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FERMENTATION OF ENZYMATICALLY SACCHARIFIED *BRASSICA COMPESTRIS* STALKS FOR FUEL ETHANOL PRODUCTION BY PICHIA STIPITIS NCIM 3498

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ABSTRACT

Ethanol production was evaluated from *Brassica compestris* stalks, a readily available potential feedstock, for production of fermentable sugars. Alkali pretreated *B. compestris* was enzymatically saccharified (50 °C, pH-5.0, 50 h) using cellulolytic enzyme cocktail (cellulase, xylanase, β–glucosidase etc.) from *Aspergillus Sp.* in the presence of non-ionic surfactant polyoxyethylene sorbitan monolaurate (Tween -20). *Aspergillus Sp.* showed cellulolytic enzyme production (FPase 7.5 U/mL, CMCase 7.5 U/mL, β-glucosidase 1.5 U/mL and xylanase 45.7 U/mL) after 96 h of incubation at 30°C using sugarcane bagasse as a carbon source. The maximum yield of monomeric sugars from delignified substrate was 560 mg/g with a saccharification efficiency of 88.37 %. The fermentation performance of *Pichia stipitis* NCIM3498 was first investigated on different sugars followed by simulated medium, which was designed to mimic the individual sugar profile in enzymatic hydrolysate. The simulated medium exhibited ethanol production (17.28 g/L) with a yield (0.48 g/g sugar utilized) and productivity (0.216 g/L/h). *B. compestris* enzymatic hydrolysate was concentrated up to 40.26 g/L sugars and subsequently fermented to ethanol in 10L fermenter. A maximum of ethanol production (15.02 g/L) was obtained with a yield (0.42 g/g sugar utilized and 0.18 g/g of dry *B. compestris* stalk) and productivity (0.20 g/L/h).

Key words: *Brassica compestris, Aspergillus sp.*, enzymatic saccharification, *Pichia stipitis*, ethanol

INTRODUCTION

Due to increased demand of energy and transportation fuel, the global oil reserves will last for about 44 years (1). With this inevitable depletion of the world's petroleum supply, there has been an increasing interest in alternative, non petroleum based source of energy. Ethanol made from biomass can be an attractive, safer and clean energy option (2-4). Among various forms of biomass, lignocellulosic biomass is particularly well suited for energy applications because of its large – scale availability, low cost and environmentally benign production (5-8). More specifically, cellulosic biomass conversion into energy production has near–zero green house gas emissions on a life cycle basis (9). An important issue regarding the bioethanol production is whether the process is economical or not. Apart from this, the cost of feedstock is an important parameter in establishing a cost effective technology. Various agro crop residues viz. sugarcane bagasse, wheat straw, corn stover, rice straw etc. have been exploited for ethanol production (3, 10-12). To the best of our knowledge, there is no research paper available on ethanol production from *B. compestris*.

B. compestris var sarso is a highly branched annual or biennial herb up to 1.5 m in height. India is a major mustard growing country of the world standing the second position in terms of total production after China contributing 28.3

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and 19.8 per cent in world acreage and production (13). The productivity of mustard has increased in India from 870 kg/hectare in 1998-99 to 941 kg/hectare in 2000-01 and was 1106 kg/hectare in 2001-02 (13). This results in huge accumulation of plant stalks annually which do not find any suitable end use and are generally burnt in the fields causing environment pollution. Therefore, this stock in the form of lignocellulosics affords a renewable and low cost raw material for bioethanol production. The presence of high content of carbohydrate fraction in its cell wall (62.10% by wt.) attributes its capacity to serve as an appropriate substrate for fuel ethanol production.

Raw, untreated biomass is extremely recalcitrant to enzymatic digestion. Therefore, pretreatment to lignocellulose substrate is necessary to improve the digestibility of the substrate into fermentable sugars. Pretreatment disrupts the cell wall and improves the amenability of enzymes to polysaccharides. The alkaline method of delignification tends to have more effect on lignin fraction and leave both hemicellulose and cellulose intact (14). Enzymatic hydrolysis is an attractive approach to render the monosaccharides from lignocellulosics accessible for fermentation (15, 2).

However the cost of cellulolytic enzymes accounts for about 60% of the total cost of a bioconversion process (16). In order to be cost competitive with molasses or starch derived ethanol, the enzymes used for biomass hydrolysis should be more efficient and less expensive. *Tricoderma Aspergillus sp.* etc. is widely studied organisms to produce cellulolytic enzymes utilizing bio waste (17). The utilization of both cellulose and hemicellulosic monomers present in lignocellulosic hydrolysate is essential for the economic production of ethanol. The enzymatic hydrolysates of lignocellulosics contain a mixture of different sugars with D-glucose and D-xylose as the major components (10, 2). Among the various pentose sugars fermenting yeast, *Pichia stipitis* has shown promise for industrial application, due to its ability to ferment wider range of sugars rapidly with a high ethanol yield and apparently produces no xylitol (18).

In this communication, enzymatic hydrolysis of alkali delignified *B. compestris* stalks was carried out using culture supernatant of *Aspergillus Sp.* for the recovery of fermentable carbohydrates. The fermentation performance characteristics of *P. stipitis* NCIM3498 were assessed by using individual sugars and the simulated medium with a particular emphasis on the sugars available in *B. compestris* enzymatic hydrolysate. The fermentation of *B. compestris* enzymatic hydrolysate was undertaken for ethanol production under batch conditions in 10L fermenter.

MATERIALS AND METHODS

Raw Material

B. compestris stalks were collected from Kurnool, Andhra Pradesh, India. Dry *B. compestris* stalk pieces having a length 0.4 m were cut into 5 cm pieces. The material was processed in to small pieces to attain particular size between 4-10 mm followed by washing with tap water until the washings were clear and colourless and then gently dried at 60 °C for overnight. This material was used throughout all the experiments.

Sugarcane bagasse waste was used as substrate for enzyme production. It was collected from Hyderabad, pretreated with 0.2 N NaOH at 110ºC for 10 min followed by washing with tap water till the pH is neutralized, gently dried at 60ºC for overnight and was used as the substrate.

Analysis of chemical composition of B. compestris

The cellulose, lignin and hemicellulose fractions of pulverised *B. compestris* stalks were determined according to Technical Association of the Pulp and Paper Institute (TAPPI) Test Methods (19).

Delignification

One kg of dry *B. compestris* stalk pulverized material was suspended in 1 N NaOH solution (1:10 ratio) and autoclaved at 121°C for 60 min. The contents were filtered with two layers of muslin cloth and the solid residue was repeatedly washed with water until the pH of the filtrate became neutral.

The residue was dried at 60°C for overnight and subsequently used for enzymatic hydrolysis experiments.

Microorganisms and media

The maintenance and culture conditions of *P. stipitis* NCIM3498 were followed as described by Nigam (18). The fermentation medium consisted of enzymatic hydrolysate supplemented with the defined media of Nigam (18). *Aspergillus sp.* was maintained on yeast extract malt agar medium, subculture at monthly and kept at 4°C.

Cellulase enzyme production

Cellulase was produced by *Aspergillus sp.* using the growth medium (per litre), de-oiled rice bran 40g, peptone 3g, yeast extract 3g, KH_2PO_4 2g, $MgSO_4$ 1g, $CaCl_2$ 1g and olive oil 2g. Erlenmeyer flasks (1 L) containing of 250 mL growth medium was inoculated with a spores suspension (1×10^4 spores) and incubated at 30°C for 96 h at 120 rpm. After incubation the culture filtrate was assayed for FPase, CMCase, β-glucosidase and xylanase and used for saccharification experiments.

Enzymatic hydrolysis

Enzymatic hydrolysis of *B. compestris* was carried out in citrate buffer (pH- 5.0, 50 mM, 50 °C) at 100 rpm at 10% solid loading. The cellulosic substrate was soaked in the citrate buffer for 1 h before adding the enzymes. Non-ionic surfactant (Tween-20; polyoxyethylene sorbitan monolaurate) was also added to the reaction mixture at a concentration of 2.5 g/L. Sodium azide was added at a concentration of 0.005 % to restrict any microbial growth during the course of enzymatic hydrolysis. The substrate soaked in citrate buffer was supplemented with enzyme cocktail from *Aspergillus sp.*: cellulase 25 FPU/g, β-glucosidase 10 IU/g and xylanase 125 IU/g of the dry substrate. Samples were withdrawn at various intervals, centrifuged and supernatant analysed for total reducing sugars released. The enzymatic hydrolysate was concentrated by evaporation to 40.26 g/L of reducing sugars in a water bath and used as a substrate for ethanol fermentation.

The extent of hydrolysis was calculated as:

$$\text{Saccharification (\%)} = \frac{\text{Reducing sugar concentration obtained x 0.90 x100}}{\text{Potential sugar concentration in the pretreated substrate}}$$

Inoculum preparation and ethanol fermentation

Inoculum was prepared by harvesting the yeast cells grown for 24 h at 30°C in the culture medium containing 10.0 g/L each of xylose and glucose with the medium defined by Nigam (18) at 150 rpm. Cells were harvested and resuspended so as to maintain the O.D at 600 nm in the range of 0.6-0.8 in a volume of 500 mL (corresponding dry weight of cell mass 2.0-2.5 g/L), which contains approximately 1.6×10^8 cells/mL.

To study the sequential sugar utilization by *P. stipitis* NCIM 3498, each carbon source viz. D-glucose, D-xylose, D-arabinose, D-mannose, D-galactose, and cellobiose was supplied to the medium of Nigam (18) at a concentration of 10 g/L. The combined effect of different carbon sources (D-glucose:D-xylose:D-arabinose:D-mannose:D-galactose:cellobiose) was investigated at a ratio of 1.0:0.5:0.25:0.25:0.25:0.25 respectively to mimic the composition of *B. compestris* enzymatic hydrolysate. These experiments were carried out at shake flask level using 250 mL fermentation medium in 500 mL capacity Erlenmeyer flask at the temperature 30 °C at 150 rpm. A 5.0 mL of the inoculum was transferred to 250 mL production medium.

Fermentation of *B. compestris* enzymatic hydrolysate was carried out in an in situ sterilizable 10 L fermenter with a working volume of 8 L. An 8 L volume of *B. compestris* enzymatic hydrolysate was taken and supplemented with the other medium ingredients as described by Nigam (18) to form a complete medium prior to sterilization at 120 °C for 20 min. After sterilization and cooling the medium, a 250 mL of inoculum was aseptically transferred to fermentation medium through peristaltic pump. The fermentation was run at agitation 150 rpm, aeration 2 L/min, temperature 30°C, and pH-5.5 automatically adjusted with 2 N HCl and 2 N NaOH. Antifoam (Silicone, Sigma Aldrich), 2.0 mL was also added to the fermentation medium to avoid the excessive foaming. Sterile syringes were used to take samples at regular time

intervals during fermentation for estimation of residual sugars, biomass and ethanol.

Analytical methods

Carboxymethyl cellulase (CMCase), filter paper activity (FPU), xylanase and β-glucosidase activities were assayed according to Saha et al. (10). One unit (U) of each enzyme activity is defined as the amount of enzyme, which produces 1 μmol reducing sugar as glucose (xylose in the case of xylanase) in the reaction mixture per minute under the above-specified conditions. β-glucosidase activity was determined by the release of p-nitrophenol from p-nitrophenol-β-D-glucoside. Total sugars were estimated by dinitrosalicylic acid method of Miller (20). Ethanol was estimated by gas chromatography (GC) (Agilent 4890D, USA) with an Poly ethylene glycol column (30 m x 0.25 mm), at 120°C, flame ionization detector at 210°C and injector at 180°C using absolute ethanol as standard. The carrier gas was nitrogen. The carrier gas was nitrogen. For yeast cell mass determination, biomass was harvested by centrifugation and washed with 0.9 % (w/v) NaCl in duplicates using 5 mL samples, dried at 105 °C for 2 h.

Results and Discussion

Chemical composition

B. compestris used in this investigation contained 45.10 % cellulose and 17.00 % pentosans, which make up the total carbohydrate content (TCC) of 62.10 % on dry solid (DS) basis (Table 1). This is comparable to TCC of other popular substrates used for bioethanol production viz. sugarcane bagasse 63 %, wheat straw 54 %, birch 73 %, spruce 63.2 % (21), corn stover 59.9 % and poplar 58.2 % (22).

Hydrolytic enzymes production

The cellulolytic enzyme production profile by *Aspergillus Sp.* is given in Table 2. The organism showed all the main enzymes required for the depolymerization of structural polysaccharides from the delignified substrate. It is well known that conjugated action of cellulases and hemicellulases results in a high sugar recovery as compared to cellulases alone. *Aspergillus sp.* is able to produce all cellulases, xylanases, and other ancillary enzymes depending on the growth conditions and substrate (23). The ever increasing cellulase production cost has further necessitated the search for alternative sources of cheap and economic agro residue for cellulase production. De-oiled rice bran, generally contain 39 % cellulose and 9 % of protein was used for the substrate for cellulases production from *Aspergillus oryzae* under shake flask cultivation conditions (24). Fungi are known to use agro residues as a carbon source for their vegetative propagation and in turn metabolites production. The fungus showed maximum cellulase after 72 h of incubation, exhibiting higher level of CMCase than FPase activity which is a normal pattern among cellulolytic organisms (23, 25, 26). The higher β-glucosidase activity could have resulted in releasing higher amount of total sugars in the *B. compestris* enzymatic hydrolysate (Figure 1). Dien et al. also observed a key role of β-glucosidase in monosaccharide yields after enzymatic hydrolysis of hot water treated corn fiber (2).

Enzymatic hydrolysis

Enzymatic hydrolysis of alkali pretreated *B. compestris* was carried out using direct culture filtrate of *Aspergillus sp.* at an enzyme preparation (cellulase, 25 FPU/g; β-glucosidase, 10 IU/g; xylanase, 125 IU/g of the dry substrate) in the presence of non-ionic surfactant, Tween-20 (2.5 g/L). Pretreatment of any lignocellulosic biomass is crucial before enzymatic saccharification. Time course of enzymatic saccharification of *B. compestris* is shown in Figure 1. The presence of all cellulolytic enzymes in a proper ratio in enzyme preparation is essential to get the satisfactory yield of monomer sugars from the plant cell wall. Saccharification of alkali pretreated *B. compestris* yielded maximum sugars (560 mg/g) with the hydrolysis efficiency of 88.37 %. This is in good agreement with the results of Kuhad et al. (17) who reported saccharification efficiency (80%) with the alkali delignified sugarcane bagasse. Sharma et al. (27) observed saccharification efficiency (57.8 %) from sunflower stalks after pretreatment with steam explosion. Saha and his coworkers (10) showed an enzymatic hydrolytic efficiency of 64 % total carbohydrates (485 mg/g) from wheat straw using commercial cellulases. The hydrolytic efficiency was found to be different from

substrate to substrate. It has been reported that the cell wall structure and components are significantly different in plants, which may influence the biomass digestibility (28). Corn fiber after hot water pretreatment when exposed for enzymatic hydrolysis using culture supernatants of *Trichoderma reesei* Rut C30 and *Aspergillus niger* NRRL 2001 gave almost 80 % yield of fermentable monosaccharides (2). Chemical pretreatment not only removes lignin but also act as swelling agent. Moreover, for the enhancement of specific surface area for better enzyme action a surfactant Tween-20 was also added. It was chosen as the surfactant as it had previously been recognized to be a good enhancer of enzymatic cellulose hydrolysis, non-toxic and suitable for biotechnical use (29). Non-ionic surfactant like Tween-20 is more effective due to its adsorption on hydrophobic surfaces mainly composed of lignin fragments (30). Enzymatic hydrolysate was not accompanied by any release of fermentation inhibitors, which is advantage over acid hydrolysis and seems to be most promising approach to get high product yields vital to economic success (10).

Ethanol fermentation

Table 3 depicts the result of ethanol production parameters using different sugars individually as well as in combination. The aim of sequential sugar utilization and simulated medium studies was to check the *P. stipitis* performance on different pentose and hexose sugars and in a mixture. The ratio of different sugars in simulated medium was designed and formulated to mimic the *B. compestris* enzymatic hydrolysate. Upon individual sugar consumption, maximum utilization of glucose and xylose (94 %) was found after 36h of incubation among all the sugars. The order of rate of percentage sugar consumption was glucose > mannaose > xylose > galactose> arabinose >cellobiose. Among the pentose sugars studied, xylose (10 g/L) showed better ethanol production as compared to galactose and arabinose after 36h of incubation at 30 °C and pH-5.5. *P. stipitis* has been observed to utilize xylose and glucose almost at the same rate but it exhibited higher ethanol yield (0.49 g/g) with glucose as compared to xylose (0.47 g/g). Hsiao et al. (31) reported the D-glucose is used in preference to D-xylose or D-xylulose by essentially all D-xylose fermenting yeasts. Among the xylose fermenting yeast, *P. stipitis* and *C. shehatae* produced ethanol from D-glucose, D-mannose, D-galactose, and D-xylose (32). Most of the yeasts are unable to ferment arabinose, galactose and cellobiose to ethanol (33). This is in contrary to *P. stipitis* NCIM3498, which is able to ferment all these carbon sources, in addition to xylose and glucose. However the growth and ethanol production rate was slow with these sources. The lowest ethanol yield was recorded when the organism was grown on cellobiose. The sugar consumption rates were found similar with arabinose and cellobiose (Table 3). The differential utilization of monosaccharides is due to metabolic differences (34).When the different sugars were used in combination, the maximum utilization rate was (90 %) lesser than individual xylose and glucose (94 % in both). The uptake of xylose and its utilization is due to its facilitated diffusion with D-glucose by the cell (35). It is because both the sugars are utilized simultaneously as a symport transport mechanism of both D-glucose and D-xylose exists in *P. stipitis*.

A typical fermentation profile of *B. compestris* stalk enzymatic hydrolysate using *P. stipitis* NCIM 3498 in batch culture utilizing 40.26 g/L total sugars is shown in Figure 2. A regular increase in ethanol production was observed till 70 h of fermentation and declined thereafter. About 86 % of the available sugars were utilized within 70 h giving an ethanol yield of 0.46 g/g sugar utilized. The higher incubation time to utilize available fermentable sugars from hydrolysate was due to the higher sugars concentration in *B. compestris* enzymatic hydrolysate (40.26 g/L). The fermentation parameters viz. ethanol yield, biomass yield, ethanol productivity etc. are summarized in Table 4. The fermentation of enzymatic hydrolysate or commercially available sugars has been reported to show better fermentation efficiencies in comparison to acid hydrolysate of lignocellulosics (10, 34). More recently, Agbogbo et al (36) found the maximum ethanol concentration from *P. stiptis* CBS 6054 was 22.7 and 24.3 g/L from 60 g/L glucose and xylose media, respectively. Among the xylose fermenting yeasts, *P. stiptis* has been promising

in utilizing better fermentable sugars present in lignocellulose hydrolysate which in turn reflects in the higher ethanol production with a greater yield. Sanchez et al. (37) reported maximum ethanol production (5.5 g/L) with *P. stipitis* followed by *Candida shehatae* (4.5 g/L) and *Pachysolen tannophilus* (1.2 g/L) from the *Paja brava* acid hydrolysate.

Enzymatic hydrolysate was free from any fermentation inhibitor which resulted in a good ethanol yield and productivity. These fermentation inhibitors affect the ethanol production efficiency of yeast (38). In comparison to simulated medium, the *B. compestris* stalk enzymatic hydrolysate exhibited less ethanol yield. This might be due to the presence of undesired compounds in hydrolysate, which affect the yeast ethanol production performance. However the presence of fermentation inhibitors is abysmally low in enzymatic hydrolysates as compared to acid hydrolysates (39).Our results were similar according to the earlier reports of Parekh et al. (40), who observed ethanol yield (0.46 g/g) upon the fermentation of SO_2 pretreated corn stover enzymatic hydrolysate with *P. stipitis* CBS 5776. Sharma et al. (27) also found a maximum ethanol yield of 0.44 g/g from enzymatic hydrolysate of sunflower stalks using *Saccharomyces cereviseae var. ellipsoideus* at 15 L fermenter scale. The ethanol yield from dry *B. compestris* stalk was 0.18 g/g, which is similar to the results of Martin et al. (39) who found same yield (0.18 g/g of dry sugarcane bagasse) from sugarcane bagasse enzymatic hydrolysate fermented with recombinant xylose utilizing *S. cereviseae* TMB 3001.

Conclusions

An important feature of *P. stipitis* NCIM 3498 is the efficient utilization of the major sugars found in complex lignocellulose hydrolysate, which indicates the potential of this strain for practical processes. The present fermenter studies with *B. compestris* stalks enzymatic hydrolysate with saccharification efficiency of 88.37 % produced 16.02 g/L ethanol with the ethanol yield (0.46 g/g sugar utilized, 0.18 g/g dry *B. compestris* stalk) and productivity (0.20 g/L/h) at 10 L fermenter level. This study proved *B. compestris* stalks as a potential, renewable and low cost biomass for ethanol production on a commercial scale.

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Table 1: Composition of *B. compestris* stalks cell wall on % dry weight basis

Component	% Dry weight
Ash	2.30
Cellulose	45.10
Pentosans	17.00
Lignin	23.24
Cold water solubility	5.40
Hot water solubility	6.66
Alcohol – benzene solubility	4.42
1% caustic soda solubility	21.70

*The data presented are averages of three independent analyses

Table 2: Hydrolytic enzymes production profile by *Aspergillus sp.* at 120 rpm, temperature 30 - □C and after 96 h of incubation.

Enzyme	Activity (U/mL)
FPase	7.5
CMCase	7.5
β – glucosidase	1.5
Xylanase	45.7

* The data presented are averages of three independent analyses

Table 3: Shake flask studies for Ethanol production from different substrates and simulated medium by *Pichia stipitis* NCIM 3498

Parameters studied	Xylose	Glucose	Arabinose	Mannose	Galactose	Cellobiose	Simulated medium
Sugar utilization (%)	94	94	70.2	92.6	76	62	90
Ethanol (g_p/L)	4.41	4.60	1.68	3.51	2.35	1.36	17.28
Biomass (g_x/L)	2.63	2.91	1.12	1.75	1.29	0.806	7.50
Ethanol yield (g_p/g_s)	0.47	0.49	0.24	0.38	0.31	0.22	0.48
Biomass yield (g_x/g_s)	0.28	0.31	0.16	0.19	0.17	0.13	0.20
Volumetric ethanol productivity (g_p/L/h)	0.122	0.127	0.046	0.097	0.065	0.037	0.216
Biomass productivity (g_x/L/h)	0.073	0.080	0.0311	0.048	0.035	0.022	0.093
Sugar uptake rate(g_s/L/h)	0.261	0.261	0.195	0.257	0.211	0.172	0.450
Specific ethanol productivity (g_p/g_x/ h)	0.046	0.043	0.041	0.056	0.050	0.046	0.025

- Individual substrate concentration 10 g/L, fermentation time 36 h
- Total sugar concentration in simulated medium 40 g/L, fermentation time 80 h
- The data presented are averages of three independent analyses.

Table 4: Fermentation parameters of the *P. stipitis* NCIM3498 when grown in the *B. compestris* stalks enzymatic hydrolysate in 10L fermented.

Parameters studied	Values
Initial sugar concentration (g_s/L)	40.26
Sugar utilization (%)	86.50
Ethanol (g_p/L)	16.02
Ethanol yield (g_p/g_s)	0.46
Biomass (g_x/L)	5.86
Biomass yield (g_x/g_s)	0.17
Volumetric ethanol productivity (g_p/L/h)	0.20
Biomass productivity (g_x/L/h)	0.07
Sugar uptake rate (g_s/L/h)	0.50
Specific ethanol productivity (g_p/g_x/ h)	0.03

* Total incubation time- 80 h. The data presented are averages of three independent analyses.

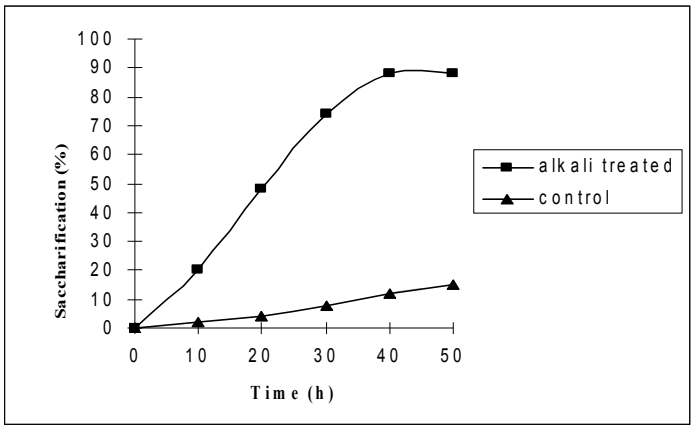

Figure 1: Enzymatic hydrolysis of *B. compestris* stalks using culture supernatants of *Aspergillus sp.* in the presence of Tween- 20. Substrate and enzyme concentration were (1: 20) and 25 FPU/g substrate, respectively

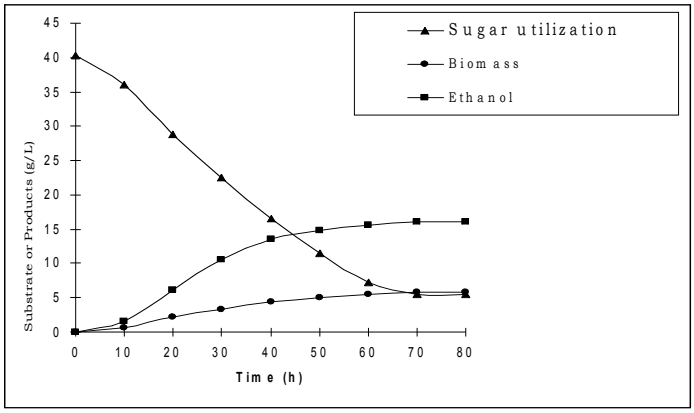

Figure 2: The time course of growth, sugar utilization and ethanol production by *P. stipitis* NCIM 3498 at 30 □C using *B. compestris* stalks enzymatic hydrolysate (pH- 5.5).

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