight was obtained from a standard plot (Duarte *et al.*, 1996). Reducing sugars and total reducing sugars were assayed by the method of Somogyi (1945) and Nelson (1944). Samples for total reducing sugars determination were treated according to Diez & Yokoya (1996).

Ethanol concentrations were analysed using a gas chromatograph CG-17A SHIMADZU, with DBwax column with 210°C injector temperature (Diez & Yokoya, 1996).

## RESULTS AND DISCUSSION

Table I and Figures 1-4 show the results of biomass, ethanol, values for total reducing sugars and reducing sugars from cultures of *Z. mobilis* CP4. When the medium with no-lipid supplement was used, ethanol production reached 13.89 g/L (Fig. 1). However a gradually raising in the ethanol production was observed when the lipid-supplement media were used. Ethanol concentrations of 15.91, 16.99 and 15.63 g/L (Figures 2-4) were found when the medium was enriched with SOD at concentration of 5, 10 and 15 mL/L respectively. These results were 14.54, 22.31 and 12.52% superior when the medium with supplementation was used. Increased values of ethanol production have also been found by other authors. Weir & Chase Jr. (1995) have reported values 12% higher and Ohta *et al.* (1981) have achieved values 20% superior when the culture media were enriched with proteolipids.

In *Z. mobilis* when both substrate metabolism and ethanol production continue while there is a cessation of cellular growth, it is said that growth is uncoupled. Joachimsthal *et al.* (1998) have reported an increased ethanol excretion in the presence of acetate while growth was negligible. Calazans *et al.* (1990) have demonstrated absence of growth during ethanol formation when there was a lack of mineral salts in the medium fermentation. This work suggested that supplementation of medium with SOD could also uncouple growth from ethanol production. This was supported by biomass values which were lower and yet ethanol concentrations were higher when the comparison is made with results of fermentation without SOD supplementation. When SOD-supplemented media were used the biomass values were 1.36; 1.23 and 1.19 g/L (Table I) at 5, 10 and 15 mL/L of SOD added to the medium, respectively. These values of biomass are 22.29; 29.71 and 32.00% lower than those where no SOD was used.

**Table 1.** Comparative kinetic parameters for sugar cane juice fermentation added with 25 mL/L of ethanol by *Z. mobilis* CP4 (28°C, 180 rpm, 48 hours).

Sugar cane juice		Lipids: 0	Lipids: 5 mL/L	Lipids: 10 mL/L	Lipids: 15 mL/L
TRS <sub>0</sub>	g/L	151,74	147,49	151,53	147,49
TRSc	g/L	48,03	45,49	47,48	41,89
RS <sub>0</sub>	g/L	15,30	14,98	13,84	14,86
RSf	g/L	55,10	70,73	71,15	70,86
[X]	g/L	1,75	1,36	1,23	1,19
[P]	g/L	13,89	15,91	16,99	15,63
Y <sub>x/s</sub>	g/g	0,036	0,029	0,026	0,028
Y <sub>p/s</sub>	g/g	0,29	0,35	0,36	0,37
Yecon.	%	16,99	20,02	20,81	19,66

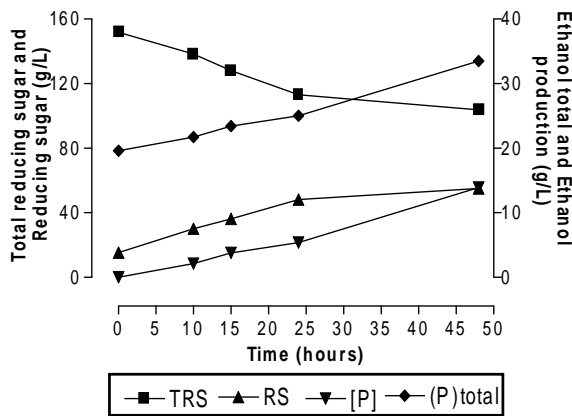
When the medium without SOD-supplementation was used, a value of 0.036 for Y<sub>x/s</sub> was found. This value was similar to that of Lee *et al.* (1981) when glucose at 100 g/L and temperature of 30°C were used. However when SOD-enriched medium with 5, 10 and 15 mL/L was used, the values of 0.029, 0.026 and 0.028 were found. These values were similar when high concentration of substrate were used (Rogers *et al.*, 1982). Lower values of Y<sub>x/s</sub> have been presented by other authors (Diez & Yokoya, 1996).

When the medium without supplementation was used a value of 0.29 for Y<sub>p/s</sub> was found. However, when enriched medium with 5, 10 and 15 mL/L was used, higher values were found for Y<sub>p/s</sub> 0.35, 0.36 and 0.37. Similar results have been achieved by Toran-Diaz *et al.* (1983) when high concentration of fructose was used. Toran-Diaz *et al.* (1983) have presented values of 0.38 and 0.34 for Y<sub>p/s</sub>. When medium was supplemented with yeast extract even higher values were found by Favela Torres & Baratti (1987).

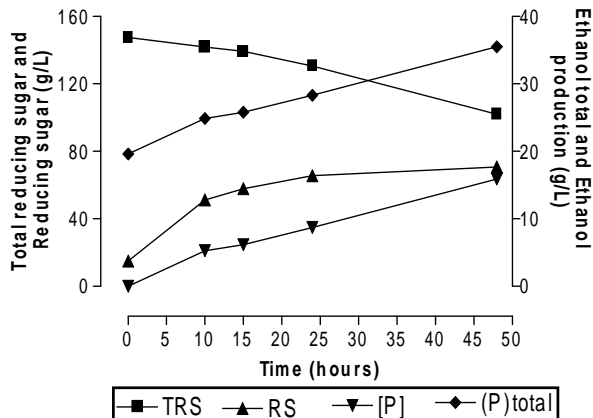
When the medium without SOD was used, a value of 16.99% for Yecon. was found. However, values of 20.02, 20.81 and 19.66% were achieved. These values are lower to that of Duarte *et al.* (1996). Duarte *et al.* (1996) have also utilised soybean oil deodourising distillate and achieved 22.40% for Yecon.. However ethanol had not been added in the culture medium.

Incomplete fermentations have been reported when high initial substrate concentrations were used (Doelle & Greenfield, 1985; Daugulis *et al.*, 1997). Such fermentations accumulated high levels of reducing sugars (Figures 1-4) (Calazans *et al.*, 1990). Lyness & Doelle (1981) have suggested that when initial sucrose concentration was high there was a low value for Yecon. due to poor utilisation of fructose rather than lack of hydrolysis of sucrose. These considerations are in agreement with our results as the final reducing sugar values are high.

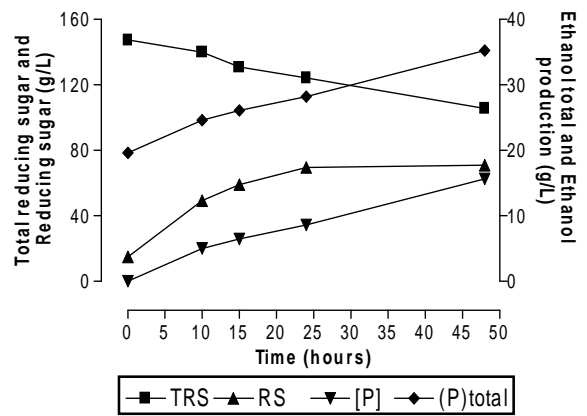
In addition to inhibition effects of ethanol (Calazans *et al.*, 1990), several authors have concluded that medium composition might also reduce the total amount of carbon available for ethanol formation and lowering fermentation efficiencies. The low efficiencies obtained in this study could probably be ascribe to the presence of certain compounds in the sugar cane juice which could be inhibitory to growth and ethanol production by *Z. mobilis* (Doelle *et al.*, 1990; Duarte *et al.*, 1996). However the results in this study, utilising sugar cane juice with supplementation of SOD, have improved the catabolic activity of *Z. mobilis* which has led to an improvement of ethanol yield.



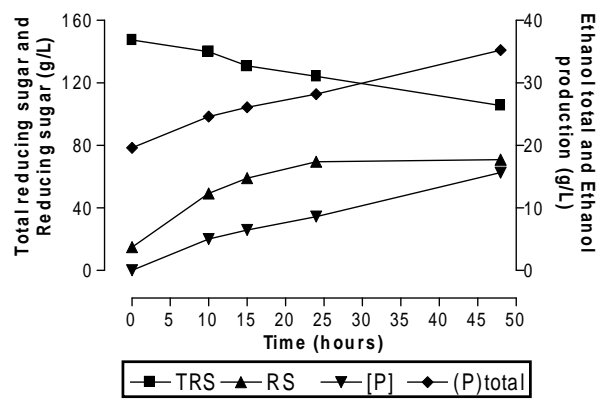
**Figure 1.** Batch fermentation of sugar cane juice medium added with 25 mL/L of ethanol by *Z. mobilis*.



**Figure 2.** Influence of SOD (5 mL/L) on batch fermentation of sugar cane juice medium added with 25 mL/L of ethanol by *Z. mobilis*.



**Figure 3.** Influence of SOD (10 mL/L) on batch fermentation of sugar cane juice medium added with 25 mL/L of ethanol by *Z. mobilis*.



**Figure 4.** Influence of SOD (15 mL/L) on batch fermentation of sugar cane juice medium added with 25 mL/L of ethanol by *Z. mobilis*.

## NOMENCLATURE

TRS= total reducing sugars; TRS<sub>0</sub>=initial total reducing sugars; TRSc=consumption of total reducing sugars; RS=reducing sugars; RS<sub>0</sub>=initial reducing sugars; RSf=final reducing sugars; [X]=biomass production; [P]=ethanol production; (P)total = ethanol total (added+production); Y<sub>p/s</sub>=ethanol yield coefficient (g ethanol/g glucose consumed); Y<sub>x/s</sub>=biomass yield coefficient (g biomass/g glucose consumed); Yecon.(fermentation efficiency) = [ethanol] / (0.511 · TRS<sub>0</sub>)

## ACKNOWLEDGMENTS

Marcia S. Tano is grateful for the financial support given by CAPES. The authors are grateful to Ieda Spacino Scarmínio for the statistical analysis.

## RESUMO

Este trabalho investigou a fermentação de caldo de cana-de-açúcar por células de *Z. mobilis* submetidas ao estresse pelo etanol e elevada concentração inicial de sacarose. Foi utilizado como suplementação a porção

saponificável do destilado da desodorização de óleo de soja (SOD) nas seguintes concentrações: 5, 10 e 15 mL/L. Os valores de produção de etanol foram respectivamente: 15,91; 16,99 e 15,63 g/L. Os valores de  $Y_{p/s}$  foram: 0,35; 0,36 e 0,37, os quais representam respectivamente: 20,69; 24,14 e 27,59%, superiores daquele cujo meio não foi suplementado.

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Received: September 29, 1999;

Revised: December 27, 1999;

Accepted: April 07, 2000.