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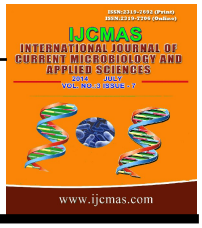

Original Research Article

Bio-ethanol production by simultaneous saccharification and fermentation using microbial consortium

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A B S T R A C T

Ethanol is an upcoming source of alternative energy. Various researches are focussing on bio-ethanol production from unutilized or waste materials. In the present study we have targeted the use of agricultural waste materials (specifically what bran, corn cob, sugarcane bagasse) for ethanol production by simultaneous saccharification and fermentation utilizing unique properties of *Thermomyces lanuginosus* SS-8 (rapid hydrolysis of lignocelluloses producing solely xylose as end product) (Reported earlier). Optimization of process parameters suggested that among all substrates taken for study, sugarcane bagasse could be saccharified most efficiently by *T. lanuginosus* SS-8. Consequently, maximal ethanol production of about 2.5 ml per gram substrate was observed upon fermentation of sugars obtained from saccharification of sugarcane. Bio-ethanol production by using a consortium of *Thermomyces lanuginosus* SS-8 and *Pichia stipitis* has been developed and significant amount alcohol production was observed.

Keywords

Saccharification Bio-ethanol production; Thermomyces lanuginosus

Introduction

With ever growing population and industrialization, demand for alternative fuel source is increasing continuously (Louime and Uckelmann, 2008). Dependence of society on primary energy sources is basis of increasing natural resource depletion and climatic changes. Major use of fossil fuels, also leads to high levels of pollution, destruction of natural landscapes and environmental habitats (Hirsch and Rittner, 2002).

In order to cater the requirements of future generation, it has become a must that a sturdy solution should be projected (Demeke et al., 2013). Bio-ethanol from the agricultural crops is commonly referred to as first-generation bio-ethanol. Conventional crop like wheat, sugarcane, corn are unable to meet the global demand of ethanol production due to their primary food and feed value. Hence lignocellulosic substances present in agricultural waste like sugarcane

bagasse, corn cob etc is very attractive for bio-ethanol production. Agricultural wastes are cost effective, renewable, and abundant in quantity. Thus a feasible solution to global energy crisis is conversion of lignocellulosic renewable resources like celluloses and hemicelluloses to ethanol (Buaban et al., 2010; Farrell et al., 2006, Mielenz, et al., 2001; Rosillo and Cortez, 1998). Starch rich biomass like potato, wheat bran, cassava, rye, barley, sugar beet can be efficiently used for biomass production. Saccharification by microbial source and simultaneous fermentation by appropriate yeast is established process for bioethanol production.

Lignocellulosic biomass, the most abundant type in the planet (Claassen et al., 1999), is potential, affordable raw material for second-generation bio-ethanol production (Wiselogel et al., 1996). Starchy grains, sugarcane, and corn cob are major raw materials for bio ethanol production used in current studies. Enhancement of production of energy from renewable sources requires optimization of process parameters. Presently research focus on the enzymatic conversion of lignocellulosic polysaccharides into monomer sugar that can be fermented into second generation ethanol (kovacs et al., 2009; Kligtik et al., 1997).

Ethanol may act as substitute of kerosene and have added advantages over other fossil fuels like emitting less CO_2, limited toxic materials and no particles (Brown et al., 1998). Furthermore ethanol production is a less energy demanding process as compared to gasoline production (Bailey et al., 1992; Ecklund, 1978). Major hurdles towards bio-ethanol production are recalcitrance of lignocellulosic biomass, inability of microorganisms to ferment all forms of sugars, etc (Bothast et al., 1999).

Thermomyces lanuginosus (formerly known as *Humicola lanuginosa*) is an imperfect fungus. It is noncellulytic, septate, reproduces asexually by forming aleurioconidia (Shrivastava et al., 2008). Some strains of *Ascomycetes* fungi have been reported earlier to produce cellulase-free thermostable β-xylanases (Singh et al., 2003; Gaffney et al. 2009; Shrivastava et al., 2009). Purification and partial characterization of the novel xylanase and its unique mode of action have been reported by our team earlier (Shrivastava et al., 2011). Cloning and expression of the unique xylanase gene in *Escherichia coli* for time effective xylanase production is also being reported in our previous studies (Shrivastava et al., 2013).

Pichia stipitis is well known xylose fermenting yeast of *Schefferomyces* genus with smooth and creamy colony having 3 to 5 µm diameter (Gupthar, 1992). It mostly occurs in hardwood forests or wet areas high in agricultural waste, moist or rainy areas rich in organic waste as well as in biomass (Blackwell et al., 2009).

In the present study the unique properties of *Thermomyces lanuginosus* SS-8 has been exploited for xylose overproduction and subsequent bio-ethanol formation by fermentation through *P. stipitis*. A new approach of bio-ethanol production by using a consortium of *Thermomyces lanuginosus* SS-8 and *Pichia stipitis* has been developed and significant amount alcohol production was observed.

Materials and Methods

Microorganisms and culture conditions

Thermomyces lanuginosus SS-8 and *Pichia stipitis* NCIM 3497 and NCIM 3498 were used for the simultaneous saccharification and fermentation. *Thermomyces lanuginosus* SS-8 was grown at 50ºC on YPSs (Yeast extract soluble starch) medium (0.4% yeast

extract, 0.1 % KH_2PO_4, 0.1 % $MgSO_4$, 0.15% soluble starch and 1.5% agar). *P. stipitis* were maintained on MGYP medium (0.3% Malt extract, 1% glucose, 0.3 % yeast extract, 0.5% peptone and 2 % agar) and grown at 28ºC. Both cultures were stored at 4ºC and revived after every 15 days.

Saccharification by *Thermomyces lanuginosus* SS-8 and xylanase activity

Hydrolysis of low value agricultural waste was carried out by *T. lanuginosus* SS-8. Medium (0.5% yeast extract, 0.1 % $MgSO_4$, 0.1% K_2HPO_4) containing 1% of sugarcane bagasse, corn cob, wheat bran, rice straw, rice husk or wheat straw. HCl treated substrates sieved through 200 µm filter was used for production of monosaccharide sugars (hexoses and pentoses).

Saccharification process was carried out in an incubator shaker at 50ºC for 72 h. Following this, amount of xylose and glucose in medium released after hydrolysis was estimated. Estimation protocol was based on calculating amount of reducing sugar present in medium. Parallel to it xylanase, amylase and cellulase activity was also estimated in production medium.

Xylanase activity was assayed using 2% (w/v) OSX (Oat Spelts Xylan) in 50 mM sodium acetate buffer, pH 5.3 at 50ºC (Biely et al. 1985). The reducing sugars liberated were quantified by the dinitrosalicylic acid (DNS) method (Miller 1959) using xylose as the standard.

The amount of reducing sugars present was measured at 540 nm in a UV–Vis spectrophotometer (Simadzu, Kyoto, Japan). Amylase and cellulase activity was also determined by same protocol by using starch and cellulose as substrate respectively.

Fermentation by *Pichia stipitis* (NCIM 3497 and NCIM 3498) and alcohol assay

Property of *P. stipitis* to ferment pentose sugar was exploited. Both strains of *P. stipitis* were studied for their ability to produce maximal ethanol. Media in the previous step was saccharified by hydrolytic enzymes produced by *T. lanuginosus* SS-8. This process produced pentose sugars. *P. stipitis* was inoculated in same medium and incubated anaerobically at 28ºC. Amount of ethanol produced was estimated after 72 h incubation.

Ethanol assay of culture filtrate was performed after fermentation process. To 1 ml of saturated culture filtrate (saturated with s-Diphenylarbazide solution), 1 ml Potassium Dichromate solution was added (5% $K_2Cr_2O_7$ in 6 N H_2SO_4). The mixture was heated at 90 ºC for 15 min and change in colour was measured by optical density at 575 nm.

Results and Discussion

Saccharification of low value agricultural waste by Thermomyces lanuginosus SS-8

T. lanuginosus could effectively hydrolyse polymers present in low value agricultural waste and produce high amount of hexose as well as pentose sugar. Among 6 substrates taken for study maximal hydrolytic enzymes were produced with sugar cane bagasse as substrate. Physical parameters and base composition of fermentation media was kept constant. For all the substrates being hydrolysed by *Thermomyces lanuginosus* SS-8 cellulase, amylase and xylanase activity was measured and amount of hexose and pentose sugar released were measured and from all the data it was observed that sugar cane was maximal hydrolysed by *T. lanuginosus* SS-8 [Fig. 1].

Fig.1 Saccharification of various low values agricultural wastes by *Thermomyces lanuginosus* SS-8

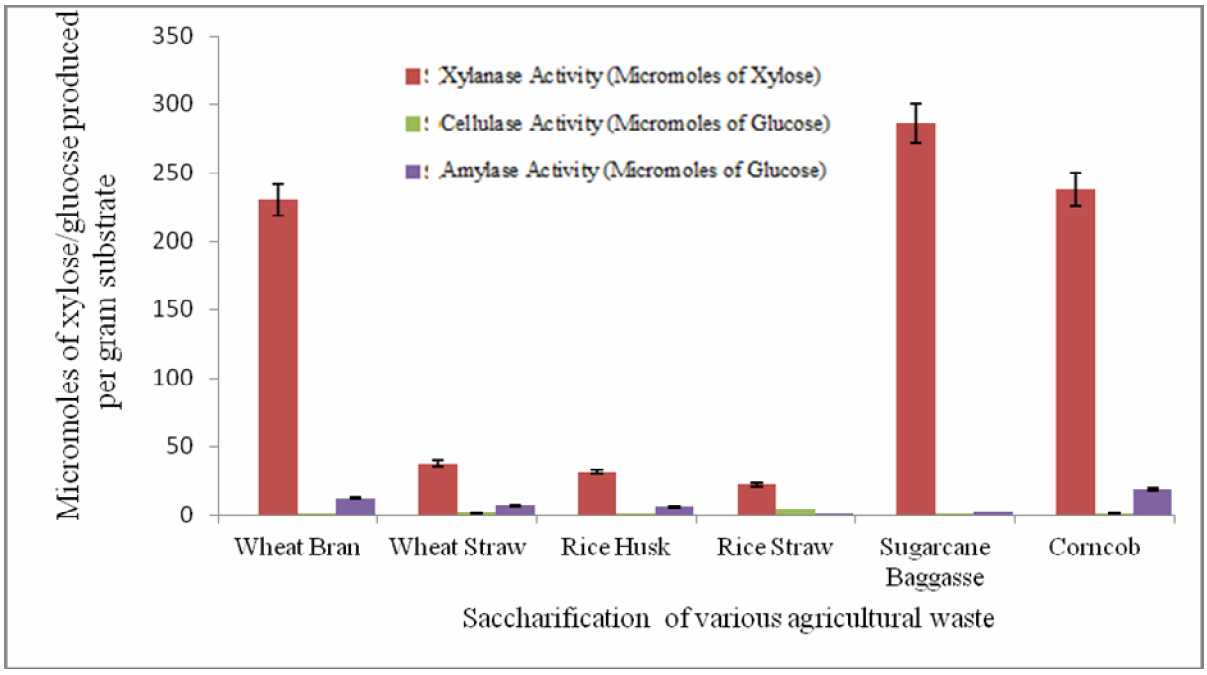
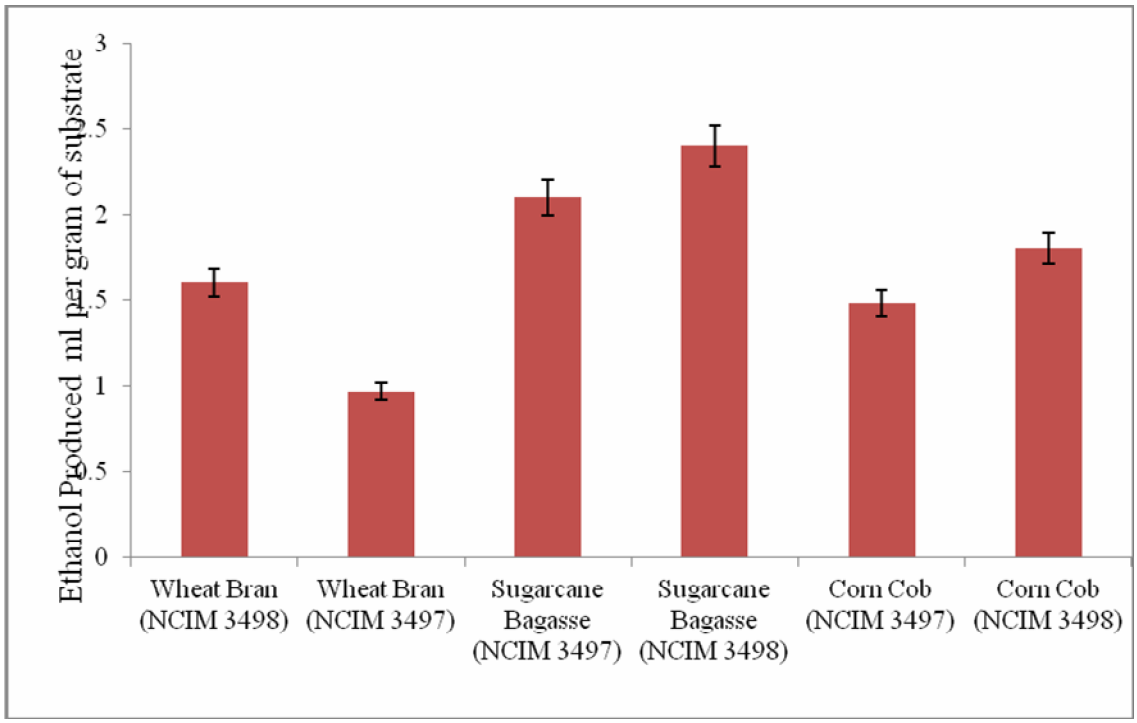


Fig.2 Ethanol production by *P. stipitis* strain NCIM 3497 and NCIM 3498



Fermentation of Saccharified agricultural waste to bio-ethanol

Fermentation of saccharified agricultural waste was carried by *P. stipitis* strain NCIM 3497 and NCIM 3498. Media containing substrates leading to substantial monosaccharide production was subjected to fermentation. Simultaneous fermentation resulted in production of significant amount of ethanol. Maximal ethanol production was observed on 3rd day of fermentation and subsequently monosaccharide sugar concentration in media decreased. Ethanol production was directly proportional to saccharification rate and thus maximal ethanol production was observed in media containing sugarcane bagasse as substrate [Fig. 2]. It also signifies that raw materials (substrates and by-products) did not have any inhibitory effect on activity of *P. stipitis*. It was also observed that both strains of *Pichia* fermented monosaccharide sugars actively. Unique property of *T. lanuginosus* SS-8 to hydrolyse lignocelluloses to broadly single monsaccharide (xylose) acted as an advantage and aided to fermentation process.

Cost effective ethanol production is the need of current world. Various processes are being developed for efficient production of ethanol at large scale. To achieve this target two basic things to be focused are maximal saccharification of the raw materials and efficient fermentation for high ethanol production. In the present work we have successfully been able to select the most competent agricultural waste and evaluated amount of ethanol produced from them at the basic set up (Petti et al. 2013; Mattam and Yazdani, 2013). Combination of *Thermomyces lanuginosus* SS-8 and *Pichia stipitis* strain NCIM 3497 and NCIM 3498 worked in consortium to produce sugar and ethanol respectively. Sugarcane and corn is widely used substrate for ethanol production (Wang et al. 2012, Wolfrun et al. 2013), and our approach towards basic optimization of ethanol production was fruitful by which we could select specific agricultural wastes on which *T. lanuginosus* and *P. stipitis* combination acted well. Further optimization of process parameters for maximal ethanol production with selected substrates is under study.

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