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Xylitol Production from Water Hyacinth (*Eichhornia crassipes*) by *Candida tropicalis* Y-27405

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This is the study which reported the use of, Water hyacinth (*Eichornia crassipes*) for xylitol production. In this study a simple reliable and efficient hydrolysis by dilute acid hydrolysis using 2% (v/v) H_2SO_4 was carried out at 121°C for 60 min. The acid pretreatment was concentrated under vacuum using rotavapor and further detoxified using overliming and subsequent activated charcoal treatment. The sugars achieved after concentration and detoxification (50±0.3g/l) was subjected to xylitol fermentation using *Candida tropicalis* Y-27405. A maximum xylitol concentration of 32.5g/l was obtained after 48 hr of fermentation, with a yield of 0.65gxylitol/g xylose and productivity of 0.67g/l/h.

Key words: Water hyacinth, *Candida tropicalis*, detoxification, Overliming, Activated charcoal treatment.

D-Xylitol has attracted worldwide interest because of its unique properties and huge potential. Xylitol known as birch sugar is obtained from the reduction of xylose. It is naturally found in the fiber of many fruits and vegetables including berries, corn husks, oats and mushrooms. It has 40% fewer calories and 75% fewer carbohydrates thus it has been used as a sugar substitute in dietary foods, especially for insulin-deficiency patients (Goli *et al*., 2012). Xylitol gained its importance in food and pharmaceutical industry, in preparation of confectioneries &chewing gums as it has tooth re-hardening and demineralization properties and decreases the levels of mutants *Streptococci*, plaque formation and dental caries, (Yuen et al., 2012). It could also prevent ear and upper respiratory tract infections and benefit pregnant and nursing women. (Prakasham et al., 2009).

Xylitol is currently being produced from lignocellulosic materials such as sugarcane baggasse, rice straw, wheat straw and plant parts like seeds, stalks and processing by-products (distiller's grain, corn soluble) (Rehman et al., 2013). Aquatic weeds can be used as lignocellulosic biomass that can contribute significantly to the future needs of the society without any competition for existing arable land and eliminate the use of food crops (Metzger et al., 2009).

Water hyacinth (*Eichhornia crassipes*) is one of the world's worst aquatic plants which cover entire water surface of rivers, dams, lakes and canals and causes the depletion of nutrients and oxygen thereby adversely affect flora and

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fauna of water (Mahmood *et al*., 2009). It grows very fast and costs billions of dollars every year for its control. Fast growth and high percentage of hemicellulose is a valued feature, therefore water hyacinth will have a great potential to be used as raw material for the production of fermentable sugars and their further conversion to value added product like xylitol, can be a novel and promising alternative (Kalhorinia *et al*., 2013)

Xylitol production by chemical methods is expensive as it requires high temperature and pressure with highly pure sugar substrates and costly chromatographic purification steps. Therefore, biotechnological production of xylitol from fermented hemicellulosic hydrolysate using microorganisms such as fungi, bacteria and yeast has become more attractive because the process occur under much milder process conditions to save on energy, substrate purification cost it is environmentally safe (Goli *et al*., 2012). *Candida* yeast was recognized as the best xylitol producers among bacteria, fungi and yeasts, which have been studied for decades for microbial production of xylitol (West, 2009). Xylose fermenting yeasts reduce xylose to xylitol by the NAD (P) H-dependent xylose reductase (XR) (Winkelhausen and Kuzmanova, 1998).

Dilute acid pretreatment process, is inexpensive, avoid corrosion and easy to perform. (Taherzadeh *et al*.,2007). Dilute acid hydrolysis is mostly used because it effectively solubilizes hemicelluloses to xylose and other sugar monomers (Chandel *et al*., 2007a). The hydrolysate formed after acid hydrolysis contain sugar monomers mostly pentoses, hexoses and lignin degradation products like acetic, formic and levulinic acid, furan derivatives and phenolic compounds, which have inhibitory effect on growth of microorganisms in further fermentation steps (Chandel *et al*., 2007b). Hence detoxification has to be carried out for removal or decreasing the concentration of inhibitors, several detoxification methods such as overliming. Several neutralization, Ion exchange resin treatment, activated charcoal adsorption were used, prior to the fermentation to remove the inhibitors and improve the efficiency of hydrolysate (Palmqvist and Hahn-Hagerdal , 2000). Overliming and neutralization was considered to be efficient for detoxification of lignocellulosic hydrolysates (Chandel et al., 2007b). By over liming with Ca $(OH)_2$/CaO, the fermentability of hemicellulosic hydrolysate was enhanced and formation of furfuryl acid from furfural was inhibited because of the removal of acid components like acetic acid and tannic acid (Perego *et al*., 1990). Activated charcoal treatment is an efficient method generally used for reducing the phenolic compounds, acetic acid, furfural and hydroxymethyl furfural found in hemicellulosic hydrolysates (Carvalheiro *et al*., 2005, Mussatto and Roberto, 2001). Activated charcoal being hydrophobic in nature removes the hydrophobic inhibitory compounds i.e., furan and phenolics more effectively.

MATERIALS AND METHODS

Raw material

Fresh water hyacinth (*Eichhornia crassipes*) was collected from natural pond in Hyderabad, Andhra Pradesh, India was thoroughly washed several times under tap water, to remove adhering dirt, and then the plant was cut into pieces of size 1-2 cm (approx), sundried and grounded to particles of size 2 mm (approx) and finally dried in a hot air oven at 65°C. The dried material was further grinded and sieved to 350µm size and stored at room temperature until used (Kalhorinia *et al*., 2013).

Analysis of chemical composition

The cellulose, lignin and hemicellulose fraction of water hyacinth (*Eichhornia crassipes*) were analyzed using standard TAPPI methods (1992).

Acid hydrolysis of water hyacinth

Five grams of powdered and dried water hyacinth (*Eichhornia crassipes*) at 1:10 (solid: liquid) ratio was taken in 250 ml Erlenmeyer flask and was subjected to dilute acid hydrolysis with 2% (v/v) H_2SO_4 at 121 °C for 60 min. Later the hydrolysate was filtered with muslin cloth, neutralized with 1N NaOH and then the total reducing sugars in the filtrate were estimated (Kalhorinia *et al*., 2013).

Concentration of hydrolysate

To increase the amount of sugars in hydrolysate of water hyacinth, concentration of the hydrolysate was carried out by vacuum using rotavapor, the temperature was maintained at 80^0C using vacuum. Concentration and fermentability

of the hydrolysate was assessed in accordance to the fermentation experiments by Dehkhoda *et al.,* (2008). Sugars, Phenolics and furans were checked before and after the concentration process.

Detoxification of acid hydrolysate

After vacuum distillation, the hydrolysate was added with calcium oxide with stirring, until the pH of the hydrolysate reaches 10.0. Then it was incubated for half an hour followed by centrifugation (3000g, 20 min) and filtration. Later the pH of the hydrolysate was brought back to pH 6 using concentrated H_2SO_4 (Chandel *et al.,*2007b). After overliming, 3.5% (w/v) of activated charcoal was added to the hydrolysates and stirred for 1 h. The mixture was again centrifuged (3000g, 20 min) and vacuum filtered (Martinez *et al.,* 2000). Sugars, phenolics and furans were estimated before and after detoxification process. The treated hydrolysate was then used for the fermentation studies.

Microorganism and maintenance

Candida tropicalis Y-27405 was procured from, The Agricultural Research Service ARS Culture Collection center (NRRL) USA. The stock culture was maintained on Yeast extract, Peptone, Xylose (YPX) agar slants containing (g/l) yeast extract, 10 ; peptone, 20 ; Xylose, 30 gL^1; and agar, 25 , pH: 5.0 and stored at 4^0 C.

Seed culture

The seed culture for xylitol fermentation was prepared by inoculating loopful of *C .tropicalis* from YPX slants in to 25ml of sterile media in 100ml Erlenmeyer flasks, consisting of 20 g/ lof Xylose, 20 g/l of peptone and 10 g/l of yeast. Inoculum was grown aerobically at 30°C on a rotary shaker at 150 rpm for 24 h.

Xylitol fermentation by *Candida tropicalis* Y-27405

Shake flask fermentation was carried out by supplementing the concentrated detoxified hydrolysate with (g/l) Yeast, 2.5; Peptone, 2.5; KH_2PO_4, 1.0; $MgSO_4$, 1.5; NH_4Cl, 0.5; $ZnSO_4$, $CaCl_2$, 0.5. Further the media was adjusted to5.0 pH and autoclaved at 110°C for 20 min. After cooling, the fermentation media was added with seed culture and incubated in an orbital shaker maintained at 30°C, 150 rpm for 96h. Sampling was done after every 12h intervals of fermentation. The collected fermented broth was centrifuged at 5000 rpm at 4°C for 10 min and the supernatant was filtered with 0.02µm filter and analyzed by HPLC for sugars and xylitol.

Analytical methods Total reducing sugars

The total reducing sugars, released after acid hydrolysis were estimated by DNS method (Miller, 1959).

Fermentation inhibitors

The fermentation inhibitors (i.e. furans and phenolics) were analyzed by spectroscopic analysis. Phenolics estimation was carried out by Folin ciocalteus method (Singleton and Rossi, 1965), and furans by Martinez *et al.,* (2000).

Dry cell mass determination

For dry cell mass determination, 10 ml of culture samples were filtered, washed and dried to a constant mass at 104°C (Yadav KS *et al.,* 2011)

Xylitol estimation

Xylitol and the sugars present in the fermented broth were estimated by high performance liquid chromatography (HPLC) fitted with Repromer Ca^{++} column (USP-L19) (9 µm. 300 x 8 mm) (Dr. Maisch GmbH, Germany). The samples were eluted with HPLC grade water at a flow rate of 0.6 ml/min at 75°C and detected with a differential refractometer (RID).

Statistical analysis

All the experiments in this study were carried out in triplicates. To assess whether there was any significant difference among the mean values of sugars, phenolics and furan derivatives before and after concentration and detoxification of hydrolysate, t-test was performed.

RESULTS AND DISCUSSION

Chemical composition of water hyacinth

When chemical composition of the dried powder of water hyacinth was determined using TAPPI method. It was found to contain 31.6±0.4% cellulose, 33.4±0.4% hemicellulose and 9.30±0.9 % lignin. The cellulose and hemicellulose together make the total holocellulose content of 65±1.6%. The cellulose and hemicelluloses content of water hyacinth reported by other researchers are compared with our results (Table 1). The results indicates that the composition of the water hyacinth varies with the growing environment.

Acid hydrolysis of water hyacinth

When acid hydrolysis of powdered and dried 5g of water hyacinth (*Eichhornia crassipes*)

was carried out at optimized conditions of 2% (v/v) H_2SO_4, 1:10 (solid: liquid) ratio at temperature 121°C for 60 min the amount of total reducing sugar released were estimated to be 28.4±1.2 g/l. Our results are in accordance with Masahiro et al (2013) who obtained 31 g l^{-1} xylose by acid hydrolysis with 2% H_2SO_4 at 121°C for 1 h from the culm of *Sasa kurilensis*. However in contrast to our result Misra et al (2013) obtained maximum xylose of 21.98 g/l from corncob, using only 1.0% (v/v) H_2SO_4, a solid to liquid ratio of 1:8 (w/v) at 121 °C for treatment time of 30 min.

Concentration of water hyacinth acid hydrolysate for xylitol production

Winkelhausen and Kuzmanova (1998) observed that lower substrate concentration inhibits both the productivity and yield of the fermentation. Hence in the present study water hyacinth hemicellulosic hydrolysate was concentrated at low temperature; under vacuum by using rotavapor to increase the fermentable sugars from 28±0.34 g/l to 52.5±0.25 g/l (Fig. 1). By concentration of hydrolysate furfural concentration reached 440±22mg/l from 300±15mg/l (Fig. 2). phenolics concentration reached to 1000±50mg/l from

Table 1. Chemical composition of water hyacinth reported by other researchers.

S.No	Cellulose	Hemicellulose	Lignin	Product	Reference
1	18	33.39	26.36	Biogas	Chanakya et al.,1993
2	17.8	43.4	7.8	Biogas	Patel *et al*.,1993
3	35	18.3	1.9	Biofuels	Abraham and Kurup,1996
4	18.4	49.2	3.55	Bioethanol	Ashish Kumar *et al*., 2009
5	34.19	17.66	12.22	Bioethanol	Deuk Joo Ahn. *et al*.,2012
6	31.6±1.3	33.4±0.8	9.30±0.9	D-xylitol	This study

Table 2. Statistical evaluation of Sugars, Phenolics and Furans before and after concentration

	Paired Differences							
			95% Confidence Interval of the Difference					
	Mean	Std. Deviation	Std. Error Mean	Lower	Upper	t	df	Sig. 2-tailed)
Pair 1	23.83333	3.60651	2.08222	14.87426	32.79241	11.446	2	.008
Pair 2	400.00000	113.60000	65.58699	117.80196	682.19804	6.099	2	.026
Pair 3	110.00000	30.92000	17.85167	-186.80954	-33.19046	-6.162	2	.025

Pair1: Sugars before Concentration: Sugars after Concentration
Pair2: Phenolics before Concentration: phenolics after Concentration
Pair3: Furans before Concentration: Furans after Concentration.

Table 3. Statistical evaluation of Sugars, Phenolics and Furans before and after detoxification

	Paired Differences							
			95% Confidence Interval of the Difference					
	Mean	Std. Deviation	Std. Error Mean	Lower	Upper	t	df	Sig. 2-tailed)
Pair 1	2.35333	3.85500	2.22569	-7.22302	11.92969	1.057	2	.401
Pair 2	960.00000	75.32000	43.48602	772.89475	1147.10525	22.076	2	.002
Pair 3	375.00000	52.80000	30.48409	243.83753	506.16247	12.301	2	.007

Pair1: Sugars before Concentration: Sugars after Concentration
Pair2: Phenolics before Concentration: phenolics after Concentration
Pair3: Furans before Concentration: Furans after Concentration.

Table 4. Fermentation parameters of Candida *tropicalis* Y-27405 using concentrated and detoxified water hyacinth acid hydrolysate containing 50.0±0.30g/L total sugars

Parameters	Candida *tropicalis* Y-27405
initial sugar (g_s/l^{-1})	50±30
sugar (g_s/l^{-1})	10.5±0.47
Xylitol(g_p/l^{-1})	32.5±1.46
Biomass(g_x/l^{-1})	7.2±0.32
Sugar Consumed (%)	79±3.52
Xylitol Yield(g_p/g_s^{-1})	0.65±0.03
Volumetric Xylitol productivity(g/l/h)	0.67±0.03
Volumetric cell mass productivity (g/l/h)	0.15±0.0067
Volumetric sugar uptake rate (g/l/h)	1.64±0.071
Fermentation Efficiency % [R]	70.8±3.1

[R] the theoretical value (0.917)(Maria F. S. Barbosa *et al*.,1988)

initial concentration of 1600±80mg/l (Fig. 3) and concentration of fermentable sugars has shown a 2-fold increase of fermentable sugars indicating that vacuum distillation has not decomposed the carbohydrates (Dehkhoda *et al*., 2008).

Detoxification of concentrated water hyacinth acid hydrolysate

To reduce the effect of inhibitors produced during the acid hydrolysis, over liming, and activated charcoal treatments were used which improved the bioconversion of the sugars into xylitol. The acid hydrolysate when treated with calcium oxide and activated charcoal brought about maximum reduction in furans from 440±22mg/l to 88±4.4mg/l (80% removal) and total phenolics from 1000±50mg/l to 200±10mg/l (80% removal) (Figs. 1-3) however, sugar concentration reduced to 50.0±0.30g/l from the initial concentration of 52.5±0.25 g/l (4.8% loss) during detoxification process. In contrast to the present study, Ge et al (2011) reported 18.3% loss of xylose. However they reported complete removal of furfural and 96.6 % removal of phenolics by overliming and charcoal treatment of corncob hydrolysate. Chandel *et al.,* (2011) reported that detoxification using an overliming process for sugarcane bagasse hydrolysate resulted in reduction of inhibitory compounds, phenolics (33.21%) and furfurals (41.75%) along with reduction of reducing sugars by 7.61%. similar trends of decrease in inhibitors present in acid hydrolysate by overliming, 51 ± 9% reduction of total furans, a 41 ± 6% reduction in phenolic compounds, and a 8.7 ± 4.5% decline in sugar was reported by Martinez *et al*., (2001). Treatment of hydrolysate with activated charcoal caused 38.7% and 57.5% reduction in furans and total phenolics, respectively (Chandel et al., 2007a). Rodrigues *et al.,* (2011) observed nearly 5% loss of xylose during the overliming of DEO hydrolysate of corn stover using Ca $(OH)_2$ which is similar to the result in the current study.

Statistical evaluation of concentration and detoxification of water hyacinth acid hydrolysate

To find the significant differences between the mean values of sugars, phenols and furans before and after concentration and detoxification t-test was performed. The results showed that in concentration part all the pair of treatments are highly significant and the t values of all the pair of treatments are positive (p<0.05) (Table 2).

To assess whether there is any significant differences among the mean values before and after detoxification, paired sample t-test was performed. There was a significant difference between the two pair of treatments i.e. phenolics and furans before detoxification and after detoxification (sig=0.002 and 0.007; p<0.05) in compare to the treatments between sugars which do not show significant difference (sig=0.401) before and after detoxification (Table 3).

Xylitol fermentation

Batch fermentation of 50g/l of sugars present in concentrated detoxified water hyacinth hemicellulosic hydrolysate, using 15% (v/v) *Candida tropicalis* Y-27405, produced maximum

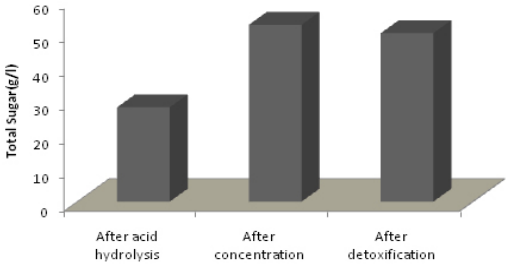

Fig. 1. Amount of Sugars in hydrolysate after acid hydrolysis, concentration and detoxification

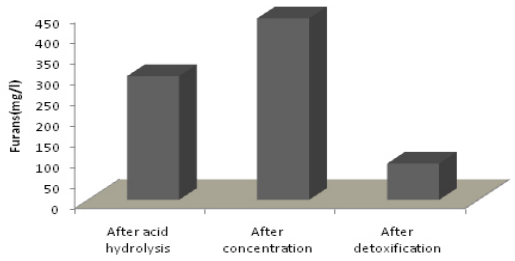

Fig. 2. Amount of furans in hydrolysate after acid hydrolysis, concentration and detoxification

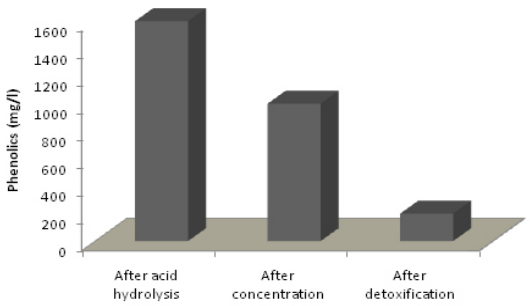

Fig. 3. Amount of phenolics in hydrolysate after acid hydrolysis, concentration and detoxification

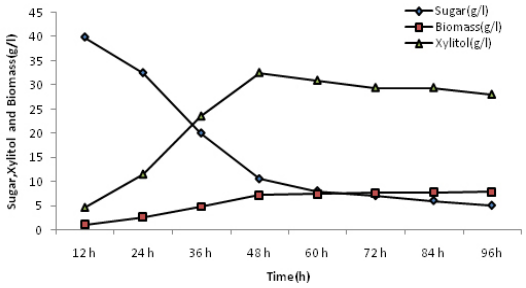

Fig. 4. The time course of growth, sugar utilization and xylitol production by *Candida tropicalis* Y-27405

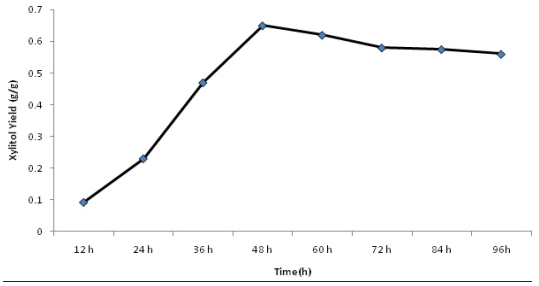

Fig. 5. Xylitol yield from concentrated detoxified hydrolysate by *Candida tropicalis* Y-27405

xylitol concentration of 32.5g/l at 48h (Fig. 4) with yield of 0.65g xylitol /g xylose (Fig.5) and productivity of 0.67g/l/h, respectively (Table 4). When xylitol production from water hyacinth in present study was compared with xylitol produced from other lignocellulosic substrates, our results are in close agreement with Masahiro et al (2013) who obtained xylitol yield (0.62 g xylitol / g xylose) at the OTR of 1.2 mmol-O_2 l^{-1} h^{-1} from the steam activated charcoal detoxified hydrolysate of culm of *Sasa kurilensis* using *Candida magnolia.* Our results are slightly more than Rodrigues et al (2011), who obtained xylitol yield of 0.61 g xylitol/g xylose with *Pichia stipitis* YS-30 by $Ca(OH)_2$ overliming and neutralization of DEO hydrolysate of corn stover with phosphoric acid. The xylitol concentration and yield reported in this study 32.5g/l &0.65g/g respectively is higher than 22.63 g/l & 0.57 g/g respectively reported by Misra et al (2013) from 40.16 g/l of xylose concentrated corncob hydrolysate in 48 h using *C. tropicalis*. Using thermotolerant *D. hansenii,* Prakasham et al (2011) reported, xylitol yield of 0.69 g/g from 20 g/l initial xylose concentration present in activated charcoal detoxified sugarcane baggasse hemicellulosic hydrolysate.

CONCLUSION

In the present study, water hyacinth (*Eichornia crassipes*), a fast growing, aquatic lignocellulosic weed was exploited for the production of xylitol using *Candida tropicalis* Y-27405 strain. When compared to the production of xylitol from other lignocellulosic substrates, water hyacinth was found to be a competent and promising substrate with a xylitol production

of 32.5g/l and a yield of 0.65g xylitol/g xylose, from 50g/l of acid pretreated, concentrated and detoxified hydrolysate. Hence water hyacinth can be used as potential lignocellulosic biomass for the production of xylitol and can contribute significantly to the future increasing food demands of the society.

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