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Research Article

SACCHARIFICATION BY FUNGI AND ETHANOL PRODUCTION BY BACTERIA USING LIGNOCELLULOSIC MATERIALS

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ABSTRACT
Lignocellulosic material is one of the most abundant, renewable and inexpensive energy resources for bioethanol production. These materials are mainly composed of three groups of polymers namely cellulose, hemicellulose and lignin. Cellulose and hemicellulose are sugar rich fractions of interest for use in fermentation processes such as ethanol production. Cellulase production by the different fungi like *Trichoderma reesei* (MTCC-4876), *Phanerochaete chrysosporium* (MTCC-787) and *Aspergillus awamori* (MTCC-6652) were studied using different substrates (rice straw, wheat straw and rice husk) by keeping the concentration constant at 5g/ 150 ml. The subculture medium was a salt solution consisting of KH_2PO_4, $CaCl_2$, etc. Fungal cells were sub-cultured in an orbital shaker (180 rpm) at 30°C for 1-2 generations (two days for each generation) and were then used as inoculums. The maximum cellulase production and saccharification observed in the presence of combination of fungi with treated rice straw. Further *Zymomonas mobilis* bacteria was used for carrying out fermentation of sugars to ethanol production. Among the three raw materials studied the ethanol yield was observed to be the highest in rice straw (9.5 g/l).

KEYWORDS: Bioethanol, lignocellulosic material, fungi, saccharification, bacteria, fermentation.

INTRODUCTION

Lignocellulose is very rich and abundant source of energy. Therefore lignocelluloses degradation is essential for maintaining the global carbon cycle. Production of ethanol or ethyl alcohol from Lignocellulosic biomass is one way to reduce both the consumption of crude oil and environmental pollution[1,2]. Primary consideration involves the production of ethyl alcohol from renewable resources and determination of the economic and technical feasibility of using alcohol as an automotive fuel blended with gasoline[3]. Ethanol represents an important, renewable liquid fuel for motor vehicles[4]. Domestic production and uses of ethanol for fuel can decrease dependence on foreign oil, reduce trade deficits, create jobs in rural areas, reduce air pollution and reduce global climate change carbon dioxide build up. In the last decade, most research has tended to focus on developing an economical and eco-friendly ethanol production process. Much emphasis is being given to the production of ethanol from agricultural and forestry residues and other forms of lignocellulosic biomass[5].The cellulosic plant material represents a source of fermentable sugars for significant use particularly non-food lignocellulosic waste products like rice straw, wheat straw and rice husk etc. Bioethanol can be produced from plentiful, domestic, cellulosic biomass resources such as herbaceous and woody plants, agricultural and forestry residues, and a large portion of municipal solid waste and industrial waste streams. To ensure that a low-cost energy feedstock is available, researchers are examining dedicated energy crops, wood and grass species that have been selected to produce high yields. Lignocellulose is an abundant material created from solar energy and renewable resources on earth, which makes them attractive for production of ethanol[6]. Lignocellulose is composed of three main fractions like cellulose (~ 45% of dry weight), hemicellulose (~30% of dry weight) and lignin (~25% of dry weight). In these waste products, cellulose and hemicelluloses are closely associated with lignin in the plant cell wall[7]. Cellulose the most abundant polymer on earth is composed of thousands of molecules of anhydroglucose linked by β (1, 4)-glycosidic bonds. Cellulose can be effectively hydrolyzed and depolymerized into fermentable sugars by the enzyme cellulase. A number of microorganisms are capable of producing extracellular cellulase enzyme and among which fungi are the widely used candidates for cellulase enzyme production. Currently most commercial cellulases are produced from *Phanerochaete* sp[8], Aspergillus sp[9] and *Trichoderma* reesei[10,11] usually used to describe a mixture of cellulolytic enzymes whose synergistic action is required for effective breakdown of substrate to its monomeric units. The action of cellulases involves the concerted action of (i) endoglucanase(s), which randomly attacks the internal, β1,4-linkages, (ii) cellobiohydrolase, which cleaves off cellobiose units from the non reducing ends of the glucan, and (iii) β 3-glucosidase, which hydrolyzes cellobiose to glucose. Pretreatment is required to alter the structures of cellulosic biomass to make more accessible to the enzymes that convert the carbohydrate polymers into fermentable sugars and to cellulase producing microorganisms. The transformation of lignocellulose into ethanol is completed in two steps: (a) Formation of fermentable sugars from celluloses and hemicelluloses and (b) Fermentation to ethanol using pentoses and hexoses liberated in the first step[12]. In conventional processes, lignins present in the raw materials and releasing fermentable sugars are eliminated by chemical and/or thermic pretreatment followed by enzymatic/acidic hydrolysis. However, biological treatments have been proposed either to replace the chemical or physicochemical treatment[13].In this work a combination of different fungi used for pretreatment and saccharification thereafter *Zymomonas mobilis* bacteria was used for carrying out fermentation. *Zymomonas mobilis* is a Gram-negative, facultative anaerobic bacterium that ferments glucose, fructose, and sucrose as carbon sources[14]. These

carbohydrates are metabolized via the same biochemical route, the Entner-Doudoroff pathway. *Z. mobilis* are rods 2-6 µm in length and 1-1.5 µm in width, flagellated but lack spores or capsules and growing in a pH range of 3.4-7.5[15].Therefore the objective of this paper is to achieve high yields of fermentable sugar and ethanol production from lignocellulosic materials using microorganisms.

MATERIAL AND METHODS

Raw Materials

- Rice straw, wheat straw and rice husk from local mill
- Each raw material was powdered and sieved into a 1 mm seiver . Powder of each raw material was used as carbon source.

Microorganisms

Trichoderma reesei, *Phanerochaete chrysosporium* and *Aspergillus awamori* were obtained from MTCC Chandigarh. These fungi produces cellulolytic enzymes that converted carbohydrate polymers into fermentable sugars . Later fermentation process *Zymomonas mobilis* was inoculated to utilize reducing sugars to ethanol.

Medium And Culture Conditions

The fungi were transferred into Nutrient broth and incubated at 30°C for activation, cultured in PDA(Potato Dextrose Agar) plates and incubated at 30°C to form colonies. Then the fungi were maintained in the above mentioned medium at 4°C. Spore count was measured with haemocytometer and adjusted to $2x10^6$ spores/ml by adjustment of optical density. After saccharification for fermentation process *Zymomonas mobilis* strain was grown in SDDL broth (glucose 20.0, yeast extract, 5.0 g L^{-1}) at 30ºC for 48 hrs. The number of viable cells 10^9 (cfu/mL) was determined by the agar plate method using Schreder agar ($MgSO_4.7H_2O$ 0.5, $(NH_4)_2SO_4$ 1.0, KH_2PO_4 1.0, yeast extract 1.0, sucrose 20, agar 15 g L^{-1}) incubated for 24 hrs at 30ºC[16]. 1000 ml of Mandels medium was prepared by adding (in gms) Urea 0.3, $(NH_4)_2SO_4$ 1.4, KH_2PO_4 2.0, $CaCl_2.2H_2O$ 0.4, $MgSO_4.7H_2O$ 0.15, bactopeptone 1.0, and yeast extract 0.25. Trace elements were also added (in mg), $FeSO_4.7H_2O$ 0.15, $MnSO_4.H_2O$ 1.6, $ZnSO_4.7H_2O$ 1.4, $CoCl_2$ 2.0. . The subculture medium was a salt solution with 2 ml/L tween-80, and 10g/L glucose added . The initial pH of the medium was adjusted to 5.5-6.0 before being autoclaved at 15lbs per sq.inch for 30 minutes[17]. Fungal cells were sub-cultured in an orbital shaker (180 rpm) at 30°C for two days and were then used as inoculums with 5g / 100 ml (Mandel's medium) of each substrate .Later on these flasks were incubated at room temperature for 3days on an orbital shaker. After five days mycelium was separated by filtration through Whatman filter paper. The filtrate was used for further studies[18].

Determination Of Total Carbohydrate

The carbohydrate content of pretreated raw materials in the culture broth was measured by Anthrone method[19].

Determination Of Reducing Sugars

Reducing sugars in pretreated raw materials in the culture broth were determined by dinitrosalicylic acid (DNS) method[20]with glucose as standard.

FPU Assay

Cellulase enzyme production was studied by FPU assay[21].

Fermentation

Culture filtrate was further inoculated with *Zymomonas mobilis* strain and allowed for fermentation for seven days[22]. After fermentation it was filtered and ethanol content was determined.

Ethanol Estimation

Determination of ethanol content was done by spectrophotometric method[23].

RESULTS AND DISCUSSION

Total sugar, reducing sugar and non reducing sugar of each raw material was determined. Initial composition of each raw material is given in the table 1.

Table 1: Initial composition of the raw materials

S.No	Raw materials	Total sugar (mg/gm)	Reducing sugar (mg/gm)	Nonreducing sugar (mg/gm)
1	Rice straw	2.41	1.83	.58
2	Wheat straw	1.75	1.68	.07
3	Rice husk	1.89	1.67	.22

Table 2 : Effect of fungal treatment on different substrates and *Zymomonas mobilis* Conc(1ml) treatment on sugar for ethanol production

S. No	Microorganisms Conc (1ml)	Total Sugar (mg/gm)	Reducing Sugar (mg/gm)	Nonreducing Sugar (mg/gm)	FPU ($IUml^{-1}$)	Ethanol(g/l)	%of ethanol
1	*T reesei* (Rice straw)	90	73.70	16.30	0.91	8.7	16
2	*T reesei* (Wheat straw)	70	57.73	12.27	0.82	2.3	10
3	*T reesei* (Rice husk)	50	41.5	8.5	0.75	4.8	14

Table 3 : Effect of fungal treatment on different substrates and *Zymomonas mobilis* Conc(1ml) treatment on sugar for ethanol production

S. No	Microorganisms Conc (1ml)	Total Sugar (mg/gm)	Reducing Sugar (mg/gm)	Nonreducing Sugar (mg/gm)	FPU ($IUml^{-1}$)	Ethanol(g/l)	%of ethanol
1	*A.awamori* (Rice straw)	70	62.7	7.3	0.91	4.1	12
2	*A.awamori* (Wheat straw)	40	32.28	7.72	0.75	2.7	10
3	*A.awamori* (Rice husk)	50	42.20	7.80	0.83	3.8	09

Table 4 : Effect of fungal treatment on different substrates and *Zymomonas mobilis* Conc(1ml) treatment on sugar for ethanol production

S.No	Microorganisms Conc (1ml)	Total Sugar (mg/gm)	Reducing Sugar (mg/gm)	Nonreducing Sugar(mg/gm)	FPU ($IUml^{-1}$)	Ethanol (g/l)	%of ethanol
1	*Phanerochaete chrysosporium* (Rice straw)	70	57.73	12.27	0.91	7.9	14
2	*Phanerochaete chrysosporium* (Wheat straw)	60	55.3	4.7	0.82	7.2	12
3	*Phanerochaete chrysosporium* (Rice husk)	40	32.28	7.72	0.75	7.4	10

Table 5 : Effect of combination of fungi treatment on different substrates and *Zymomonas mobilis* Conc(1ml) treatment on sugar for ethanol production

S.No	Microorganisms Conc (1ml)	Total Sugar (mg/gm)	Reducing Sugar (mg/gm)	Nonreducing Sugar(mg/gm)	FPU ($IUml^{-1}$)	Ethanol (g/l)	%of ethanol
1	*Treesei*, *A.awamori* and *Phanerochaete chrysosporium* (Rice straw)	120	112.50	7.5	0.96	9.5	25
2	*Treesei*, *A.awamori* and *Phanerochaete chrysosporium* (Wheat straw)	106	95.40	10.60	0.91	8.3	20
3	*Treesei*, *A.awamori* and *Phanerochaete chrysosporium* (Rice husk)	85	77.45	7.55	0.85	8.9	18

Powdering the substrate increases the surface area and the pore size of the particle necessary for the absorption of moisture and penetration of microbes. Autoclaving for sterilization has affected and resulted in increase in sugar content. With fungal treatment increase in the yield of sugars was observed. When compared to individual fungal treatment, the combination of fungi given high yield of sugars. It has been reported that the bacteria *Zymomonas mobilis* which gives a high ethanol yield, tolerates high ethanol concentrations and can ferment arabinose and xylose[24,25]. Effect of bacterial treatment on sugar is represented simultaneously in the tables 2, 3, 4, and 5. Overall yield of ethanol (25%) was highest with the Rice straw treatment (Table 5).

CONCLUSION

Bioethanol conversion from Lignocellulosic materials holds great potential due to the widespread availability, abundance and relatively low cost of cellulosic materials. In this article Zymomonas *mobilis* was used for ethanol production. Many laboratories around the world are involved in research on the different aspects of natural biodegradation of lignocellulosic materials. Consequently, processes that use microorganisms are being developed to explore the potential for their biotechnological application because high cost of cellulase is one of the major hinderence to make the process commercialized. This can be reduced by adopting cellulase producing microorganisms like *Trichoderma reesei*, *Phanerochaete chrysosporium* and *Aspergillus awamori*. Even though the progress achieved, more effort is needed for providing low cost and high production of bioethanol by using of potential microorganisms to have significant industrial impact.

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