SCREENING OF *ZYMOMONAS MOBILIS* AND *SACCHAROMYCES CEREVISIAE* STRAINS FOR ETHANOL PRODUCTION FROM CASSAVA WASTE

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ABSTRACT

The efficient ethanol production using Zymomonas mobilis (NRRL B808) and Saccaromyces cerevisiae (NRRL Y898) in utilizing the sago industry waste (Tippi), was studied in the liquid state fermentation process. The fermentation parameter for ethanol production was optimized for various pH and temperature ranges. Both strains of Z.mobilis and S.cerevisiae were selected for ethanol production potential at optimized conditions because Z.mobilis can tolerate in higher sugar concentration (more than 15%) and also S.cerevisiae is higher ethanol tolerant. Ethanol production at pH 6.0 and 36 h (residence time) of fermentation was the highest in the Z.mobilis mediated fermentation (5 % more) than the ethanol production rate of S.cerevisiae.

Keywords: *Cassava, Ethanol, fermentation, Zymomonas mobilis, Saccharomyces ceresiviae.*

INTRODUCTION

Agricultural crop residues are among the major sources of the total biomass with considerable production of food feed and fuel [1-3]**.** Several processes have been designed to use waste in many biotechnological processes in the production of fermentation products or in biomass production. All these process depend on the case of degradation of waste[3-5]. The waste of tapioca (*Manihot esculenta,* Crantz.) after the major portion of the tuber is used for sago and starch production is rich in carbohydrate and various types of micro-organism proliferate in it[6]. The free sugars and the sugars formed by enzymatic saccharification can be used in many biotechnological processes for single cell protein or ethanol production[7, 8]. Simultaneous saccharification and fermentation of several starchy substrates for ethanol production have been reported by many workers [3,7].

Currently *S. cerevisiae* is used all over the world as the major ethanol producing micro-organism. Despite its extensive use, it has a number of disadvantages[9]. Out of the various micro-organisms tested to find on alternate organisms, *Z. mobilis* has emerged as promising organism for ethanol production. This study has demonstrated that it has a number of advantages and it seems to be most suitable for continuous culture[10, 11]. The present work reports the efficiency of the bacterium *Z. mobilis* and that of the yeast *S.cerevisiae* on ethanol production utilizing the sago industry solid waste (Tippi) in simultaneous saccharification and fermentation process in liquid state[12]. The effects of initial pH and reducing sugar content of the tippi hydrolysate on the fermentation process were studied[7].

EXPERIMENTAL

Microorganisms and growth: *Z. mobilis* and *S.cerevisiae* cultures were grown on broth of maintenance medium [7, 13]. The composition of the maintenance medium contained glucose 20g/l, yeast extract 10g/l, and KH_2PO_4 2g/l. Cultures were maintained in agar slants containing 15g/l of agar into maintenance medium at 28°C for two days.

Preparation of inoculum: To prepare the starter culture, 50 ml of the maintenance medium was taken in 250 ml Erlenmeyer flask, sterilized at 121ºC for 20 min and inoculated with a loopful of *Z. mobilis* or *S. cerevisiae* cultures. The flasks were incubated at 28°C. Log phase culture of *Z. mobilis* (24 h) and *S. cerevisiae* (36 h) used for further studies.

Preparation of slurry: The solid waste (tippi) of sago industry using peeled tubers of tapioca was collected from Varalakshmi sago industry in Salem district, Tamilnadu. The air dried tippi was ground in a Willey Mill to pass through 0.5 mm mesh. The composition of the dried tippi was estimated by standard procedures. It contains 48 % starch, 13 % cellulose, 9 % hemicellulose 11 % lignin 1.1% free reducing sugar, 2.8 % proteins, 40% total organic carbon and total nitrogen 0.46%. Similar results were reported by earlier workers (ref). 10 g of tippi powder was dissolved in 100 ml distilled water. 11.2 mg/g alpha amylase 30 mg/g $CaCl_2$, were added to the slurry. Liquefication was carried out at pH 6.0 and 75°C for 90 min. The slurry was constantly agitated till it was completely liquefied and the period of liquefication was 1.5 h. After liquefication, the pH of the slurry was lowered to 4.5 using 1N HCl and saccharification of the slurry was carried out using the optimum concentration (5 mg/g) of the AMG with mild agitation for 22 h at a temperature of 55^{o}C. 10 ml of inoculum either Z. *mobilis* or *S. cerevisiae* in the broth of mid log phase culture was added to cooled saccharified slurry. The pH values were brought to 6.0 using 0.1N NaOH as the pH of slurry before adjustment was 4.5. Simultaneously saccharification and fermentation (SSF) was carried out at room temperature with three replicate samples for each pH levels studied. The conical flasks were kept in a rotary shaker (Remi, India) at120 rev/min. The samples were collected at an interval of 12 h and studies were carried out upto 48 h for *Z.mobilis* and upto 60 h for *S. cerevisiae*. This procedure has been devised by us following the procedure adopted by Nelliah[13], modifying the procedure.

Analytical method: Total reducing sugar was estimated by 3,5-dinitrosalicylic acid method of Miller[14]. Ethanol was determined by the dichromate reduction method of Caputi *et al*[15].The biomass was estimated by dry cell weight vs. turbidity method[16]. The kinetic parameters of ethanol fermentations were calculated by Abate *et al*[7,17]**.**

RESULTS AND DISCUSSION

***Effect of pH on ethanol production by Z. mobilis and S. cerevisiae*:** Tippi hydrolysate fermentation medium, adjusted to pH 5.0, 5.5, 6.0 and 6.5 was used to investigate the effect of H^+ ion concentration on ethanol production by *Z. mobilis* and *S. cerevisiae*. The above pH range is the optimum condition for the growth of the mentioned organisms. The maximum ethanol production (0.93% v/v) and sugar utilization (73.1%) was observed at pH 6.0 by *Z. mobilis*. *S. cerevisiae* produced maximum ethanol production (0.88 % v/v) and sugar utilization (71.8 %) at the same pH. Moreover, no marked difference in ethanol production and sugar utilization was observed at pH 6.5. However, the decrease in pH 5.5 and below suppressed the ethanol production and sugar utilization ability of these two microbes, the corresponding ethanol production and sugar utilization were 0.84% & 0.81% (v/v) and 67.5%, 64.6% respectively by *Z.mobilis*. In *S. cerevisiae* the ethanol production and sugar utilization were 0.78 & 074% (v/v) and 61.5, 58.3% respectively. Therefore, pH 6.0 was considered optimum and used for further experimentation in both of the organisms. These obtained results were found to be better than the previous workers [7,13] .

***Effect of temperature on ethanol production by Z. mobilis and S. cerevisiae*:** The thermal tolerance of the microbe in fermentation medium at various temperatures *viz.* 25°C, 30°C, 35°C, 40°C was tested (Table 1). These temperatures were chosen to find out the optimum condition for their growth. The results show that maximum ethanol (0.93 and 0.88 % (v/v)) and sugar utilization (73.1 and 71.8%) by *Z. mobilis* and *S. cerevisiae* at 35°C whereas further increase in temperature was inhibitory to ethanol production. Similar results have been earlier reported by Panesar *et al* [7, 18] at the screening of *Z.mobilis* strains for ethanol production from molasses. Thus, the temperature 35°C was considered as optimum for further study.

***Batch Fermentation*:** Cassava waste hydrolysate obtained from saccharification using commercial amylase and amyloglucosidase enzymes under the optimum conditions was fermented either by *Z.mobilis* or *S.cerevisiae*. The residual reducing sugars, biomass and ethanol concentration are given in Table 2a & 2b.

***Reducing sugars*:** The maximum reducing sugars yield obtained after saccharification of tippi hydrolysate with α amylase and AMG was 39.73 g/l. Submerged fermentation with *S.cerevisiae* was slightly faster than that with *Z.mobilis* initially. At the end of 48 h in *S.cerevisiae* fermentation 38% residual sugars were left in the fermenting tippi hydrolysate, while *Z.mobilis* fermentation had only 27% residual reducing sugars.

***Biomass*:** Biomass of both *Z.mobilis* and *S.cerevisiae* increased throughout the fermentation period. The maximum biomass obtained by *Z.mobilis* was only 60% of that of *S.cerevisiae* (Table 2a & 2b). The biomass formed by the bacterium was much lower than by the yeast which might be useful from the point of view of waste production[5].

***Ethanol*:** The concentration of ethanol produced during the course of fermentation of tippi hydrolysate by *Z. mobilis* was higher than that produced by *S.cerevisiae.* The final ethanol concentration obtained in 36 h fermentation period of *Z. mobilis* was 9.26 g/l while *S. cerevisiae* produced 8.73 g/l ethanol in the same period (Table 2a &2b). These results are in conformity with those of Dabas *et al*[19] who obtained 6 – 6.4% (v/v) ethanol in 36 h at 30°C from 25% wheat mash saccharified by a combination of α amylase and amyloglucosidase and fermented by two strains of *S. cerevisiae*.

The fermentation characteristics and some of the parameter of the fermentation kinetics of these two organisms were compared in Table 3. Saccharification of cassava waste by amylase and amyloglucosidase enzyme saccharification resulted with 68.7% saccharification and reducing sugar yield of 39.73 g/l. The final ethanol concentration obtained in 36 h fermentation period of *Z. mobilis* was 9.26 g/l while *S.cerevisiae* produced 8.73 g/l ethanol in the same period. The biomass of *S. cerevisiae* (1.87 g/l) was comparatively more than that of *Z. mobilis* (1.53 g/l). The highest ethanol conversion 23.3% representing 46% of the theoretical yield was obtained by *Z. mobilis* in enzyme saccharification and fermentation. These observations were corroborated by the reports of previous workers[20, 21].

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Table-1: Effect of temperature on ethanol production by *Zymomonas mobilis* and *Saccharomyces cerevisiae*

Temperature ° C	Sugar utilization (%)		Ethanol production (%)	
	Z. mobilis	*S. cerevisiae*	*Z. mobilis*	*S. cerevisiae*
25	62.3	59.3	0.71	0.67
30	68	64	0.84	0.79
35	73.10	71.8	0.93	0.88
40	64	59	0.81	0.76

Table-2: Submerged fermentation of Cassava waste hydrolysate (100 g/l) by pure cultures of *Zymomonas mobilis* and *Saccharomyces cerevisiae.* Results are mean ± standard error of three replicates

Enzyme	Parameters (g/l)	*Z. mobilis*					*S.cerevisiae*				
		Period of fermentation (Hours)									
		0	12	24	36	48	12	24	36	48	60
Saccharification	Reducing sugar	39.73 ±1.5	26.12 ±1.2	23.5 ±1.6	20.2 ±2.0	10.7 ±1.1	25.96 ±1.4	22.1 ±1.6	17.9 ±0.9	15.6 ±1.3	11.21 ±0.6
	Biomass		0.97 ±0.02	1.41 ±0.09	1.52 ±0.01	1.78 ±0.12	0.93 ±0.08	1.63 ±0.11	1.87 ±0.11	2.62 ±0.16	2.99 ±1.4
αAmylase and Amyloglucosidase	Alcohol		8.6 ±0.7	8.73 ±0.5	9.26 ±0.6	8.41 ±0.5	6.66 ±0.4	8.38 ±0.6	8.78 ±0.4	8.71 ±0.4	8.71 ±0.5

Table-3: Effect of enzyme saccharification on the alcoholic fermentation of cassava by *Z. mobilis* and *S.cerevisiae.* Results are mean ± standard error of three replicates

Organisms	Sugar after Saccharification	Percent Saccharification	Fermentation time (Hours)	Ethanol (g/l)	Biomass (g/l)	Volumetric Ethanol productivity ($gl^{-1}h^{-1}$)	Specific Ethanol productivity ($gl^{-1}h^{-1}$)	Ethanol conversion %	Per cent theoretical yield
Z. mobilis	39.73 ± 2.9	68.76 ± 2.8	36	9.26 ± 0.8	1.52 ± 0.1	0.26 ± 0.02	0.17 ± 0.01	23.3 ± 2.4	46 ± 3.4
S. cerevisiae	39.73 ± 2.9	68.76 ± 2.8	36	8.73 ± 0.7	1.87 ± 0.2	0.24 ± 0.01	0.13 ± 0.01	21.97 ± 1.8	43 ± 2.8

REFERENCES

1. R.Zhang ,G.H.M.El.Mashad, K. Hartman, F.Wang, G.Liu, C.Choate and P.Gamble., *Bioresource Tech.*, **98,** 929 (2007).
2. R.Moral, J.M.Caselles, M.D.P.Mureia, A.P.Espinosa B.Rufete and C.Paredes, *Bioresource Tech.*, **96**, 158 (2005)
3. M.Balat, H.balat and C.Oz, Prog. Energy Comb.Sci., **34**, 551 (2008).
4. G.A.Baddi, M. Hafidi, J.Cegarra, J.A.Alburquerque, J.Gonzalvez, V.Gilard, J.C. Revel, *Bioresource Tech*., **93**, 285 (2004).
5. A.Ghofar, S.Ogawa and T.Kokugan, *J. Biosci. Bioengi*., **100**, 606 (2005).
6. V.B.Manilal, C.S.Narayanan and C. Balagopalan, *World J. Microbio. Biotechnol.*, **7,** 185 (1991)
7. K.Pramanik and D.E.Rao, *IE(I) Journal*, **85**, 53 (2005).
8. F.A.Aderemi and F.C.Nworgu , *American-European J. Agri. Environ. Sci*., **2**, 308 (2007).
9. D.Saigal, *Indian J. Microbiol.*, **33**, 159 (1993).
10. P.L.Rogers, K.J.Lee, M.L.Skotnicki and D.E.Tribe, *Adv. Biochem. Engg. Biotechnol.*, **23,** 37 (1982).
11. P.Gunasekaran and K.Chandraraj, *Indian J. Microbiol.*, **30,** 107 (1999).
12. A.O.Ubalua, *Afr.J.Biotechnol*., **6**, 2065 (2007).
13. H.Nelliah, Ph.D. thesis, Studies on ethanol production from cassava starch using *S.cerevisiae* and *Z.mobilis*, Madurai Kamaraj University, Madurai, 1988.
14. G.L.Miller, *Anal. Chem*., **31**, 426 (1959).
15. A.Caputi, M.Veda and T.Brown, *American J. Enol. Viticul.*, **19**, 160 (1968).
16. H.W.Doelle and P.F.Greenfield, *Appl. Microbiol. Biotechnol.*, **22**, 405 (1985).
17. C.Abate, D.Callieri, E.Rodriguoz and O.Garro, *Appl. Microbiol. Biotechnol.*, **45**, 580 (1996).
18. P.Panesar, S.S.Marwaha, S.S.Gill and R.Rai, *Indian J. Microbiol.*, **41**, 187 (2001).
19. R.Dabas , V.K.Verma and K.Chaudhary, *Indian J. Microbiol*, **37**, 49 (1997).
20. N.Lark, Y.Xia, G.Guogin, C.S.Gong and G.T.Tsao, *Biomass and Bioenergy*, **12**, 135 (1997).
21. M.Sairam and G.Seenayya, *Biotechnol. Lett.*, **11**, 589 (1989).

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Every great advance in science has issued from a new audacity of imagination.

-**John Dewey**