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Potential utilization of sorghum field waste for fuel ethanol production employing *Pachysolen tannophilus* and *Saccharomyces cerevisiae*

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ABSTRACT

In this study, we demonstrate that the sorghum field waste, sorghum stover could be used to produce fuel grade ethanol. The alkaline treatment of 2% NaOH for 8 h removed 64% of lignin from sorghum stover. Maximum of 68 and 56 g/L of ethanol yield were obtained by *Saccharomyces cerevisiae* (MTCC 173) and *Pachysolen tannophilus* (MTCC 1077) from sorghum stover under optimized condition, respectively. pH and temperature were optimized for the better growth of *S. cerevisiae* and *P. tannophilus*. A total of 51% and 48% more ethanol yield was obtained at initial sugar concentration of 200 g/L than 150 g/L by *P. tannophilus* and *S. cerevisiae*, respectively. Respiratory deficiency and ethanol tolerance of the organisms were studied. This investigation showed that 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.

1. Introduction

Production of biofuels from renewable energy sources is gaining importance in the light of rising fossil fuel prices, depleting oils reserve and increasing "green house effect" associated with the use of fossil fuels. Ethanol accounts for 90% of total-biofuels production and used in different parts of the world. Moreover, global crude oil production is predicted to decline. The projected gasoline and diesel consumption world wide is about 1829 and 1614 billion litres in 2020, respectively (Johnson, 2002). But our petroleum reserves are finite. Ethanol can be used as a motor fuel in several forms. Today, all cars can be run on a mixture of 90% gasoline and 10% ethanol without adjusting the engine. New cars even use mixtures containing up to 20% ethanol. There are also new engines available that can run on pure ethanol and so called flexible fuel vehicles that are able to use mixtures of 0–85% ethanol in gasoline (E85). The addition of ethanol to gasoline (gasohol) which reduces emission of carbon monoxide (>18%), hydrocarbons (>18%) and eliminates the use of lead, benzene, butadiene (anti-knocking agents), has widely been enforced in recent years. Demand for ethanol as an alternative fuel source has steadily increased (Zaldivar et al., 2001) due to efforts in decreasing the overall amount of green house gases emitted into the atmosphere, dwindling fossil fuel resources and increased gasoline prices (Dawson and Boopathy, 2006).

The production of ethanol from sugars or starch from sugarcane and cereals, respectively, impacts negatively on the economics of the process, thus making ethanol more expensive compared with fossil fuels. Since the cost of raw materials can be as high as 40% of the ethanol cost, recent efforts have been focused on utilizing lignocellulosic materials for the production of ethanol to lower production costs (Wyman, 1999). Lignocellulose which is the principal component of the plant cell wall and accounts for about 50% of the biomass in the world with an estimated annual production of $10–50 \times 10^{12}$ kg (Classen et al., 1999) and mostly wasted in the form of post-harvest agricultural residues and wastes of food processing industries (Mtui, 2009). This natural and potentially cheap and abundant polymer (lignocellulose) is composed of three main fractions: cellulose (~45% of dry weight), hemicellulose (~30% of dry weight) and lignin (~25% of dry weight) and found as agricultural waste (wheat straw, corn stalks, rice straw, sugar cane bagasses), industrial waste (pulp and paper industry), forestry residues, municipal solid waste etc., (Wiselogel et al., 1996). Various industrial and lignocellulosic wastes including cassava (Amutha and Gunasekaran, 2000), jack fruit seeds (Mariappan and Murugesan, 2005), aquatic weeds, corn field wastes (Sathesh-Prabu and Murugesan, 2010), pine apple wastes (Ban-Koffi and Han, 1990) can be used as a substrate for the production of ethanol.

The total crop residue production in the world is estimated at 3.8 billion mg of which 74% are of cereals, 8% of legumes, 3% of oil crops, 10% of sugar crops and 5% of tubers (Lal, 2005). Sorghum stover, the non-edible plant parts that are left in the field after harvest, is produced in the ratio (straw/grain) of 1.5 (Lal, 1995). India is the second largest producer of sorghum (10–11 million ton/yr)

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worldwide. It occupies around 11 million ha in the semi-arid regions of the country (Kleih et al., 2007) and generates 15 tons stover per ha. Hence the present investigation was undertaken to evaluate the potential of sorghum field waste, sorghum stover for the production of ethanol employing ethanologenic microorganisms.

2. Methods

2.1. Collection of raw material

Sorghum (*Sorghum bicolor* L) stover (SS) was collected from the field after harvest from Theni, Tamil Nadu. After cleaning to remove soils, SS was sun dried for 2 days and afterwards dried at 80 °C for 48 h in hot air oven. Dried samples were then pulverized into very fine powder so as to pass through a 35-mesh sieve and stored in air tight container for further study.

2.2. Analysis of physico–chemical properties of substrates

The physical and chemical characteristics of SS like moisture, ash, calcium, magnesium, phosphorus, sulphate, nitrogen, crude protein, cellulose, hemicellulose, lignin, neutral detergent fibre, acid detergent fibre, pentose (arabinan, xylan) and hexose (glucan, galacton) sugars were estimated by following standard protocols (Gupta, 2000; Muthuvel and Udayasooriyan, 1997; Sadasivam and Manickam, 1991). The carbohydrate content of the substrate was determined by measuring the pentose and hexose sugars in high-performance liquid chromatography (HPLC) system (Shimadzu, Japan). For this, the samples were neutralized with $Ca(OH)_2$, centrifuged at 5000*g* for 10 min and filtered through 0.22-μm Millipore filters before analysis. The used mobile phase used was 10 mM NaOH at a flow rate of 1 ml/min. Monomeric sugars at concentrations of 0, 25, and 50 mg/L were used as standards. All the sample analyses were carried out in triplicate.

2.3. Collection and maintenance of microbial cultures

Two yeast cultures, *Saccharomyces cerevisiae* (MTCC 173) and *Pachysolen tannophilus* (MTCC 1077) for ethanol fermentation were procured from Microbial Type Culture Collection Centre (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. *S. cerevisiae* was grown in YEPD medium (yeast extract, 3 g; peptone, 10 g; dextrose, 20 g and agar, 20 g per litre) and *P. tannophilus* in malt yeast agar medium (malt extract, 3 g; yeast extract, 3 g; peptone, 5 g; glucose, 10 g and agar, 20 g per litre). Both the cultures were grown in their respective medium at room temperature (R.T. = 26 ± 2 °C) for 48 h and subcultured at 30 days interval.

2.4. Optimization of temperature and pH

For optimization of temperature and pH, 100 μL from 24 h cultures of *S. cerevisiae* and *P. tannophilus* was inoculated into 100 mL of respective broth and incubated at different temperatures (R.T., 30, 35 and 40 °C) and with different pH (4.5, 5, 5.5 and 6). Growth was measured by spectrophotometrically using UV–Visible spectrophotometer (Genesys 2, NY) at OD_{530} at 6 h interval for 24 h.

2.5. Pretreatment of substrates

SS was delignified by alkaline treatment. The substrate (4 g) was added with different concentrations of NaOH (0%, 1%, 2%, 3%, 4%) and subjected to heat treatment at 15 Lb pressure for 1 h and afterwards it was kept at R.T for 8 h. Then the residues were removed from the solutions by filtering through cheesecloth. The residues were then triple rinsed with hot distilled water and neutralized with acetic acid. After neutralization, the residues were oven dried at 50 °C for over night. Finally, the residue was weighed. The weight difference is equivalent to the amount of lignin removed. The best pretreatment was then used for further enzymatic hydrolysis. The delignified substrates were further subjected to the action of cellulase enzyme (extracted from *Aspergillus niger*; SRL, Mumbai) in the ratio of 20 FPU:1 g (Filter Paper Units enzyme:g substrate) and incubated at 50 °C for saccharification for 24 h. Amount of saccharification was estimated in terms of release of reducing sugars by DNS method (Miller, 1959). The clear solution containing sugars was collected by centrifugation (6000*g*, 10 min) and used for fermentation.

2.6. Fermentation

Inoculum (10% v/v) was prepared by following the method of Nellaiah and Gunasekaran (1992). The entire fermentation medium was prepared in such a way that the hydrolysate medium having 150 and 200 g/L of total sugars. The medium was supplemented with urea (0.16% w/v) and $(NH_4)_2PO_4$ (0.12% w/v) as suggested by Bulawayo et al. (1996). Fermentation was carried out under optimized condition for each organism in the table top fermenter (2.5 L, Minifors, Switzerland) with the working volume of 1 L. For pH control, the acid and alkaline reservoir bottles filled with 0.1 N HCl and 0.1 N NaOH, respectively. Hundred milligrams of chloramphenicol (Himedia, Mumbai) was added into the medium to control the bacterial contamination. Five drops of silicone oil (Loba, India) was added into the fermenter to control the foaming formed during fermentation. Thermostat (Technico, Chennai) was used for cooling. The stirrer speed was maintained at 120 rpm. Fermentation was carried out for 96 h and samples were withdrawn at every 24 h interval for the estimation of ethanol by dichromate reduction method (Caputi et al., 1968) and residual sugar by DNS method. The alcohol content was also determined by the determination of the specific gravity using a standard alcohol density table (AOAC, 1980).

2.7. Determination of respiratory deficiency

Respiratory deficiency was calculated by employing the standard method (Ogur et al., 1957). The appropriately diluted samples drawn from broth during growth and fermentation were plated 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 (Bajaj et al., 2001).

2.8. Ethanol tolerance studies

Ethanol tolerance of *S. cerevisiae* and *P. tannophilus* was determined by measuring the growth spectrophotometrically using UV–Visible spectrophotometer (Genesys 2, NY) at OD_{530} in the presence of exogenously added ethanol (0–7% v/v).

2.9. Statistical analysis

Data were subjected to one-way analysis of variance followed by Tukey test and two-way analysis of variance (ANOVA) using SPSS-11 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