BIOCONVERSION OF HEMICELLULOSE HYDROLYSATE OF SWEET SORGHUM BAGASSE TO ETHANOL BY USING *PICHIA STIPITIS* NCIM 3497 AND *DEBARYOMYCES HANSENII* SP.

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Production of ethanol from concentrated D-xylose solutions and hemicellulose hydrolysate of sweet sorghum bagasse was achieved by using *Pichia stipitis* NCIM 3497 and an isolated yeast *Debaryomyces hansenii* sp. These yeasts were capable of producing ethanol from solutions containing 800 g/L D-xylose, and the optimum sugar concentration was found to be 150 g/L at pH 4, 30°C, with a production time of 72 hours. These yeasts were capable of utilizing multiple sugars. Hemicellulose hydrolysates of sweet sorghum bagasse were obtained by dilute acid hydrolysis and autohydrolysis including steam explosion treatment. The hydrolysate was treated by an over-liming process for detoxification and pH adjustment. Ethanol yield from hemicellulose hydrolysate was found to be higher than that of synthetic medium containing D-xylose. These yeasts can be used in production of ethanol from concentrated hemicellulose hydrolysates containing high pentose sugars obtained while treating lignocellulosic biomass at high substrate concentrations.

Keywords: D-xylose; Sweet sorghum bagasse; Hemicellulose hydrolysate; Fermentation; Pichia stipitis NCIM 3497; Debaryomyces hansenii sp.; Ethanol.

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INTRODUCTION

Industrial production of ethanol from lignocellulosic hydrolysates requires the use of microorganism capable of utilizing the different types of sugars present in it (Balat et al. 2008; Bettiga et al. 2009). Jeffries et al. (2000) reviewed the ethanol production from xylose by yeasts and pointed out that xylose fermentation using yeasts has many advantages over fermentation using bacteria. Yeasts have higher ethanol tolerance than bacteria. It is easier to harvest and recycle yeast cells than bacterial cells from the fermentation broth, and yeast fermentation is resistant to contamination from bacteria and viruses. Among the yeasts used for xylose fermentation only six species produce significant amounts of ethanol and only three of them namely *Candida shehatae, Pachesolen tannophilus, and Pichia stipitis* have been studied extensively. *Pichia stipitis* is one of the natural xylose-fermenting yeast strains and it has been shown to be the most useful species for direct fermentation of xylose to ethanol (McMillan 1993; Ghosh et al. 2003). Like other natural pentose fermentors it produces ethanol under oxygen-limited conditions (Gong et al. 1981). Many studies have selected *Pichia stipitis* as the best yeast among microorganisms producing ethanol from xylose, and research has confirmed the

ethanol concentration in the media up to 57 g/L, lower production of xylitol from sugars, and higher ethanol tolerance than other xylose fermenting yeasts (Toivola et al. 1984; DuPreez et al. 1989; Slininger et al. 1991). Several research groups have performed genetic modification studies of these microorganisms for improved product yield and performance. *Pichia stipitis* NRRL-7124 showed 80.4 ± 0.55% efficiency in conversion of sugars in the wheat straw hydrolysate to ethanol with a yield of 0.41 ± 0.01 g/g (Nigam 2001).

Genetically modified yeasts such as *Saccharomyces cerevisiae* 1400 (*pLNH*33) (Kotter and Ciriacy 1993), *Saccharomyces cerevisiae* 1400 (*pLNH*32) (Ho et al. 1998), and *Saccharomyces cerevisiae* TMB3662 (Bettiga et al. 2009), have been studied for the fermentation of xylose. Recombinant *Saccharomyces* expressing xylose isomerase from *Opinomyces, Xylulokinasse* from *Saccharomyces* and sugar transporter genes from *Pichia* have showed the ability to ferment both xylose and glucose with an ethanol yield of 0.48 g/g (Madhavan et al.2009). Genetically modified bacteria like *Escherichia coli*, *Klebsiella oxytoca, Zymomonas mobilis*, etc., are used to produce ethanol from xylose. The transformant *E. coli* gave a yield of 39.2 g/L ethanol from 80 g/L xylose (Ohta et al. 1990), *K. oxytoca* M5A1 produced 45 g/L ethanol from 100 g/L xylose (Ohta et al. 1991), and *Z. mobilis* ZM4 (pZB5) produced 62 g/L ethanol from a mixture of 1:1 mixture of glucose and xylose with a total concentration of 130 g/L (Joachimsthal et al. 1999).

Economical production of ethanol from lignocellulose hydrolysate requires high concentrations of fermentable sugars in it. High osmolality of the media like molasses prevents the action of ethanologens, as most of these microorganisms used for ethanol production are incapable of performing at high sugar and salt concentrations. Fermentation of molasses requires the conditioning of molasses using neutralizing agents to remove the inhibitory effects of molasses on yeast activity, and it also needs the addition of nutritional factors for the yeast growth (Sanchez et al. 2008). Brooks (2008) showed that *Saccharomyces cerevisiae* R-8 could grow in a D-glucose solution having a concentration of 400 g/L at 42^0C and, *Saccharomyces kluvveri* K-6 could give a maximum ethanol concentration of 46.03 g/L. Fed-batch fermentation of high sugar containing solutions can produce higher ethanol concentrations as high as 147 g/L (Alfenore et al. 2004). There are a few studies (Osho 2005; Brooks 2008) showing the growth and ethanol production in concentrated D-glucose solutions, but few studies have addressed the fermentation of concentrated sugar solution of D-xylose or any other pentose sugars.

Only less than one percent of the microorganisms present on the Earth have been studied well, and this implies the importance of searching for new microorganisms for the conversion of lignocellulosic biomass to value added products. Warnick et al. (2002) isolated *Clostridium phytofermentans* sp. nov, which can utilize a wide variety of sugars and directly convert cellulose to ethanol. The strain difference among the *Saccharomyces* species showed differences in tolerance to fermentation inhibitors and thereby in growth and ethanol production in hydrolysates of spruce, barley straw and wheat straw (Almeida et al. 2009).

Post-hydrolysis treatment of biomass hydrolysate is required to improve the fermentability of it by adjusting its pH and to remove the toxic compounds from it. An

over-liming process in which the pH of the hydrolysate is increased to 9-10 using $Ca(OH)_2$ and then adjusting the pH to 5.5 by using H_2SO_4 was found to increase the fermentability of the hydrolysates. The detoxifying effect of over-liming is due to the precipitation of toxic compounds and instability of some toxic compounds at high pH (Palmqvist et al. 2000). Adding an initial high yeast inoculum during the fermentation of lignocellulosic hydrolysate was found to decrease the effect of fermentation inhibitor and ethanol toxicity over ethanol yield (Chung et al. 1985; Nishikawa et al. 1988). Fermenting microorganisms like *Saccharomyces cerevisiae, Pichia stipitis, Zymomonas mobilis*, and *Candida shehatae* showed the assimilation of lignocellulosic degradation products like vanillin and furaldehyde into them, and prolonged incubation of these microbes in them partially recover the effect of these inhibitors on fermentation of lignocellulosic hydrolysates (Delgenes et al.1998).

The study presented here was aimed at isolating efficient pentose fermenting microbes and using them for production of ethanol from hemicellulose fraction of sweet sorghum bagasse.

EXPERIMENT

Materials

Sweet sorghum bagasse used was obtained from Directorate of Sorghum Research (DSR), formerly known as National Research Centre for Sorghum (NRCS), Hyderabad, India. Biomass pretreatments were done to obtain the hydrolysate for fermentation, using a batch treatment reactor equipped with temperature and pressure controls. Yeasts capable of utilizing xylose were isolated from different sources, including compost piles, forest soils, and wetlands. *Debaryomyces hansenii* sp. producing ethanol from pentose sugars was isolated from wetlands. *Pichia stipitis* NCIM 3497 used in the study was obtained from National Collection of Industrial Microorganisms (NCIM) of National Chemical Laboratory (NCL), Pune, India. The biochemicals used for the media preparation and fermentation study were purchased from Himedia Laboratories, Mumbai, India.

Methods

Isolation of yeasts for xylose fermentation

The screening and isolation of the yeasts for pentose fermentation to ethanol was carried out by following the method of Nigam et al. (1985): Soil samples from compost piles, forest soils, and wetlands were used as inoculum for media containing D-xylose (30 g/L) as carbon source and yeast extract (10 g/L) as nitrogen source at a pH of 5 and temperature 30 ^{o}C. Xylose-utilizing microbes were subjected to carbon assimilation test using Yeast Nitrogen Base without amino acids and ammonium sulphate (1 g/L). Ethanol production was confirmed by using Gas Chromatography (GC). Ethanol-producing colonies were used for further isolation and purification procedures, and the isolates that showed high ethanol yield were subjected to optimization studies. The isolate that gave the best results was sent for identification to the Institute of Microbial Technology

(IMTECH), Chandigargh, India. Yeast cultures were stored at 0 ^{o}C in medium containing 150 g/L D-xylose sugar.

Fermentation of D-xylose to ethanol

The studies were performed in 250 mL screw-capped conical flasks in an incubator equipped with temperature and mixing controls. The media did not undergo any deoxygenation procedures in order to avoid any anaerobic conditions, and was closed tightly to avoid any further air transfer during fermentation. The D-xylose sugar concentration in the fermentation broth was varied from 30 g/L to 800 g/L. Yeast extract was added as nitrogen source at a concentration of 1 g/L, and 1 mL of trace metal mixture was added as nutrient supplement. The composition of trace metal mixture is given in the Table 1. The pH values of the media used were varied from pH 2 to pH 7, while temperature was set to 30 ^{o}C. Ethanol tolerance was studied by method followed by Novak et al. (1981). Ethanol at different concentrations was added into the media, and the growth of the selected yeast was monitored at 600 nm (OD_{600}), taking medium without yeast inoculation as initial blank. Ethanol concentration added into the media varied from 10 g/L to 150 g/L with 10 g/L increase in concentration. Ethanol concentration was estimated using Nucon 5700 Gas Chromatograph equipped with Chromosorb -101 glass column and flame ionization detector (FID) (Varma et al., 1984).

Table 1. Composition of Trace Metal Mixture used for Fermentation

Metal compound	Weight per 1000 mL distilled water
Calcium Chloride	1000 mg
Cobalt Nitrate	125 mg
Copper Sulphate	1 mg
Ferric Chloride	150 mg
Manganese Chloride	180 mg
Magnesium Sulphate	250 mg
Zinc Sulphate	30 mg

Production of hemicellulose hydrolysate of sweet sorghum bagasse

The bagasse used, Sweet Sorghum Variety 84 (SSV 84), was selected based on the compositional analyses of bagasse samples from 20 sweet sorghum genotypes. The selected bagasse was pulverized using a biomass pulverizer to the size of less than 180 µm and pretreated in a batch reactor to obtain hemicellulose fraction into the hydrolysate. Substrate concentrations were varied from 100 g/L to 250 g/L, and the hydrolysates having higher reducing sugar concentration were used for fermentation studies. Dilute acid hydrolysis and autohydrolysis including steam explosion were used to produce the hydrolysate. Dilute sulphuric acid (5 to 50 g/kg) was used for acid hydrolysis at temperature ranges from 120 ^{o}C to 200 ^{o}C.

Ethanol production from hemicellulose hydrolysate of sweet sorghum bagasse

The hydrolysate obtained by biomass hydrolysis was treated with calcium hydroxide for detoxification and neutralization. Over-liming was applied for detoxification and neutralization process to improve the fermentability of dilute acid hydrolysate (Martinez et al. 2001). The supernatant from over-liming treatment was used

for yeast inoculation after autoclaving. Samples of hydrolysate prior to yeast inoculation was used for reducing sugar estimation using DNS reagent (Miller, 1959). Yeast extract (1 g/L) was added in to the hydrolysate as the nitrogen source. Ethanol production was estimated in 24 hour intervals, and the study stopped when ethanol concentration started to decline from the previous day.

RESULTS AND DISCUSSION

Identification of the isolated yeast

The xylose fermenting yeast was originally isolated, purified, and sent for identification to the Institute of Microbial Technology. The isolated yeast was identified as *Debaryomyces hansenii* sp. at the Microbial Type Culture Collection (MTCC) of the Institute of Microbial Technology (IMTECH), Chandigargh. The yeast showed some variation from other isolates of *Debaryomyces hansenii* present at MTCC due to strain variations, and further molecular level characterizations are needed to identify and confirm the strain variations. *Debaryomyces hansenii* is a well-known (Breuer and Harms 2006) halotolerant yeast used for production of polyols including xylitol, arabitol, and ethanol using different substrates (Girio et al. 2000). It also showed growth improvement under stress conditions of temperature and salinity (Papouskova and Sychrova 2007). This yeast was used for xylitol production from hardwood hemicellulose (Parajo et al. 2006), rice straw hemicellulose (Mayerhoff et al. 2004), and brewery spent waste hemicellulose (Carvalheiro et al. 2007).

Ethanol production from D-xylose

The yeasts were capable of producing ethanol from a solution containing up to 800 g/L D-xylose. Increasing the sugar concentration decreased both the activity of the yeast and the ethanol concentration in the medium. The optimum sugar concentration giving maximum ethanol concentration by *Pichia stipitis* NCIM 3497 was found to be 150 g/L, which resulted in an ethanol concentration of 40.21 g/L, showing a theoretical maximum of 52.56 percent. *Pichia stipitis* NCIM 3497 was found to be capable of producing ethanol from a variety of sugars, including D-glucose, cellobiose, D-mannose, and D-xylose. *Debaryomyces hansenii* sp. was found to be producing ethanol from D-glucose, D-mannose, and D-xylose, but unable to produce ethanol from cellobiose, even though it grew on it. These yeasts were able to utilize L-arabinose but not able to produce ethanol from it. The data obtained while using 30 g/L of different sugars for fermentation using these yeasts is given in the Figs. 1 and 2.

Pichia stipitis NCIM 3497 produced more ethanol from all sugar concentrations and was found to be better than *Debaryomyces hansenii* sp. The optimum pH was found to be pH 4 under increased sugar concentrations. The highest ethanol concentration was obtained at pH 4, and the product concentration decreased both above and below this pH level. *D. hansenii* sp. also showed the same product formation characteristics at different pH values, and the values of *P. stipitis* NCIM 3497 are shown in the Fig. 3

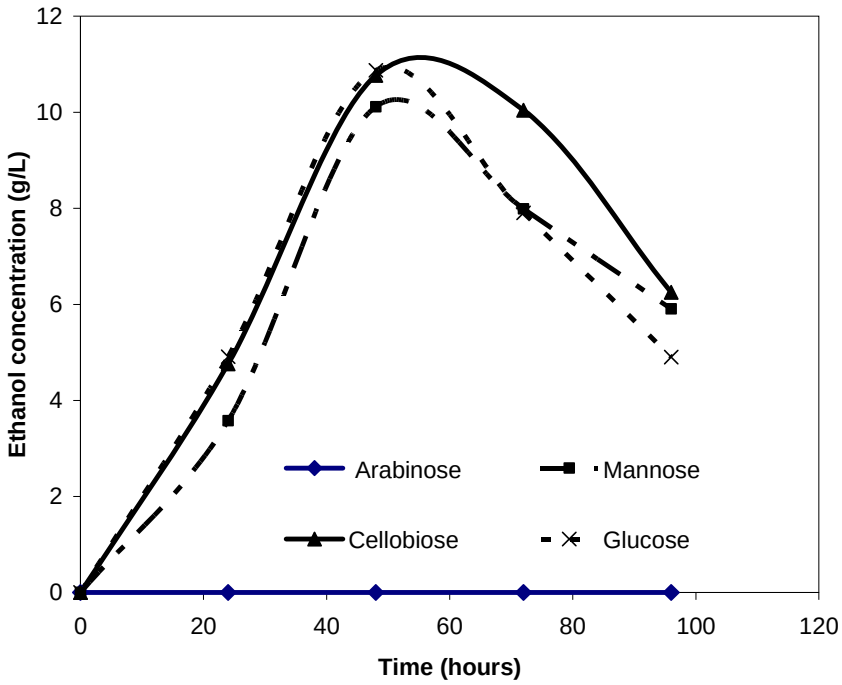

Figure 1. Ethanol production from different sugars by using *Pichia stipitis* NCIM 3497

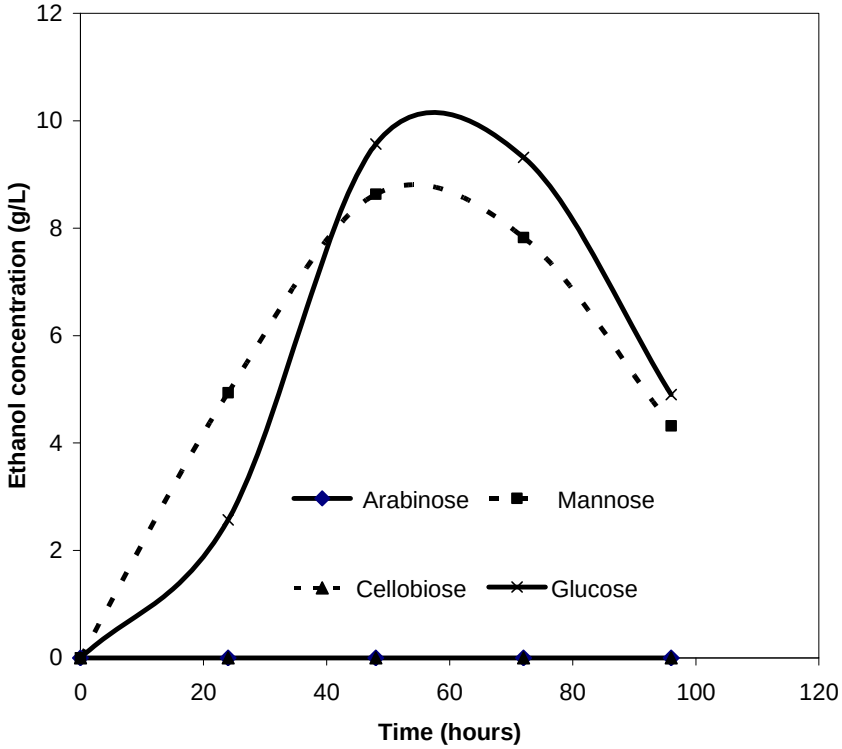

Figure 2. Ethanol production from different sugars by using *Debaryomyces hansenii* sp.

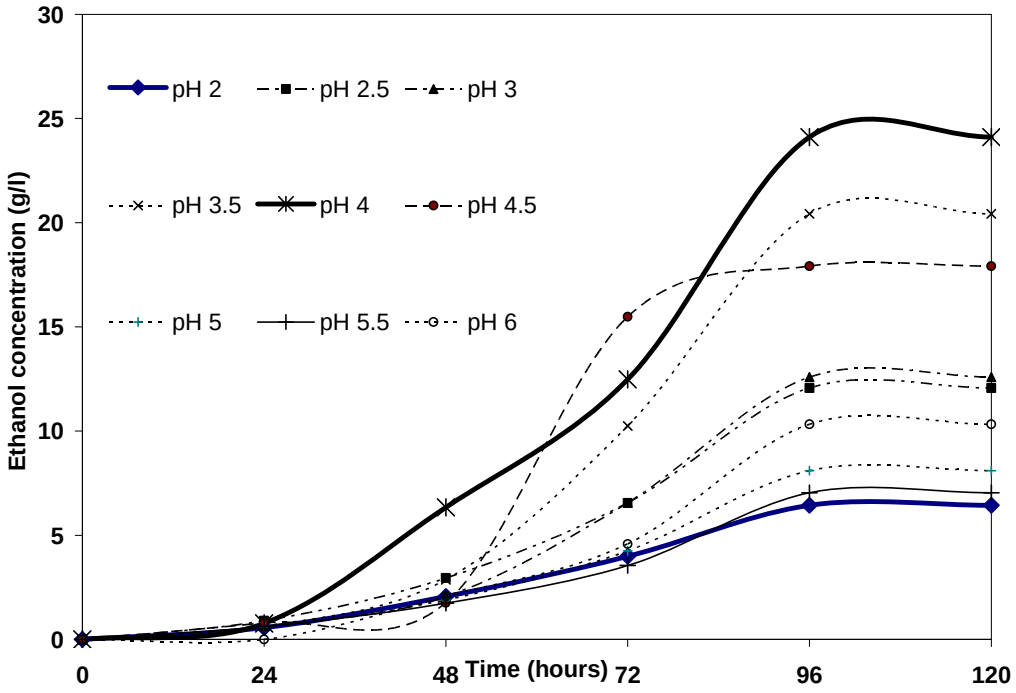

Figure 3. Production of ethanol by *P. stipitis* NCIM 3497 from 150 (g/L) D-xylose under different pH conditions

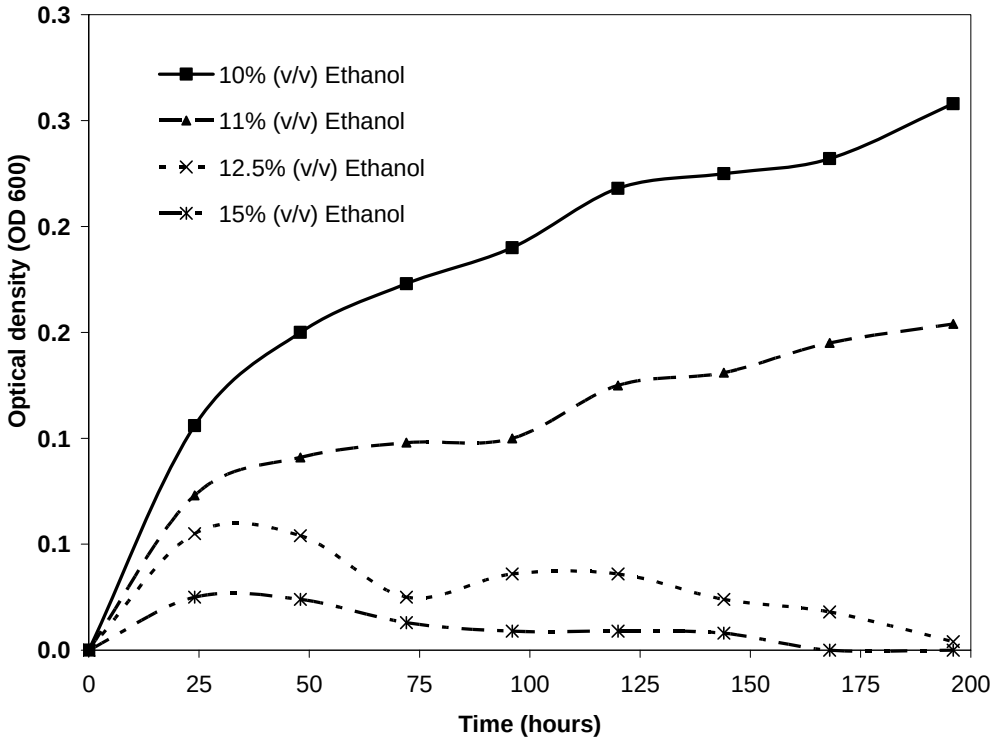

Figure 4. Growth of *P stipitis* NCIM 3497 in presence of different ethanol concentrations

The maximum ethanol concentration of 40.21 g/L was obtained from 150 g/L xylose sugar solution at 30 ^{o}C and 72 hours. The yeasts were capable of growth in medium with ethanol concentration of up to 110 g/L. Ethanol concentrations higher than 110 g/L prevented the growth of these yeasts in D-xylose medium. Further optimization of temperature and other nutrient supplementations may increase the productivity of these yeasts.

Comparison of yeasts based on activity

The yeasts were compared based on their activity on D-xylose and its fermentation to ethanol. Among the yeasts *Pichia stipitis* NCIM 3497 was found to be better than *Debaryomyces hansenii* sp. in growth and ethanol production in D-xylose. The results are shown in the Fig. 5 and Table 1. *P. stipitis* NCIM 3497 reached its stationary phase in growth and ethanol production within 48 and 72 hours of inoculation.

Ethanol production by *D. hansenii* sp. reached its maximum concentration of 37.08 g/L at 144 hours of inoculation. Yeasts used for comparison in activity were taken from higher sugar concentrations and gave higher ethanol concentrations than yeasts taken from lower sugar concentrations. This observation implies that the yeasts were adapting to higher sugar concentrations and improve their activity when the osmolality is reduced.

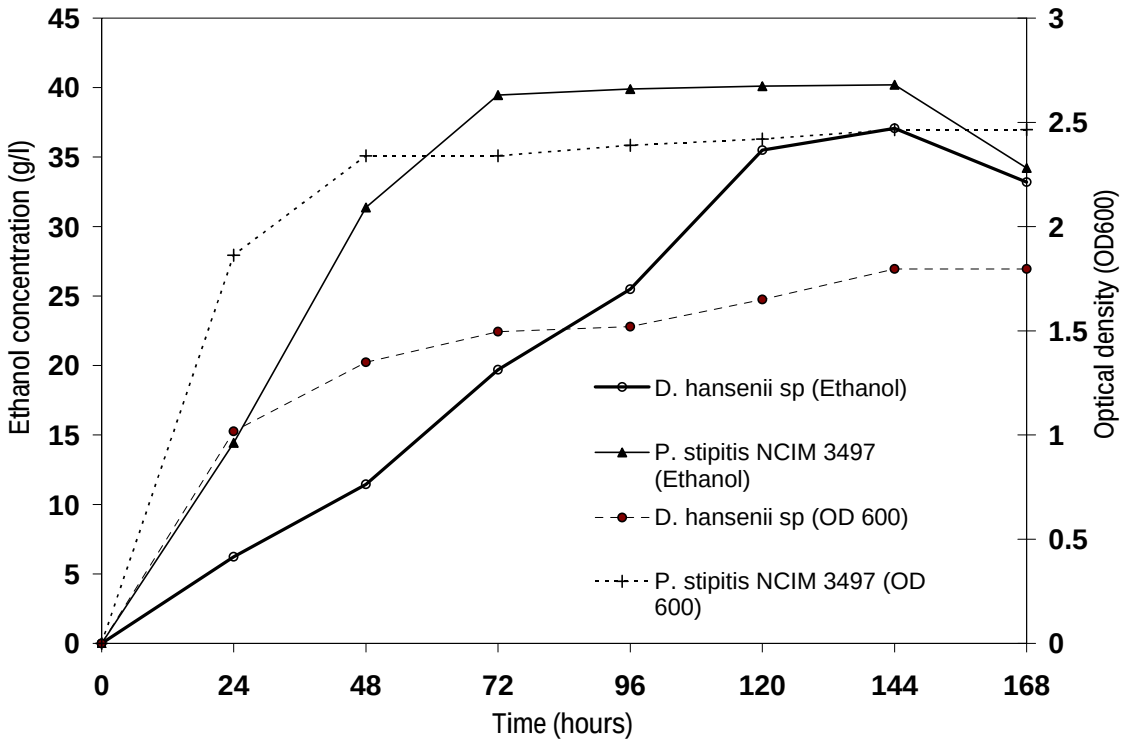

Figure 5. Ethanol production from 150 g/L D-xylose using *D. hansenii* sp. and *P. stipitis* NCIM 3497 under optimum conditions

Table 2. Comparison of Ethanol Production at Higher D-xylose Concentrations by *Debaryomyces hansenii* sp. and *Pichia stipitis* NCIM 3497

Time (Hrs)	D-xylose concentration (g/L)									
	400	500	600	700	800	400	500	600	700	800
	Ethanol Yield (g/L)									
	***Debaryomyces hansenii* sp.**					***Pichia stipitis* NCIM 3497**				
0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	2.82	0.80	4.47	2.35	2.18
48	1.62	1.19	0	0	0	6.21	0.89	5.72	2.80	2.47
72	2.82	3.47	0.97	0	0	14.09	2.56	7.79	3.17	3.09
96	4.03	4.79	1.65	0.86	0.99	16.79	6.20	9.90	4.47	3.47
120	6.23	6.2	2.4	1.1	1.3	19.63	7.70	11.15	4.72	3.57
144	8.35	7.8	3.1	1.5	2.1	20.33	8.30	12.03	4.82	3.86
168	10.05	10.58	3.66	1.88	2.60	20.46	10.06	12.90	5.42	4.26
192	18.23	13.43	6.37	5.21	4.01	19.38	7.27	13.80	5.96	4.37

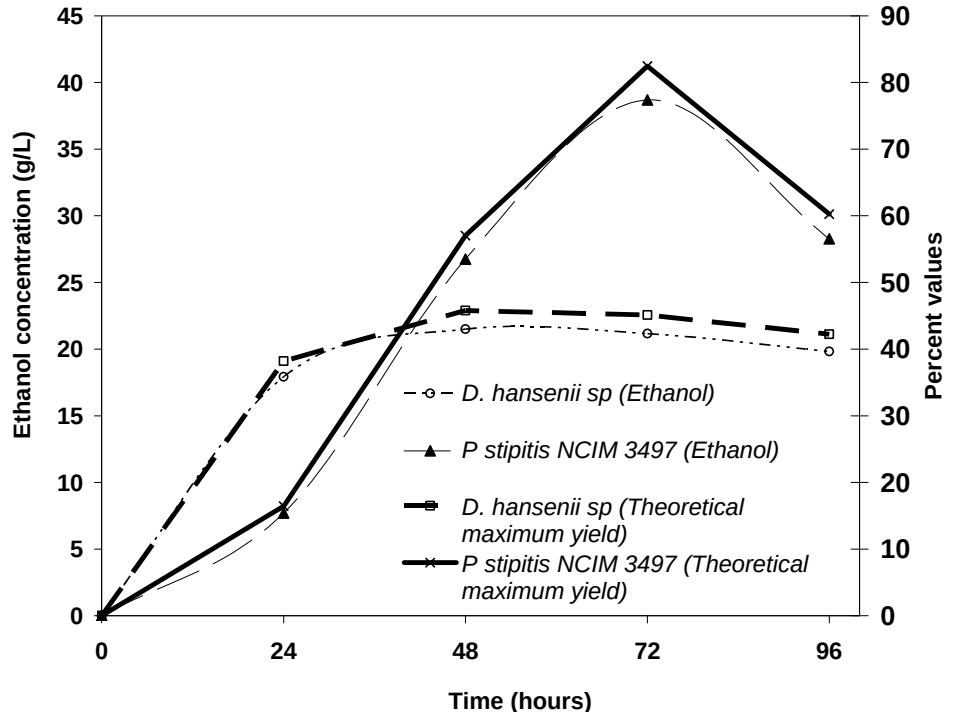

Figure 6. Ethanol production from hemicellulose hydrolysate of sweet sorghum bagasse using yeasts

Ethanol production from hemicellulose hydrolysate

The hemicellulose hydrolysate of sweet sorghum bagasse was fermented to ethanol by using pentose fermenting yeasts *Pichia stipitis* NCIM 3497 and *Debaryomyces hansenii* sp. The highest ethanol concentration of 38.7 g/L was produced by *Pichia stipitis* NCIM 3497 from hydrolysate obtained by treating sweet sorghum

bagasse at 250 g/kg substrate concentration and 5 g/kg sulphuric acid input at 140 ^{o}C for 30 minutes, having a reducing sugar concentration of 92.02 g/L. The maximum theoretical yield of ethanol production was found to be 82.5 per cent. This was higher than the yield obtained when using pure xylose in the synthetic media for fermentation using the same microorganisms. This increase in yield may be attributed to the presence of other components from the sweet sorghum bagasse. Detailed process and material characterization is needed to understand the exact mechanism of improvement in the fermentation yield while using hemicellulose hydrolysate of sweet sorghum bagasse.

Proper aeration with increased sugar concentration would increase the efficiency of ethanol production from hemicellulose hydrolysates. The selected yeasts have great potential to be used in ethanol production from hemicellulose fraction of lignocellulosic biomass. Molecular level characterization of *Debaryomyces hansenii* sp. will help to identify the yeast more exactly.

CONCLUSIONS

1. Hemicellulose hydrolysate obtained while treating sweet sorghum bagasse powder at high solids loading is a concentrated sugar solution containing different sugars with pentoses as major component. Production of ethanol from these solutions is possible only with the use of osmotolerant and pentose fermenting yeast strains such as *Pichia stipitis* NCIM 3497 and *Debaryomyces hansenii* sp., which can ferment other sugars also present in the hydrolysate.

2. These yeasts are capable of utilizing different sugars and produce ethanol from D-xylose, D-glucose, and D-mannose. The yeast *Pichia stipitis* NCIM 3497 was capable of producing ethanol from Cellobiose also. These yeasts were displayed osmotolerance in synthetic media containing D-xylose sugar concentration up to 800 g/L, and the optimum sugar concentration for maximum ethanol concentration was found to be 150 g/L. The highest theoretical ethanol yield of 82.5 percent with an ethanol concentration of 38.7 g/L was obtained while using dilute acid hydrolysate of sweet sorghum bagasse having a total reducing sugar concentration of 92.02 g/L.

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REFERENCES CITED

Alfenore, S., Cameleyre, X., Benbadis, L., Bideaux, C., Uribelarrea, J. L., Goma, G., Molina-Jouve, C., and Guillouet, S. (2004). "Aeration strategy: a need for very high

ethanol performance in *Saccharomyces cerevisiae* fed-batch process," *Applied microbiology and Biotechnology* 63, 537-542.

Almeida, J. R. M., Karhumaa, K., Bengtsson, O., and Gorwa-Grauslund, M. (2009). "Screening of *Sacchromyces cerevisiae* strains with respect to anaerobic growth in non-detoxified lignocellulose hydrolysate," *Bioresource Technology* 100, 3674-3677.

Balat, M., Balat, H., and Oz, C. (2008). "Progress in bioethanol processing," *Progress in Energy and Combustion Science* 34, 551-573.

Bettiga, M., Bengtsson, O., Hahn-Hagerdal, B., and Gorwa-Grauslund, M. F. (2009). "Arabinose and xylose fermentation by recombinant *Saccharomyces cerevisiae* expressing a fungal pentose utilization pathway," *Microbial Cell Factories*. doi:10.1186/1475-2859-8-40. (http://www.microbialcellfactories.com).

Breuer, U., and Harms, H. (2006). "*Debaryomyces hansenii* – An extremophilic yeast with biotechnological potential,"*Yeast* 23, 415-437.

Brooks, A. A. (2008). "Ethanol production potential of local yeast strains isolated from ripe banana peels," *African Journal of Biotechnology* 7 (20), 3749-3752.

Carvalheiro, F., Duarte, L. C., Medeiros, R., and Girio, F. M. (2007). "Xylitol production by *Debaryomyces hansenii* in brewery spent grain dilute-acid hydrolysate: Effect of supplimentation," *Biotechnology Letters* 29, 1887-1891.

Chung, I. S., and Lee, Y. Y. (1985). "Ethanol fermentation of crude acid hydrolysate of cellulose using high-level yeast inocula," *Biotechnology and Bioengineering* 27(3), 308-315.

Delgenes, J. P., Moletta, R., and Navarro, J. M. (1998). "Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by *Saccharomyces cerevisiase, Zymomonas mobilis, Pichia stipitis,* and *Canadida shehatae*," *Enzyme and Microbial Technology* 15, 220-225.

Du Preez, J. C., van Driessel, B., and Prior, B. A. (1989). "D-xylose fermentation by *Candida shehatae* and *Pichia stipitis* at low dissolved oxygen levels in fed-batch cultures," *Biotechnology letters* 11(2), 131-136.

Ghosh, P., and Ghose, T.K. (2003). "Bioethanol in India: Recent past and emerging future," *Advances in Biochemical Engineering/Biotechnology* 85, 1-27.

Girio, F. M., Amaro, C., Azinheira, H., Pelica, F., and Amaral-Collaco, M. T. (2000). "Polyols production during single and mixed substrate fermentation in *Debaryomyces hansenii*," *Bioresource Technology* 71, 245-251.

Gong, C., S., Chen, L., F., Flickinger, M., C., and Tsao, G., T. (1981). "Conversion of hemicellulose carbohydrates," *Advances in biochemical engineering and technology* 20, 93-118.

Ho, N. W. Y., Chen, Z., and Brainard, A. P. (1998). "Genetically engineered *saccharomyces* yeast capable of effective co-fermentation of glucose and xylose," *Applied and Environmental Microbiology* 64(5), 1852-1859.

Jeffries, T. W. and Jin, Y. (2000). "Ethanol and thermotolerance in the bioconversion of xylose by yeasts," *Advanced in applied Microbiology* 47, 221-268.

Joachimsthal, E., Hagget, K. D., and Rogers, P. L. (1999). "Evaluation of recombinant strains of *Zymomonas mobilis* for ethanol production from glucose/xylose media," *Applied Biochemistry and Biotechnology* 77, 147-157.

Kotter, P., and Ciriacy, M. (1993). "Xylose fermentation by *Saccharomyces cerevisiae*," *Applied Microbiology and Biotechnology* 38(6), 776-783.

Madhavan, A., Tamalampudi, S., Srivastava, A., Fukuda, H., Bisaria, V. S., and Kondo, A. (2009). "Alcoholic fermentation of xylose and mixed sugars using recombinant *Saccharomyces cerevisiae* engineered for xylose utilization," *Applied Microbiology and Biotechnology* 82, 1037-1047.

Martinez, A., Rodriguez, M. E., Wells, M. L., York, S. W., Preston, J. F., and Ingram, L. O., (2001). "Detoxification of dilute acid hydrolysates of lignocellulose with lime," *Biotechnology Progress* 17, 287-293.

Mayerhoff, Z. D. V. L., Roberto, I. C., and Silva, S. S. (2004). "Xylitol production from rice straw hemicellulose hydrolysate using different yeast strains," *Biotechnology Letters* 19, 407-409.

McMillan, J., D. (1993). "Xylose fermentation to ethanol: A review," Published by NREL. ISBN B0006QLMFU. (http://www.nrel.gov)

Miller, G.L. (1959). "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Analytical Chemistry* 31, 426-428.

Nigam, J. N., Ireland, R. S., Margaritis, A., and Lachance, M. A. (1985). "Isolation and screening of yeasts that ferment D-xylose directly to ethanol," *Applied and Environmental Biotechnology* 50(6), 1486-1489.

Nigam, J. N. (2001). "Ethanol production from wheat straw hemicellulose hydrolysate by *Pichia stipitis*," *Journal of Biotechnology* 87(1), 17-27.

Nishikawa, N. K., Sutcliff, R., and Saddler, J. N. (1988). "The influence of lignin degradation products on xylose fermentation by *Klebsiella pneumoniae*," *Applied Microbiology and Biotechnology* 27, 549-552.

Ohta, K., Alterthum, F., and Ingram, L. O. (1990). "Effects of environmental conditions on xylose fermentation by recombinant Escherichia coli," *Applied and Environmental Microbiology* 56, 463-465.

Ohta, K., Beall, D. S., Mejia, J. P., Shanmugamm, K. T., and Ingram, L. O. (1991). "Metabolic engineering of *Klebsiella oxytoca* M5A1 for ethanol production from xylose and glucose," *Applied and Environmental Microbiology* 57, 2810-2815.

Osho, A. (2005). "Ethanol and sugar tolerance of wine yeasts isolated from fermenting cashew apple juice," *African Journal of Biotechnology* 4(7), 660-662.

Palmqvist, E. and Hahn-Hagerrdal, B. (2000). "Fermentation of lignocellulosic hydrolystates.1: Inhibition and detoxification," *Bioresource Technology* 74, 17-24.

Papouskova, K. and Sychrova, H. (2007). "The co-action of osmotic and high temperature stresses results in a growth improvement of *Debaryomyces hansenii* cells," *International Journal of Food Microbiology* 118, 1-7.

Parajo, J. C., Dominguez, H., and Dominguez, J. M. (2006). "Production of xylitol from raw wood hydrolysates by *Debaryomyces hansenii* NRRL Y-7426," *Bioprocess and Biosystems Engineering* 13, 125-131.

Sanchez, O. J., and Cardona, C. A. (2008). "Trends in biotechnological production of fuel ethanol from different feedstocks," *Bioresource Technology* 99, 5270-5295.

Slininger, P. J., Branstrator, L. E., Bothast, R. J., Okos, M. R., and Ladisch, M. R. (1991). "Growth, death, and oxygen uptake kinetics of *Pichia stipitis* on xylose," *Biotechnology and Bioengineering* 37(10), 973-980.

Toivola, A., Yarrow, D., van den Bosch, E., van Dijken, J. P., and Scheffers, W. A. (1984). "Alcoholic fermentation of D-xylose by yeasts," *Applied and Environmental Microbiology* 47(6), 1221-1223.

Varma, R., Sawant, U. D., and Karanth, N. G. (1984). "Estimation of ethanol in fermentation broth by solvent extraction and gas chromatography," *Enzyme and Microbial Technology* 6(5), 233-235.

Warnick, T. A., Methe, B. A., and Leschine, S. B. (2002). "Clostridium phytofermentans sp. nov., a cellulolytic mesophile from forest soil," *International Journal of Systematic and Evolutionary Microbiology* 52, 1155-160.

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