BIOCONVERSION OF WATER HYACINTH HYDROLYSATE INTO ETHANOL

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The fast growing aquatic weed water hyacinth, which is available almost year-round in the tropics and subtropics, was utilized as the chief source of cellulose for production of fuel ethanol via enzymatic hydrolysis and fermentation. Fungal cellulases produced on-site by utilizing acid-alkali pretreated water hyacinth as the substrate were used as the crude enzyme source for hydrolysis of identically pretreated biomass. Four different modes of enzymatic hydrolysis and fermentation were trialed in the present study for optimization of the yield of ethanol. Two common yeasts viz., *Saccharomyces cerevisiae* and *Pachysolen tannophilus*, were used for fermentation of hexose and pentose sugars in the hydrolysate. Significant enhancement of concentration (8.3 g/L) and yield (0.21 g/g) of ethanol was obtained through a prefermentation hydrolysis-simultaneous saccharification and fermentation (PH-SSF) process, over the other three processes viz., separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and single batch bioconversion (SBB) by utilizing fungal culture broth with and without filtration as crude enzyme source.

Key words: Aquatic weed; Water hyacinth; Enzymatic hydrolysis; Hydrolysate; Bioconversion; Fermentation; Ethanol

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INTRODUCTION

In view of alarming environmental consequences arising out of fossil fuel combustion and concerns about petroleum supply and escalating prices of crude oil, the research for renewable bio-fuels has been stepped up sharply in the last decade. Bio-ethanol from cellulosic biomass (lignocellulosic wastes) has attracted much attention as a plausible alternative to petroleum-based transport fuels due mainly to its low cost share of raw material. Biomass having less competition with food crops would be realistically more appropriate for bio-ethanol production. As such, lignocelluloses as agricultural, industrial and forest residues or weeds have been identified as potential alternative, sustainable sources of bio-fuel and other value–added products.

Water hyacinth [*Eichhornia crassipes* (Mart.) Solms], a noxious aquatic weed has ever been creating menace in many tropical and sub-tropical freshwater habitats due to its faster growth rate than any other vascular macrophytes. This nuisance aquatic weed detrimentally affects commerce by way of clogging irrigation channels, vitiating commercial fishing, causing economic hardship, and seriously interfering with recreation. The vast quantity of weed biomass can be disposed off in an eco-friendly way through its utilization as a cheap feedstock in an innovative process of bioconversion into fuel ethanol. The conversion of cellulose from lignocellulosics to ethanol is more challenging than conversion of soluble carbohydrates from food crops. In general, the former process involves two steps: (a) conversion of cellulose to fermentable sugar monomers by either acid/alkali hydrolysis or by enzymatic hydrolysis, and (b) microbial fermentation of the

resulting sugars to ethanol (Asenjo et al. 1991). Abundantly available biomass of the undesirable weed water hyacinth was proven as an acceptable candidate for bio-ethanol production in previous studies which adopted either separate hydrolysis and fermentation (SHF) processes (Abraham and Kurup 1996; Aswathy et al. 2009; Kahlon and Kumar 1987; Nigam 2002) or simultaneous saccharification and fermentation (SSF) processes (Mishima et al. 2008). As the high cost of commercial enzyme is the major bottleneck in the enzymatic hydrolysis of cellulose to sugar, some of the studies have utilized on-site-produced enzyme. Our laboratory had previously optimized a simple pretreatment method for obtaining cellulose-rich water hyacinth, a cost-effective medium for cellulolytic enzyme production mainly by *Trichoderma reesei* (Mukhopadhyay and Nandi 2001) and the hydrolysis of pretreated water hyacinth with crude enzyme (Mukhopadhyay et al. 2008).

In the present study, four different modes of enzymatic hydrolysis and fermentation process were given trial to assess their viability for production of fuel ethanol in a cost effective manner through utilization of pretreated water hyacinth hydrolysate as the sole carbon source for yeast fermentation. The efficiency of the processes was compared in terms of yield of ethanol (g/g biomass) per unit biomass used for its production.

EXPERIMENTAL

Substrate and Pretreatment

Water hyacinth (*Eichhornia crassipes*) was collected from local ponds and lakes. It was sundried, and roots were discarded, as they have been reported to absorb heavy metal pollutants from water bodies (Dos Santos and Lenzi 2000). Weed plants (without roots) were then pretreated successively with 0.1 N H_2SO_4 and 1% (w/v) NaOH, as described previously (Mukhopadhyay and Nandi 2001). Pretreated water hyacinth washed to neutrality, oven-dried to a constant weight, and then milled to powder, was used separately as substrate both for cellulase production as well as for enzymatic hydrolysis. The composition, in terms of cellulose, hemicellulose, and lignin, in normal and pretreated water hyacinth (PWH) biomass were determined previously (Mukhopadhyay et al. 2008).

Enzyme Production and Enzymatic Hydrolysis

For cellulase and β-glucosidase production, *Trichoderma reesei* ATCC 26921 and *Aspergillus phoenicis* ATCC 52007 were obtained by courtesy of the U.S. Department of Agriculture, Peoria, Illinois. Enzymes were produced by growing the fungi separately for 8 days at 31±1°C in a simple liquid medium (4.2 g/L $(NH_4)_2SO_4$, 2 g/L KH_2PO_4, 0.05 g/L yeast extract, 2ml/L Tween-80, 2% (w/v) poultry manure containing 1.6 % total N, pH 4.8) containing 40g/L PWH biomass as the chief C source after previous optimization (Mukhopadhyay and Nandi 2001). *Aspergillus phoenicis* ATCC 52007, a β-glucosidase mutant, was used for obtaining a β-glucosidase-rich enzyme source. The crude culture supernatants separately from *T. reesei* and *A. phoenicis* were blended in such a way as to have an enzyme source with FPase and β-glucosidase in the ratio of 1 : 1.2. Enzymatic hydrolysis of PWH was carried out by incubating presterilized biomass (40 g/L, pH 5.0) with filter (G_4 Sintered glass) sterilized crude enzyme blend of *T. reesei* and *A. phoenicis* at 45°C and for 72 h with agitation at 150 rpm, as optimized previously (Mukhopadhyay *et al*., 2008). Samples were aliquoted separately from fungal culture broths after 8 days incubation for enzyme production and from hydrolysates of PWH after its 24 h partial hydrolysis and 72 h hydrolysis with crude enzyme blend for

their GLC analysis. The sugar monomers (glucose, xylose, arabinose, mannose, and galactose) released by enzymatic hydrolysis of PWH were determined and quantified by a Hewlett-Packard 5890 series ll gas chromatograph equipped with glass columns (1.83 m x 2 mm) containing 3% SP- 240 Supelcoport (100-200 mesh) and a flame ionization detector (FID). A recording integrator HP 3396 A was used to determine peak areas. The injector and detector were kept at 220°C and 250°C, respectively, and the column oven was operated isothermally at 200°C (for alditol acetates of neutral sugars). Nitrogen was used as a carrier gas at a flow rate of 27.3 ml/min.

Yeasts for Fermentation

A glucose fermenting yeast, *Saccharomyces cerevisiae* MTCC 171 and a glucose and xylose fermenting yeast, *Pachysolen tannophilus* MTCC 1077 were procured from Institute of Microbial Technology, Chandigarh, India.

The stock cultures were maintained on Malt extract-Yeast extract-Glucose-Peptone (MYGP) agar (3 g/L malt extract, 3 g /L yeast extract, 15 g/L glucose, 10 g /L peptone, and 20 g/L agar, pH 6.0) slants and stored at 4°C.

Preparation of Yeast Inoculum

Yeasts from agar slants were suspended aseptically in 100 ml liquid MYGP medium (pH 5.0) and incubated at 30 ± 1°C for 24 h and at 25 ± 1°C for 48 h, respectively, for *S. cerevisiae* and *P. tannophilus*, with agitation at 150 rpm. These suspension cultures of yeasts were then used as inocula for fermentation.

Ethanol Production from Pretreated Water Hyacinth

Four different modes of enzymatic hydrolysis (saccharification) and fermentation processes with or without prefermentation hydrolysis were trialed to optimize the process for better production of ethanol from pretreated biomass (PWH).

Separate hydrolysis and fermentation (SHF)

The hydrolysate of PWH obtained after 72 h enzymatic hydrolysis was centrifuged, and the supernatant after filter (G_4 Sintered glass) sterilization was used as the sole C source in fermentation medium. Nutrients of yeast fermentation (YF) medium (without C source) were added to the supernatant to have a basal medium (pH 5.0) composition of 5.0 g/L yeast extract, 5.0 g/L peptone, 5 g/L NH_4PO_4, 0.2 g/L $MgSO_4$, and 7 H_2O. Suspension (2% v/v) of *S. cerevisiae* (10^8 cells/ml) was then inoculated to the broth to initiate the fermentation process and incubated at 30°C for 48 h with agitation at 150 rpm for the first 24 h. The broth was then reinoculated with suspension (3% v/v) of *P. tannophilus* (6 x 10^7 cells/ml) and incubated further at 25°C for another 48 h (Fig.1) without agitation, as optimized previously (Mukhopadhyay and Mukherjee 2008).

Simultaneous saccharification and fermentation (SSF)

The SSF reaction mixture consisted of a previously sterilized PWH (40g/L), blend of crude culture supernatants of *T. reesei* and *A. phoenicis* (pH 5.0), basal YF medium, and 2% (v/v) inoculum of *S. cerevisiae* to give the same concentration as that of the above SHF experiment. The reaction of SSF was carried out at 37°C for 72 h in order to undergo both hydrolysis (saccharification) and fermentation simultaneously. Inoculum (3% v/v) of *P. tannophilus* was then added to the SSF mixture and incubated at a temperature of 25°C for another 48 h, keeping other conditions identical (Fig.2) as followed in SHF.

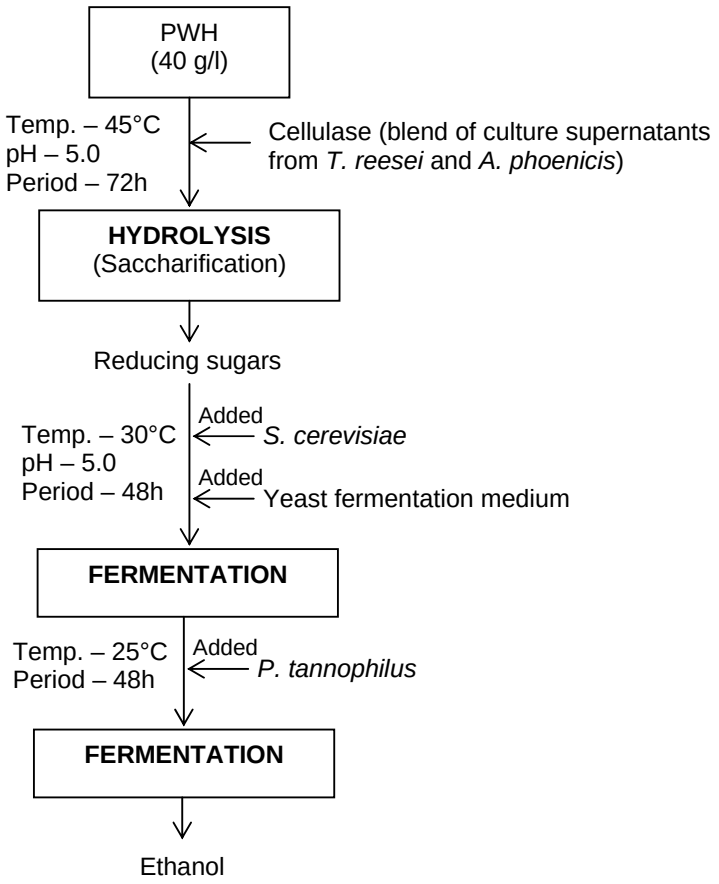

Fig. 1. Schematic representation of separate hydrolysis and fermentation (SHF) of pretreated water hyacinth (PWH) biomass

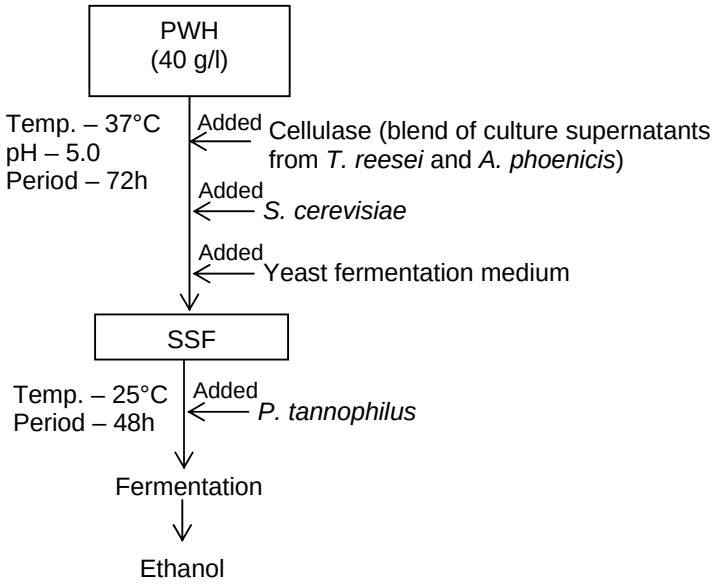

Fig. 2. Schematic representation of simultaneous saccharification and fermentation (SSF) of pretreated water hyacinth (PWH) biomass

Single-batch bioconversion (SBB)

In this mode, the entire fungal culture broth was heat-inactivated after 8 days of enzyme production, then cooled to 30°C and enriched with basal YF medium to allow fermentation to run in this broth for a total period of 96 h by successive inoculation with *S. cerevisiae* and with *P. tannophilus*, respectively, at a 48 h interval (Fig. 3), keeping other conditions identical as in SHF.

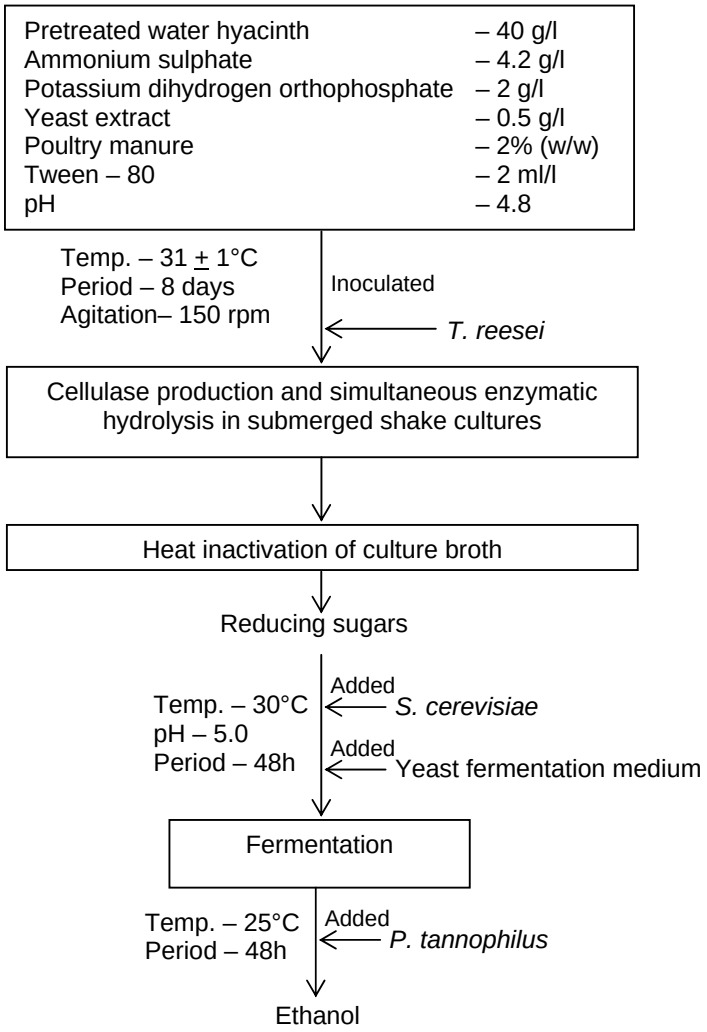

Fig. 3. Schematic representation of single-batch bioconversion (SBB) of pretreated water hyacinth (PWH) biomass

Prefermentation hydrolysis-simultaneous saccharification and fermentation (PH-SSF)

In this process the presterilized PWH (40g/L) was first partially hydrolysed at 45°C with a blend of culture supernatants as in SHF, but only for 24 h instead of 72 h duration (Fig.4). To this, was further added whole culture (used for enzyme production) broths of *T. reesei* and *A. phoenicis* (without filtration), together with 2% (v/v) suspension of *S. cerevisiae* and basal YF medium. Saccharification (hydrolysis) and fermentation were then allowed to take place simultaneously (SSF) for 48 h at 37°C in this fermentation broth, which contained 40 g/L PWH on an average. The temperature

was then adjusted to 25°C to continue fermentation for another 48 h by reinocubating with 3% (v/v) *P. tannophilus*.

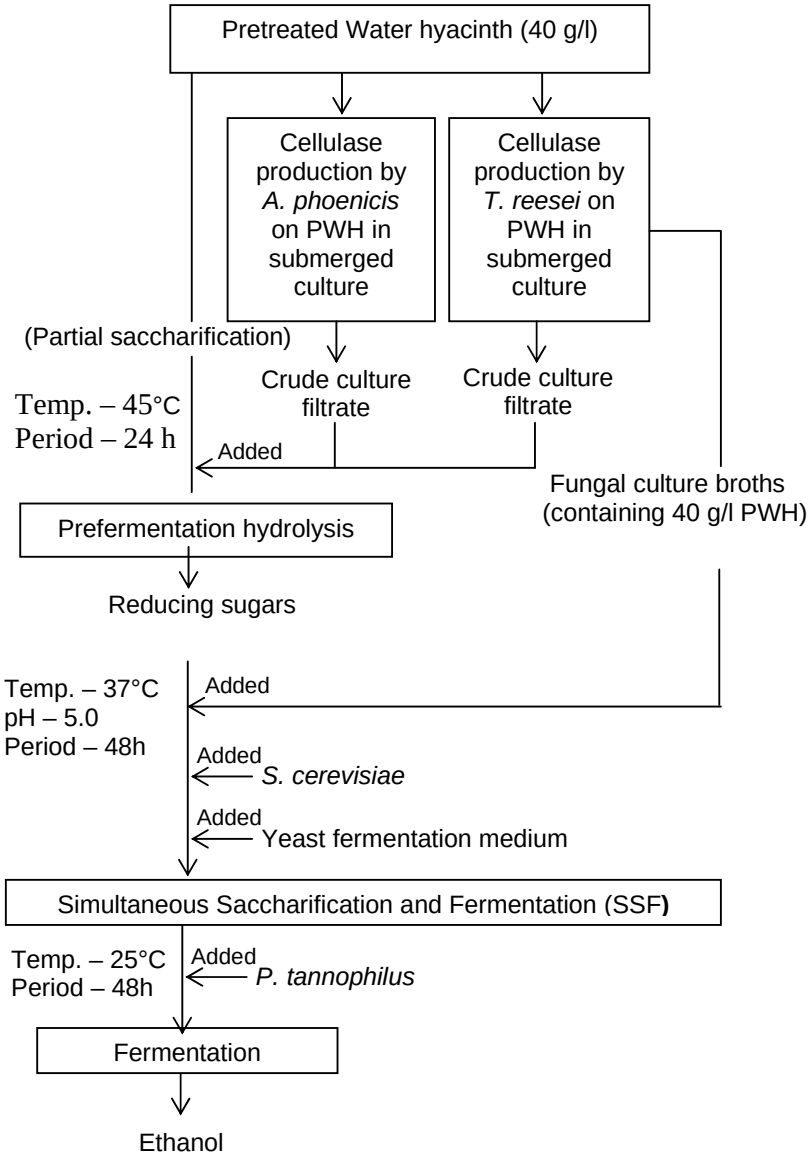

Fig. 4. Schematic representation of prefermentation hydrolysis – simultaneous saccharification and fermentation (PH-SSF) of pretreated water hyacinth (PWH) biomass

Estimation of Ethanol Concentration

At the end of each mode of fermentation, the reaction broth was subjected to fractional distillation at 78 to 79 ^{o}C to collect ethanolic solution. The concentration of ethanol was then determined spectrophotometrically by chemical oxidation with acidic dichromate solution, following mainly the method of Caputi et al. (1968), and expressed in g/L. The concentration of substrate (PWH) was maintained identical at 40 g/L throughout the experiments, both for enzyme production as well as for enzymatic hydrolysis in all four modes, as such, and the yield of ethanol per unit biomass was calculated from the total substrate loading (40 g/L PWH) used in each process and

expressed as g/g PWH. All the experiments were performed in triplicate, and the results were presented as means ± standard deviation. The significant difference (at 5% level) among the fermentation modes was determined by one way analysis of variance (ANOVA), using Microsoft Excel.

RESULTS AND DISCUSSION

Enzyme Production and Enzymatic Hydrolysis

Water hyacinth (without root) successively pretreated with acid (0.1 N H_2SO_4) and alkali (1% w/v NaOH) was used in identical concentration (approximately equivalent to 1 % cellulose) as source of cellulose both for inducing production of cellulolytic enzymes by fungi as well as for enzymatic hydrolysis. Pretreatment of water hyacinth was found to increase its cellulose content from 37.8 % to 67.8 % of dry weight in our previous investigation (Mukhopadhyay *et al*. 2008). The cellulose and residual hemicellulose in pretreated water hyacinth (PWH) were enzymatically hydrolysed to fermentable oligosaccharides and monosaccharides with crude fungal enzymes produced on-site in liquid state fermentation. Due to poor yield of extramycelial β-glucosidase (degrading cellobiose to glucose), the enzyme preparation from *T. reesei* was supplemented with enzyme from *A. phoenicis*, a β-glucosidase mutant compatible with *T. reesei* (Awafo et al. 1996). The enzyme blend of these two fungal cultures was used as crude for hydrolysis of pretreated water hyacinth in an effort to simplify the process. GLC analyses revealed (Table 1) that both the polysaccharides (cellulose and hemicellulose) of the PWH were hydrolysed to produce sugar monomers (glucose, xylose, arabinose, mannose, and galactose) in the fungal culture broth used as enzyme source as well as in the PWH hydrolysates after 24 h and 72 h enzymatic hydrolysis.

Table 1. Sugar Compositions of Fungal Culture Broths and of PWH Hydrolysates after Enzymatic Hydrolysis for 24 h and 72 h

Sugars	Sugar concentration (g/L)*			
	Fungal culture broths		PWH hydrolysates after enzymatic hydrolysis	
	T. reesei	*A. phoenicis*	for 24 h	for 72 h
Glucose	10.4 ± 1.05	2.1 ± 0.6	3.8 ± 0.3	12 ± 0.5
Xylose	2.0 ± 0.5	0.3 ± 0.25	1.8 ± 0.15	3.2 ± 0.15
Arabinose	0.81 ± 0.1	0.07 ± 0.15	1.2 ± 0.5	2.05 ± 0.15
Mannose	0.97 ± 0.1	0.05 ± 0.15	0.15 ± 0.02	0.57 ± 0.19
Galactose	0.36 ± 0.15	0.0	0.12 ± 0.02	0.46 ± 0.04
Total sugars**	14.54	2.52	7.07	18.28

* Mean ± Standard deviation
** Glucose + Xylose + Arabinose + Mannose + Galactose

Ethanol Production from Pretreated Water Hyacinth

The common yeast *S. cerevisiae* was employed for fermentation of hexose sugars in the hydrolysate. Since xylose was also present as the second predominant sugar, *P. tannophilus* was used in addition to make the biomass-to-ethanol process more economical. In our previous study, the maximum fermentation with these two yeasts was optimized by incubation of the fermentation mixture with *S. cerevisiae* for 48 h at 30°C followed by *P. tannophilus* for another 48 h at 25°C, with agitation only for first 24 h

(Mukhopadhyay and Mukherjee 2008). After a thorough survey of literature on different processes of hydrolysis and fermentation (Abraham and Kurup 1997; Barron et al. 1995; Brookes and Ingram 1995; Motwani et al. 1996; Palmqvist et al. 1996; Zayed and Meyer 1995), four principal modes using crude enzyme produced on-site had been trialed to develop a simple and cost-effective process for better recovery of ethanol from water hyacinth in laboratory scale.

In the SHF process, the reaction broth after 72 h enzymatic hydrolysis of PWH showed a sugar concentration of 18.28 g/L (Table 1) before initiation of fermentation, which produced an average ethanol concentration of 4.5 g /L at the end of the process (Table 2). The yield of ethanol was, therefore, 0.25g/g sugars and 0.11 g/g PWH from a total biomass loading of 40 g/L PWH, whereas Abraham and Kurup (1997) obtained a yield of 0.11 g/g alkali pretreated water hyacinth through SHF.

Simultaneous saccharification and fermentation (SSF) represents a single step process in which fermentable sugars get released by enzymatic hydrolysis of PWH and are simultaneously exploited by yeasts for fermentation in the same medium. As such, sugars produced by hydrolysis would not be possible to monitor separately. An average ethanol concentration of 5.2 g/L was obtained in this process which corresponds to a yield of 0.13 g/g PWH (Table 2) on the basis of biomass**.** Mishima et al. (2008), on the other hand, reported an ethanol yield of 0.14 g/g dry substrate through SSF of pretreated water hyacinth using commercial cellulase and *S. cerevisiae*.

Single batch bioconversion (SBB) entails a variation of SHF in which fermentation is allowed after termination of enzyme production along with enzymatic hydrolysis of PWH in the same culture broth. The reducing sugars in this broth (Table 1) after fermentation produced ethanol at a concentration of 5g/L with a yield of 0.34g/g. On the basis of pretreated biomass (PWH) loading this yield was observed to be 0.13 g/g PWH (Table 2). Zayed and Meyer (1996) obtained an ethanol yield of 0.11g/g delignified wheat straw using an identical process of SBB.

In the PH-SSF process, partial hydrolysis of PWH for 24 h yielded reducing sugars of 7.07 g/L (Table 1), to which was further added sugars (17.06 g/L) from the fungal culture broths (Table 1). This constituted an average sugar concentration of 12 g/L in the fermentation broth, where two yeasts were inoculated successively. After 96 h fermentation, an average ethanol concentration of 8.3 g/L with an yield of 0.69g/g sugar was obtained from this broth. As the biomass concentration of fungal culture broth was 40 g/L, so, the average biomass loading of the fermentation broth was maintained at 40g/L, yielding thereby ethanol of 0.21 g/g PWH, which represented significant ($p<0.05$) enhancement over the above three processes (Table 2) trialed in the present study. This ethanol yield was found to be equivalent to 0.31 g/g cellulose in the pretreated water hyacinth. The PH-SSF mode of fermentation is clearly distinct from the other three processes wherein the entire fungal culture broth was added to partially hydrolysed PWH. In this way enzyme bound to lignocellulosic residue (PWH) could also become available for better saccharification to continue SSF more efficiently, as evidenced by Lee et al. (1995) and Wayman et al. (1992). Moreover, it enriched the partially hydrolyzed substrate (PWH) in fermentation medium with additional fermentable sugars from the culture broth. Motwani et al. (1996) reported a yield of butandiol of 0.15 g/g pretreated water hyacinth by using commercial cellulase and *Klebsiella oxytoca* in a process of prefermentation hydrolysis-combined hydrolysis and fermentation (PH-CHF) which corresponds to our present process of PH-SSF. The yield of ethanol per unit biomass of pretreated water hyacinth obtained through PH-SSF in the present study was comparable to or even better than those reported earlier (Abraham and Kurup 1997; Aswathy et al. 2009; Motwani et al. 1996; Mishima et al.

2008). Moreover, unlike other studies, the present study used crude fungal enzyme (mainly cellulases) produced on site by utilising the identical biomass (PWH) as used for enzymatic hydrolysis. Besides cost-effectiveness, the use of the same lignocellulosic biomass maintained uniformity in the quality of the fermentable sugars both in the enzyme preparation and in the hydrolysate.

Table 2. Concentration (g/L) and Yield g/g PWH) of Ethanol Produced under Different Modes of Enzymatic Hydrolysis and Fermentation

Modes of fermentation	Concentration g/L*	Yield g/g dry substrate*
SHF	$4.5^{a} \pm 0.3$	$0.11^{a} \pm 0.007$
SSF	$5.2^{a} \pm 0.2$	$0.14^{a} \pm 0.007$
SBB	$5.0^{a} \pm 0.2$	$0.13^{a} \pm 0.006$
PH-SSF	$8.3^{b} \pm 0.5$	$0.21^{b} \pm 0.011$
CD at 5% level	1.72	0.045
*Results are mean of three replications ± SD. Different letter in the superscript denotes significant difference at 5% level.		

CONCLUSIONS

1. The yield of ethanol recovered from acid-alkali pretreated water hyacinth through enzymatic hydrolysis and fermentation of PH-SSF process was significantly higher than that recovered through SHF, SSF, and SBB processes.
2. The use of crude fungal cellulases produced on PWH in liquid state fermentation would be a cost-effective approach towards enzymatic hydrolysis of identical biomass of PWH instead of using commercial cellulases.
3. Fed-batch addition of fungal culture broth containing crude enzyme with fermentable sugars was found to be more effective for a better yield of ethanol.
4. The aquatic menace water hyacinth, which is currently being used in waste water treatment for its unique ability to absorb heavy metal pollutants, could also be utilized as abundant cheap feedstock for the production of fuel ethanol.

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