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ORIGINAL RESEARCH ARTICLE

Antimicrobial Strategies for Bacterial Contaminants during Ethanol Production from Cellulosic Crop Residues

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ABSTRACT

Ethanol productions from cellulosic crop yield were obtained by *Saccharomyces cerevisiae* and *Pachysolen tannophilus* from sorghum stovar under optimized condition respectively, pH and temperature were optimized for the better growth of *S. cerevisiae* and *P. tannophilus*. A total of 51per cent and 48 per cent more ethanol yield was obtained at initial sugar concentration of 200 g/L. The cellulose fraction of lignocelluloses can be converted to ethanol by either simultaneous saccharification and fermentation or separate enzymatic hydrolysis and fermentation processes. Valuable process mechanisms about the sorghum stovar, paddy straw as water hyacinth waste utilization and inexpensive potent substrate for the production of eco-friendly liquid fuel. The ethanol productivity was higher in *Saccharomyces cerevisiae* and *Zymomonas mobilis* from the substrate of Sorghum stovar (32.01 g^{-1} and 39.11 g^{-1}) followed by water hyacinth (27.70 g^{-1} and 28.04 g^{-1}). Low ethanol yield was recorded in paddy straw (25.03 g^{-1} and 26.06 g^{-1}) respectively.

Key words: cellulosic crop residues, Bioethanol, Bacterial contamination, *Saccharomyces cerevisiae, Zymomonas mobilis.*

1. INTRODUCTION

Bioethanol is the most promising factor nowadays and for feature generation in invader to meet out energy curses and to repair the environment free from pollution. New attempts are made to develop technologies to sharpen the process of bioethanol production to get maximum benefit from non-food crops and wastes generates from industrial side. The demand of non-renewable energy resources, the environmental concern over the burning of fossil fuels, and the recent price rises and instability in the international oil markets have all combined to stimulate interest in the use of fermentation processes for the production of alternative bio-fuels. In 2008 India imported 128.15 million metric tons of crude, constituting 75% of its total petroleum consumption for that year. By 2025, India will be importing 90% of its petroleum. In an effort to increase its energy security and independence, the Government of India in October of 2007 set a 20% ethanol blend target for gasoline fuel to be met by 2017 [1] Several environmental impacts are directly related to biomass energy production and consumption.

Cellulose, a linear polymer of glucose accounts for 35 to 60 per cent of dry mass. This polymer is rigid and difficult to break. However on hydrolysis the polysaccharide is broken down to the sugar molecule this is called saccharification. Hemicellulose 20 to 40 per cent of dry mass this consists of short highly branched chain of xylose and arabinose (5 carbon sugar), glucose, galactose and mannose (6 carbon). Hemicelluloses, this is branched amorphous nature is relatively easy to hydrolysed. Cellulosic ethanol contributes little to the greenhouse effect and has a five times better net energy [2]. When used as a fuel, cellulosic ethanol releases less sulfur, carbon monoxide, particulates, and greenhouse gases. Cellulosic ethanol should earn producers carbon reduction credits, which is about 3 to 20 cents per gallon [3]. Pre-treatment method is done to remove the lignin and hemicelluloses, part to reduce cellulose crystallinity, and increase the porosity of the materials. Dilute-acid hydrolysis has been successfully developed for pre-treatment of cellulosic materials. This pretreatment method

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gives high reaction rates and significantly improves cellulose hydrolysis [4]. Depending on the substrate and the conditions used, up to 95% of the holo cellulosic sugars can be recovered by dilute-acid hydeolysis from the cellulosic feedstock [5].

The cellulose fraction of lignocelluloses can be converted to ethanol by either simultaneous saccharification and fermentation (SSF) or separate enzymatic hydrolysis and fermentation processes. Saccharification and fermentation is more favoured because of its low potential costs [6]. In India, the vast majority of ethanol is produced from sugarcane molasses, a by-product of sugar. In the future, ethanol may also be produced directly from sugarcane juice [7].

Sugarcane ethanol would be slightly more cost effective to produce at 33 Rs/litre. But, this assumes that the Indian sugar and alcohol industry would be capable of producing it by 2017. Currently, corn is easier and less expensive to process into ethanol when compared to sugarcane. The department of energy estimates that it costs about $2.20 per gallon to produce cellulosic ethanol, which is twice than ethanol from corn. Enzymes that destroy plant cell wall tissue cost 30 to 50 cents per gallon of ethanol compared to 3centsper gallon for corn. The department of energy hopes to reduce production cost to $1.07 per gallon by 2012 to be effective. However, biomass is cheaper to produce from corn, because it requires fewer inputs, such as energy, fertilizer, herbicide and is accompanied by less soil erosion and improved soil fertility. Additionally, non fermentable and unconverted solids left after making ethanol can be burned to provide the fuel needed to operate the conversion plant and produce electricity. Energy used to run corn-based ethanol plants is derived from coal and natural gas.

Rice straw is one of the abundant cellulosic waste materials in the world. It is annually produce about 731 million tonnes which are distributed in Africa (20.9 million tonnes), Asia (667.6 million tonnes), Europe (3.9 million tonnes), America (37.2 million tonnes) and Oceania (1.7 million tonnes). This amount of rice straw can potentially all are to produce rarely about 205 billion liters of bio-ethanol per year, which is the largest quality from a single biomass feedstock [8].

Another interesting feed stock is the water hyacinth (*Eichhornia crassipes*). Water hyacinth is a native plant of Brazil but has been naturalized in many tropical/temperate countries. It is regarded as a nuisance because of its remarkable growth rate in aquatic ecosystem particularly in channels and ponds. Although, the water hyacinth is considered by many as an invasive pest, it could be useful as a source of biomass, because it is abundant and easy to cultivate. Previous potential studies have addressed the potential use of water hyacinth as biomass [9,10,11].

These studies indicate that water hyacinth is a promising raw material for ethanol production. Using the water hyacinth as a cellulose biomass would be a low cost method for ethanol production. The saccharification of water hyacinth was carried out by hydrolyzing it in 1% sulpuric acid at 121°C for 1 h. The addition of yeasts from hydrospheric environments could constitute a cost efficient way of producing ethanol from the water hyacinth hydrolysate. A *Zymomonas mobilis*, yeast strain 484 produced 22.4 ml of ethanol/kg of dried water hyacinth [12].

As a fuel, ethanol is mainly of interest as a petrol additive or substrate because ethanol-blended fuel produces a cleaner more complete combustion that reduces green house gas and toxic emissions. As a consequence of the surge in demand for biofuels, ethanol producing microorganisms such as yeast and bacterium *Zymomonas mobilis* are of considerable interest due to their potential for industrial scale bioethanol production. Although, bioethanol has traditionally been produced in batch fermentation with the yeast Saccharomyces cerevisiae, there are advantages in using *Zymomonas mobilis* as an alternative for bioethanol production. In comparison to yeast, Z. *mobilis* grows and ferments rapidly, without the requirement for the controlled supply of oxygen during fermentation, and has a significantly higher ethanol product rate and yield. Most importantly, it has a high tolerance for ethanol.

Large number of publication stated that the optimum temperature for enzymatic hydrolysis is at 40-50°C, while the microorganisms with good ethanol productivity and yield do not usually tolerate this high temperature. This problem has usually been tackled by applying thermotolerant microorganisms such as *Kluyveromyces marixianus*, Candida lusitaniae and *Zymomonas mobilis* or mixed cultures of some microorganisms like *Brettanomyces clausenii* and *Saccharomyces cerevisiae* [13,14]. Another method to stand against this problem was reported to add a pre-hydrolysis step e.g. 50°C for 24 hrs, followed by the addition of microorganisms at a suitable lower temperature [15].

2. MATERIALS AND METHODS

2.1. Collection of raw material

Cellulosic was collected from the field after harvest from Chidambaram, Tamil Nadu. After cleaning to remove soils, cellulosic wastes was sun dried for 2 days and afterwards dried at in hot air oven. Dried samples were then pulverized into very fine powder so as to store in air tight container for further study.

2.2. Collection and maintenance of microbial cultures

The cultures used for fermentation process were *Saccharomyces cerevisiae*, isolated from fermented cashew apple juice and maintained in the Department of Microbiology, Annamalai University *and Zymomonas mobilis* (MTCC-2427) was obtained from Microbial Type Culture Collection, Gene Bank, institute of Microbial Technology (IM Tech) Chandigrah. These cultures were maintained in Yeast Peptone Dextrose (YPD) and RM agar slant at 30±2°C with monthly transfer.

2.3. Enzymatic hydrolysis

Twenty five grams each of the powdered crop residues was mixed with 100 ml of water in 250 ml Erlenmeyer flask. The crude enzyme source obtained from *Aspergillus fumigatus*, *Penicillium digitatum* and *Trichoderma reesei were* use at 1 ml, 2 ml and 3 ml level of the pretreament of cellulosic crop residues (28±2°C)

2.4. Fermentation medium

The cellulosic hydrolysate obtained after the saccharification with acid \ alkali \ enzyme was filtered and centrifuged to remove unhydrolysate , the pH of the supernatant was adjusted to 5.0 with 10% ammonium hydroxide solution before inoculum. In the case of alkali hydrolysate, the pH of the supernatant was adjusted to 5.0 with 10% sulphuric acid solution before inoculation. The 100ml clear supernatant was then enriched with 0.2 % urea as nitrogen source and the fermentation was carried out at 28-30°C using *Saccharomyces cerevisiae, Zymomonas mobilis* and their combination (4%w/v).

2.5. Minimum inhibitory concentration for bacteria

MIC of the antibiotics was tested in Muller Hinton broth for bacteria and yeast by Broth macro dilution method [16]. The antibiotics powder (1.0 mg ml^{-1}, 1.5 mg ml^{-1}, 2.0 mg ml^{-1} and 2.5 mg ml^{-1}) was dissolved in 5% DMSO to obtain stock solutions. Fermentation medium was prepared at different concentrations and 50 µl of standardized suspension of the test organism was transferred on to test tube. The control tube contains only organism and devoid of antibiotics. The culture tubes were incubated at 40°C for 24 h. The lowest concentrations which did not show any growth of tested organisms on conical flask containing fermentation medium was determined as minimum inhibitory concentration **(**MIC).

Determination of respiratory deficiency

Respiratory deficiency was calculated by employing the standard method. The appropriately diluted samples drawn from broth during growth and fermentation were platted on respective medium. The colonies appearing after 72 h were over layered with 20 mL of tetrazolium agar (9 g of agar boiled in 600 mL of phosphate buffer (0.02 M, pH 7) and added with 0.6 g of 2, 3, 5-triphenyl tetrazolium chloride) and further incubated at 30 _C for 2 h. Small white respiratory deficient colonies were counted. In each case, a minimum of 2000 colonies were examined.

Statistical analysis

Data were subjected to one-way analysis of variance followed by Tukey test and two-way analysis of variance (ANOVA) using IRRI STAT package to determine the level of significance of variations in all the treatments caused by the variables studied. All experiments were completed in triplicate and data were expressed as mean. Probability (P) values of <0.05 were considered significant.

3. RESULTS AND DISCUSSIONS

Decreased ethanol production rates and reduced ethanol yields resulting from lactic acid produced during fermentation or acetic acid in the medium have also been reported [17,18]. Hence, present initiated preliminary ideas for the use of the antibiotics Peni G, Virginiamycin and Streptomycin for the production of bioethanol at a low level of concentration.

Amutha and Gunasekaran [19] obtained ethanol yield of 46.7 g l^{-1} from one litre liquefied cassava starch (150 g) using co-immobilized system containing *Saccharomyces diasticus* and *Zymomonas mobilis*. The yield increase worked out to be 25 per cent more than non -immobilized system.

Sathesh-Prabu and Murugesan [20] demonstrate that the sorghum field waste, sorghum stovar could be used to produce fuel grade ethanol. The alkaline treatment of 2 percent NaOH for 8 hrs removed 64 per cent of lignin from sorghum stovar. Maximum of 68 and 56 g/L of ethanol yield were obtained by *Saccharomyces cerevisiae*

and *Pachysolen tannophilus* from sorghum stovar under optimized condition, respectively, pH and temperature were optimized for the better growth of *S. cerevisiae* and *P. tannophilus*. A total of 51per cent and 48 per cent more ethanol yield was obtained at initial sugar concentration of 200 g/L than 150 g/L by *P. tannophilus* and *S. cerevisiae*, respectively. Sorghum field waste could be effectively used for the production of fuel ethanol to avoid conflicts between human food use and industrial use of crops.

In the current study, the ethanol tolerance by *Zymomonas mobilis* and *Saccharomyces cerevisiae* was maximum at 10 per cent (27.88 and 26.21 g^{-1}) followed by 8 per cent (24.67 and 23.45 g^{-1}), 6 per cent (22.47 and 21.32 g^{-1}) and 2 per cent (21.65 and 20.50 g^{-1}). The ethanol yield was high at 200 g of initial sugars in hydrolysed sorghum stovar when compared to 150 g of initial sugars. The ethanol yield was maximum after 96 hours in *Zymomonas mobilis* (0.391 g^{-1}) at 200g of initial sugars of hydrolyzed sorghum stovar. The ethanol yield was higher in *Saccharomyces cerevisiae* and *Zymomonas mobilis* from the substrate of Sorghum stovar (0.321 g^{-1} and 0.391 g^{-1}) followed by water hyacinth (0.277 g^{-1} and 0.284 g^{-1}). Low ethanol yield was recorded in paddy straw (0.253 g^{-1} and 0.253 g^{-1}) respectively. The fermentation efficiency was higher in *Saccharomyces cerevisiae* and *Zymomonas mobilis* from the substrate of Sorghum stovar (55.80 g^{-1} and 57.26 g^{-1}) followed by water hyacinth (53.47 g^{-1} and 56.41 g^{-1}). The ethanol productivity was higher in *Saccharomyces cerevisiae* and *Zymomonas mobilis* from the substrate of Sorghum stovar (32.01 g^{-1} and 39.11 g^{-1}) followed by water hyacinth (27.70 g^{-1} and 28.04 g^{-1}). Low ethanol yield was recorded in paddy straw (25.03 g^{-1} and 26.06 g^{-1}) respectively. The study has dual benefits in terms of managing the agro-residues and producing the green energy. However, the cost of the enzyme to be used for hydrolysis (highly preferred process) must be reduced in order to make the process economically feasible. This present study proved valuable process mechanisms about the sorghum stovar, paddy straw as water hyacinth waste utilization and inexpensive potent substrate for the production of eco-friendly liquid fuel so called bioethanol.

Table 1: Effect of Antimicrobial strategies against bacterial contamination in cellulosic crop residues

S. No	Antibiotics	Concentration (mg l^{-1})	Bacteria	*Zymomonas mobilis*	Yeast
1	Penicillin	1.0	+++	++	-
		1.25	++	+	-
		1.50	-	-	-
		2.0	-	-	-
		2.5	-	-	-
2	Virginiamycin	1.0	+++	++	-
		1.25	++	+	-
		1.5	-	-	-
		2.0	-	-	-
		2.5	-	-	-
3	Tetracycline	10	++	+	-
		20	+	-	-
		30	-	-	-
		40	-	-	-
4	Streptomycin	1	+++	++	-
		1.25	++	+	-
		1.50	-	-	-
		2.0	-	-	-
		2.5	-	-	-

Table 2: Consumption of sugar and ethanol yield by *Saccharomyces cerevisiae* and *Zymomonas mobilis* from paddy straw hydrolysed

Saccharomyces cerevisiae								*Zymomonas mobilis*							
Initial sugar								Initial sugar							
200g				150g				200g				150g			
Time (h)	Sugar reaming (g)	Ethanol (g/l)	Ethanol yield (g/l)	Time (h)	Sugar remaining (g)	Ethanol (g/l)	Ethanol Yield (g/l)	Time (h)	Sugar reaming (g)	Ethanol (g/l)	Ethanol yield (g/l)	Time (h)	Sugar reaming (g)	Ethanol (g/l)	Ethanol yield (g/l)
24	110.65	9.86	0.049	24	83.67	7.98	0.039	24	112.58	10.67	0.053	24	79.56	8.25	0.041
48	53.49	11.56	0.057	48	33.19	8.12	0.040	48	55.39	16.99	0.084	48	33.29	9.98	0.049
72	39.67	25.63	0.128	72	25.45	18.89	0.094	72	41.89	27.43	0.137	72	27.49	30.26	0.151
96	28.12	50.68	0.253	96	13.67	28.67	0.143	96	30.47	53.21	0.266	96	14.68	40.25	0.201

Table 3: Consumption of sugar and ethanol yield by *Saccharomyces cerevisiae* and *Zymomonas mobilis* from sorghum stovar hydrolysed

Saccharomyces cerevisiae								*Zymomonas mobilis*							
Initial sugar								Initial sugar							
200g				150g				200g				150g			
Time (h)	Sugar reaming (g)	Ethanol (g/l)	Ethanol yield (g/l)	Time (h)	Sugar remaining (g)	Ethanol (g/l)	Ethanol yield (g/l)	Time (h)	Sugar reaming (g)	Ethanol (g/l)	Ethanol yield (g/l)	Time (h)	Sugar reaming (g)	Ethanol (g/l)	Ethanol yield (g/l)
24	100.25	11.25	0.056	24	80.23	10.96	0.054	24	99.31	25.86	0.129	24	76.23	13.56	0.067
48	48.72	24.32	0.121	48	36.27	24.95	0.124	48	43.54	45.56	0.227	48	31.27	26.64	0.133
72	33.78	48.37	0.241	72	27.78	46.25	0.233	72	30.23	56.82	0.284	72	23.78	50.72	0.251
96	32.63	64.23	0.321	96	15.98	60.63	0.303	96	27.01	78.38	0.391	96	10.98	66.46	0.332

Table 4: Consumption of sugar and ethanol yield by *Saccharomyces cerevisiae* and *Zymomonas mobilis* from water hyacinth

Saccharomyces cerevisiae							*Zymomonas mobilis*						
Initial sugar							Initial sugar						
200g				150g			200g				150g		
Time (h)	Sugar reaming (g)	Ethanol (g/l)	Ethanol yield (g/l)	Time (h)	Sugar remaining (g)	Ethanol (g/l)	Ethanol Yield (g/l)	Time (h)	Sugar reaming (g)	Ethanol (g/l)	Ethanol yield (g/l)	Time (h)	Sugar reaming (g)	Ethanol (g/l)	Ethanol yield (g/l)
24	105.47	10.17	0.050	24	81.11	8.56	0.042	24	108.54	15.98	0.079	24	77.99	11.59	0.057
48	52.12	19.64	0.098	48	34.02	15.34	0.076	48	49.78	27.12	0.135	48	32.65	20.88	0.104
72	36.65	30.86	0.154	72	26.65	27.12	0.135	72	38.98	35.82	0.179	72	25.39	40.62	0.203
96	30.78	55.48	0.277	96	14.43	30.98	0.154	96	32.15	56.99	0.301	96	12. 73	60.31	0.284

Table 5: The average kinetic parameters for ethanol production with initial sugar concentration of 200 gl^{-1}

S. No	Parameters	*Saccharomyces cerevisiae*			*Zymomonas mobilis*		
		Paddy straw	Sorghum stovar	Water hyacinth	Paddy straw	Sorghum stovar	Water hyacinth
1	[A] Final ethanol concentration(P)	50.68	64.23	55.48	53.21	78.38	59.99
2	[B] Ethanol(Yp/s)	0.253	0.321	0.277	0.266	0.391	0.284
3	[C] Volumetric ethanol productivity(Q)	0.551	0.669	0.577	0.554	0.816	0.651
4	[D] Fermentation efficiency	50.48	55.80	53.47	52.26	57.26	56.41
5	[E] Ethanol productivity	25.03	32.01	27.70	26.06	39.11	28.40
6	[F] Sugar utilized (%)	85.94	83.68	84.61	84.76	86.49	83.92

[A] Grams of ethanol per litter, [B] Grams of ethanol per grams of sugar, [C] Grams of ethanol per litter per hour, [D] % of theoretical maximum from sugar substrate, [E] Grams of ethanol per litter per100g of sugar, [F] (Residual sugar/initial sugar) ×100

REFERENCE

1. The Economic Times. 2009a. "India Approves New Policy to Promote Biofuels." *The Economic Times*. Available at: http:// economictimes.indiatimes.com/ articleshow/5374944.cms. Website accessed January 2010.
2. Searchinger, T., R. Heimlich, et al. 2008. "Use of U.S. Croplands for Biofuels Increases Greenhouse Gases through Emissions from Land-Use Change." *Science.* 319: 1238-1240.
3. Ewing, M. and S. Msangi. 2009. "Biofuels Production in Developing Countries: Assessing Tradeoffs in Welfare and Food Security." Environmental Science and Policy 12(4):520-524.
4. Palmqvist, E., Hahn-Hägerdal, B., 2000. Fermentation of lignocellulosic hydrolyzates, inhibition and detoxification. Bioresource Technology 74, 17-24.
5. Lee, Y.Y., Z. Wu and R. Torget. 2000. Modeling of counter current shrinking-bed reactor in dilute acid total hydrolysis of lignocellulosic biomass. Bioresour. Technol., 71: 29-39.
6. Duff, S. J. B., Moritz, J. W. & Andersen, K. L. (1994) Simultaneous hydrolysis and fermentation of pulp mill primary clarifier sludge. Can. J. Chem. Engng, 72, 1013-20.
7. Alexander kiyoshi mino.2010. Ethanol production from sugarcane in india: viability, constraints and implications ph.d thesis.

8. Heitzman, J. and R.L. Worden. 1996. *India: a country study*. Federal Research Division, Library of Congress, Washington D.C. Bioresource Technology 74, 17-24.
9. Abraham M, Kurup GM (1996). Bioconversion of tapioca (*Maniho tesculenta*) waste and water hyacinth (*Eichhornia crassipes*) influence of various Physico-chemical factors. J. Ferment. Bioeng. 82: 259-263.
10. Nigam JN (2002). Bioconversion of water-hyacinth (*Eichhornia crassipes*) hemicellulose acid hydrolysate to motor fuel ethanol by xylose–fermenting yeast. J. Biotechnol. 97: 107-116.
11. Rahman MM, Chowdhury AA, Khan MR, Islam A (1986). Microbial production of biogas from organic wastes. J. Ferment. Technol. 64:45-49.
12. Ogawa Masami G.O, IshidaYukinari Usui and Naoto Urano 2008 Ethanol production from the water hyacinth *Eichhornia crassipes* by yeast isolated from various hydrospheres. African Journal of Microbiology Research Vol.(2) pp. 110-113, May, 2008.
13. Esteghlalian A, Hashimoto AG, Fenske JJ, Penner MH. Modelling and optimization of the diluted-sulfuric-acid pretreatment of corn stover, poplar and switchgrass. Bioresour Technol 2007; 59:129-36.
14. Kaar WE, Holtzapple MT. Using lime pretreatment to facilitate the enzymatic hydrolysis of corn stover. Biomass Bioenergy 2000; 18:189-99.
15. Ohgren K, Galbe M, Zacchi G. Optimisation of steam pretreatment of SO2-impregnated corn stover for fuel ethanol production. Appl Biochem Biotechnol 2005; 124:1055-67.
16. Eriksson, T., J. Berjerson and F. Tjerneld. 2002. Mechanism of surfactant effect in enzymatic hydrolysis of lignocelluloses. Enzyme and Microbiol Technol., 31: 358-364.
17. Abbott DA, Ingledew WM. Buffering capacity of whole corn mash alters Concentrations of organic acids required to inhibit growth of *Saccharomyces cerevisiae* and ethanol production. Biotechnol Lett 2004; 26:1313-6.
18. Graves T, Narendranath NV, Dawson K, Power R. Interaction effects of lactic acid and acetic acid at different temperatures on ethanol production by *Saccharomyces cerevisiae* in corn mash. Appl Microbiol Biotechnol 2007; 73:1190-6.
19. Amutha, R. and P.Gunasekaran. 2001. Production of ethanol from liquefied cassava starch using co immobilized cells of *Zymomonas mobilis* and Saccharomyces diastaticus. J Biosci Bioeng., 92:560–4.
20. Sathesh-Prabu, C., A.G.Murugesan. 2011. Potential utilization of sorghum field waste for fuel ethanol production. J. Bioresource Tech 102, 2788–2792.