

Enhancement of Sugar Content of *Cyperus* Sp. through Cellulolytic Enzymes for Bioethanol Generation

Rashi Vishwakarma,^{a,*} and Rintu Banerjee^a

Cyperus (family: Cyperaceae), which contains a notable amount of carbohydrate, has a versatile nature, which makes easy to cultivate. The main objective of the present investigation was to evaluate the effect of enzymatic saccharification on *Cyperus* sp. for improved yield of reducing sugar and bioethanol production. The cellulosic residues after hydrolysis with cellulase from *Trichoderma reesei* Rut C30 yielded 456 mg/g of reducing sugars. A pH of 5, temperature 50 °C, solid to liquid ratio 1: 5, enzyme concentration 20 IU/ml and incubation time 6 h were found to be most effective for the saccharification of *Cyperus* sp. and saccharification efficiency of 74.2 % was achieved. The biomass conversion to yield ethanol after fermentation with *Saccharomyces cerevisiae* for 36 h was 6.49 %. The recirculation studies with laccase showed that while maintaining the enzyme activity, delignification was considerable to 4 cycles. This study highlights the use of *Cyperus* sp. as an alternative source of bioethanol.

Keywords: *Cyperus* sp.; Saccharification; Pretreatment; Reducing sugars

Contact information: a: PK Sinha Center for BioEnergy, IIT Kharagpur, West Bengal 721306, India;

*Corresponding author: v.rashi245@gmail.com

INTRODUCTION

The current high cost of lignocellulolytic enzymes is a major bottleneck in the economic bioconversion of lignocellulosic biomass to fuels and chemicals. The recent thrust in bioconversion of agricultural and industrial wastes to chemical feedstock has led to extensive studies on cellulolytic enzymes produced by fungi and bacteria. A lot of emphasis had been given to screening of the agricultural wastes for release of sugars produced by hydrolysis of lignocellulosics. Vlasenko et al. (1993) investigated 30 potential cellulosic raw materials for saccharification by *Penicillium* cellulases. It was found that several factors affect the enzymatic hydrolysis rate, such as substrate concentration, cellulase activity, and reaction conditions (temperature, pH, as well as incubation time).

Cellulose is a potentially valuable resource for fibre, fuel and feed. Investigations into ability of microbes to degrade native and modified cellulose so far have revealed that only a few fungi possess ability to degrade native cellulose. Fungal lignocellulolytic enzyme systems are secreted at high levels, making them the most promising starting points for further development of highly efficient lignocellulolytic enzyme systems (Liu et al., 2013). Pretreatment of lignocellulose biomass is crucial for achieving effective hydrolysis of substrates as enzymatic hydrolysis of native lignocelluloses produces less

than 20% glucose from the cellulose fraction (Zhang and Lynd, 2004). The selection of a pretreatment method has an impact on the subsequent enzymatic hydrolysis (Merino and Cherry, 2007; Romani et al., 2010). Varnai et al. (2010) examined how pretreatments change the structure of the lignin and concluded that complete delignification did not seem essential to achieve hydrolysis. Some pretreatments simply change the location of the lignin which can enhance the hydrolysis without the removal of the lignin (Chandra et al., 2007; Varnai et al., 2010).

Waste biomass and plant sources that are not used for food purpose are especially suitable alternative sources for bioethanol generation. *Cyperus* (family: Cyperaceae) was used in this study for production of bioethanol. It is generally found in tropical and subtropical region and considered as most troublesome weed which can be found anywhere at the bank of water bodies, in the field with crops plant, and its versatile nature makes it easy to cultivate. The major focus of this work is to study the various factors which influence the saccharification process, thus enhancing the yield of reducing sugars for superior ethanol yield. It also presents the relatively unexplored *Cyperus* sp. of grass as an alternative source for production of bioethanol.

EXPERIMENTAL

Materials

Substrate

Grass was collected locally from local fields of Kharagpur, India. The vegetative part was air dried and crushed to powder.

Biochemical composition analysis of grass

Lignin content was measured by the titrametric method (Hussain et al., 2002) and cellulose content was estimated by “semimicro determination of cellulose” (Updegroff, 1969) method. All experiments were done in triplicates.

Enzymes

Hyperactive laccase enzyme was produced from the *Pleurotus* sp (Bhattacharya and Banerjee, 2008) and cellulase was produced from *Trichoderma reesei* RUT C30 (Das et al., 2008). Crude enzyme extract was used for all the experiments.

Methods

Enzymatic pretreatment, saccharification and recycling of laccase

Enzymatic pretreatment of *Cyperus* sp. was performed in Erlenmeyer flasks containing substrate and required volume of laccase. Samples were withdrawn periodically and the solid residues were used for lignin estimation. Recirculation studies upto 4 cycles were studied. Fresh enzyme was added to the recovered enzyme while maintaining the enzyme volume and activity.

Delignified substrate was washed with distilled water and then air dried overnight at 80 °C and was subsequently used for saccharification. Individual sets of experiments were placed to study the effect of enzyme concentration, solid to liquid ratio, temperature, incubation time and pH. Sample aliquots were taken periodically and centrifuged, and the supernatants were analyzed for reducing sugar.

The saccharification efficiency was calculated as follows:

Saccharification efficiency = (reducing sugars × 0.9 × 100)/ total sugars present in the pretreated substrate.

Assay of enzymes and Effects of various parameters on reducing sugar content

Cellulase activity was measured by dinitrosalicylic acid method (Miller et al., 1960) to determine enzyme titre. The individual effects of each of enzyme concentration, substrate concentration (solid loading), incubation time, temperature, and pH were studied on the content of reducing sugars while keeping the other values constant.

Bioethanol fermentation

Suspension culture of *S. cerevisiae* (24 h) was inoculated into 25 mL of the fermentation medium (in 100 mL Erlenmeyer flasks) containing saccharified solution of *Cyperus sp.* from the previous step. Yeast extract (2 g/L) was added as an additive to the culture medium for enhanced growth. The fermentation was carried out at 37 °C under shaking condition for 48 h. Ethanol was estimated spectrophotometrically by potassium dichromate method.

RESULTS AND DISCUSSION

Biochemical analysis

Biochemical estimations of cellulose, hemicelluloses and lignin were made of raw grass (Table 1). The ash content was found to be 5.98 %. The overall sugar yield obtained from pretreatment and enzymatic hydrolysis was considered as a major indicator for ethanol production. Pretreatment with laccase from *Pleurotus sp.* at 40 °C for 8 h yielded 4.03 % of lignin content. Lignin content below 12 % does not significantly affect the enzymatic digestibility (Chang et al., 2001). Lignin content, crystallinity, and acetyl content of the biomass limits the enzyme digestibility (Chang and Holtzapple, 2000).

Table 1. Biochemical Analyses (%) in Raw and Pretreated Biomass of *Cyperus sp.* Biomass

	Raw (%)	Delignified (%)
Cellulose	20.76	30.07
Hemicellulose	12.93	15.87
Lignin	11.88	4.03
Ash	5.98	

Irfan et al. (2011) have studied enzymatic hydrolysis of the pretreated biomasses (sugarcane bagasse and wheat straw) with commercial enzymes and indigenously

produced enzyme from *Trichoderma reesei* grown under solid state fermentation that showed 14.1 % saccharification rate on account of alkaline hydrolysis.

The saccharification efficiency of the pretreated *Cyperus* sp. in the present study was found to be 74.2 %.

Enzymatic delignification and recycling

Delignification of grass was performed using hyperactive laccase followed by lignin estimation. In the preliminary experiments, selection of initial parameters such as pH 7.0, 40 °C, solid to liquid ratio 1:6, enzyme concentration 500 IU/ mL and incubation time 8 h showed maximum degree of delignification. Considerable delignification was achieved for upto 4 cycles with fresh enzyme being added to the recovered enzyme while maintaining the enzyme volume and activity (Fig.1).

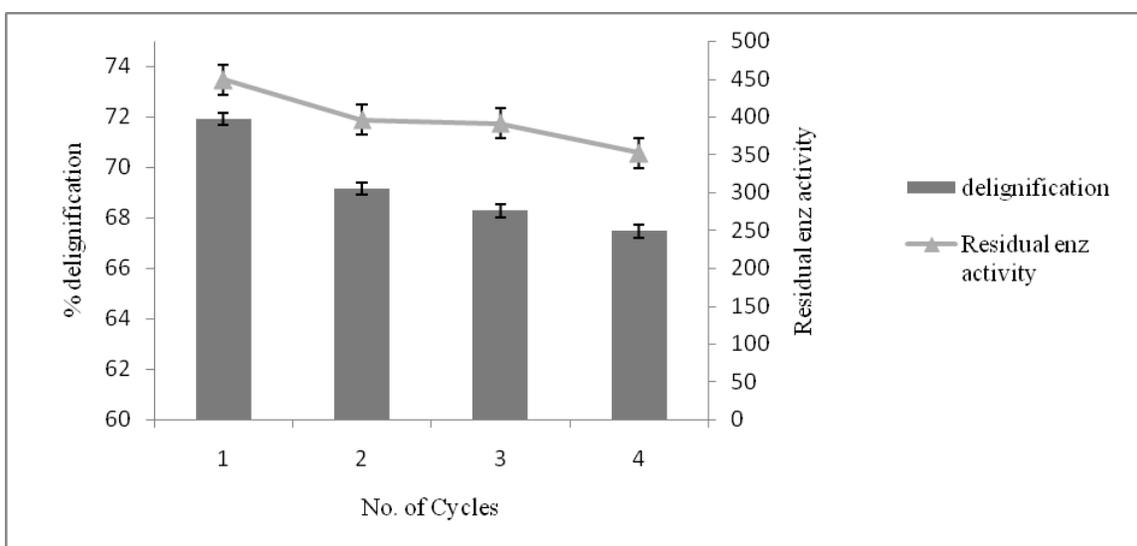


Fig. 1. Effect of recirculation of enzyme on delignification and enzyme activity

Enzymatic hydrolysis by cellulase from T. reesei RUT C30

Pretreated *Cyperus* grass sample was hydrolyzed by cellulase from *T. reesei* (20 IU/mL/g substrate) at pH 5, 50 °C for 8 h. The reducing sugar concentration reached 431.775 mg/g substrate. Xylose and arabinose have been detected by Chen et al. (2008), showing the presence of xylanase in *T. reesei* cellulase. They also found a high amount of cellobiose in the cellulosic hydrolysate, indicating relatively low cellobiase activity in *T. reesei* cellulase. It is known that there are many factors that influence the enzymatic hydrolysis of cellulose in lignocellulosic feedstocks, such as cellulose crystallinity, cellulose degree of polymerization, lignin barrier (content and distribution), hemicellulose content, and porosity (Alvira et al., 2010). It has been suggested that adsorption is one of the major requirements for efficient enzymatic hydrolysis of insoluble cellulose. Cellulase enzymes are very specific in their action, producing virtually no glucose degradation products. Cellulases (of fungal or bacterial origin) are in fact a mixture of enzymes which act in concert and synergistically (Mosier et al., 1999). However, cellulose hydrolysis in aqueous media suffers from slow reaction rates because

the substrate (cellulose) is a water insoluble crystalline biopolymer. Therefore, the enzymes have to accomplish the hydrolytic decomposition via first adsorbing on the cellulose surface, partially stripping the individual polymer chains from the crystal structure, and then cleaving the glycoside bonds in the chain (Lynd et al., 2002; Zhang and Lynd, 2004). Adsorption sites of crystalline cellulose are very limited due to the tight packing arrangement of cellulose fibrils, which not only excludes the enzymes but also largely excludes water (Lynd et al., 2002; Zhang and Lynd, 2004). Pretreatment methods increase the surface area accessible to water and cellulases are expected to generate improvements in hydrolysis kinetics and conversion of cellulose to glucose (Gollapalli et al., 2002; Sun and Cheng, 2002; Zhang and Lynd, 2004). However there is no clear understanding of the mechanism by which these adsorbed enzymes promote the enzymatic degradation of cellulose. Substrate factors such as the degree of crystallinity of cellulose and the type and content of lignin may also influence the efficiency of hydrolysis.

Effects of enzyme concentration on saccharification

Enzymatic hydrolysis of pretreated *Cyperus* sp. sample using different enzyme dosages showed maximum amount of reducing sugar at 20 IU/mL at the end of 8 h. Further increase in enzyme dosage did not yield a corresponding increase in reducing sugars (Fig. 2). The accessibility of cellulase to the limited adsorption sites on crystalline cellulose structure is generally believed to play an important role in determining the cellulose hydrolysis rate.

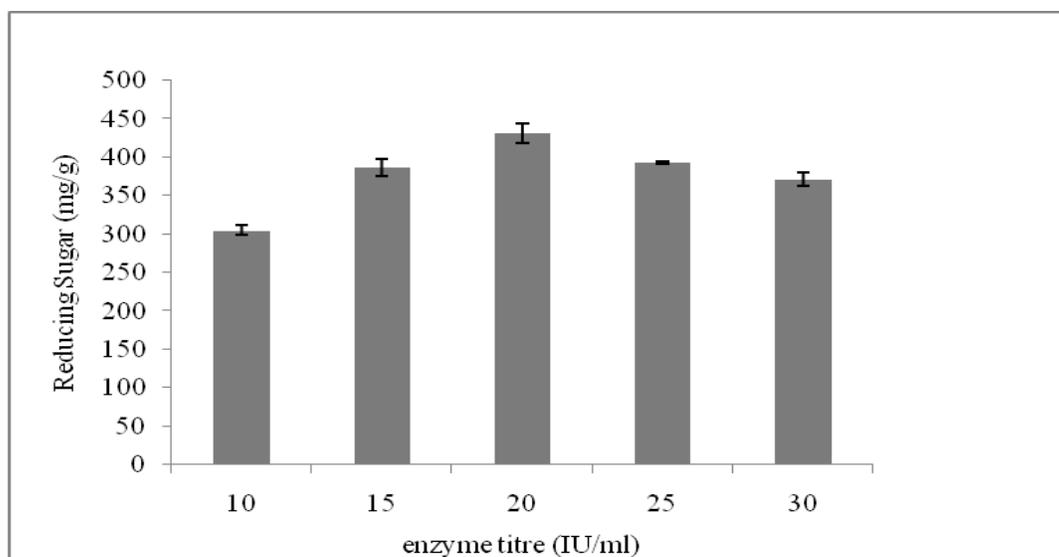


Fig. 2. Effect of enzyme titre on the yield of reducing sugars of *Cyperus* sp. (Values held constant: solid loading: 20 %; pH: 5; temp.: 50° C; incubation time: 8 h)

Effects of incubation time on saccharification

Incubation time is a major factor that can affect saccharification and in turn the hydrolysis yield. Enzyme concentration was fixed at 20 IU/mL. An incubation time of 6 h showed highest reducing sugar yield (456.01 mg/g), which decreased considerably at 8

h and 10 h to 409 mg/g and 387 mg/g, respectively (Fig. 3). The results could imply that the available enzyme adsorption sites became saturated, thus leading to decline in the catalytic turnover rate.

Cellulase reflected a decreasing trend in saccharification for longer incubation time. Melo et al. (2007) reported that the enzyme level declined with prolonged incubation. This could be due to loss of moisture or denaturation of the enzyme resulting from variation in pH due to release of sugars. Singh et al. (2009) reported that the decrease of enzyme activities may be due to the accumulative effect of cellobiose.

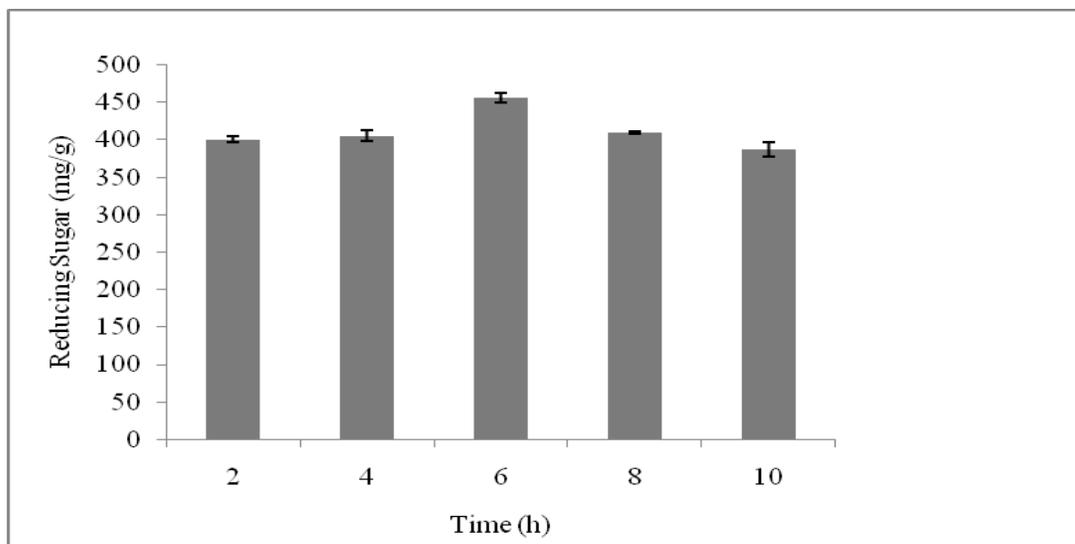


Fig. 3. Effect of incubation time on the yield of reducing sugars of *Cyperus* sp. Values held constant: enzyme titre : 20 IU/mL; solid loading : 20 %; pH : 5; temp.: 50° C

Effects of substrate concentration on enzymatic hydrolysis

The effects of substrate concentration on enzymatic hydrolysis were investigated at a fixed enzyme dosage of 20 IU/mL. Little difference in reducing sugar concentration was observed with the substrate concentration ranging from 15 to 20 % (Fig. 4). The reducing sugar at 15 % solid loading was 407 mg/g, just a little higher than that at 452 mg/g substrate concentration at 20 % solid loading. Previous investigations have shown that high substrate concentration usually resulted in lower hydrolysis yield due to product inhibition (Ramos *et al.*, 1993), enzyme inactivation (Reese, 1980) and reactivity decrease of the substrate (Sinitsyn *et al.*, 1991). Sufficient enzyme dosage and low substrate concentration are possible explanations for the little difference in hydrolysis yield observed at different substrate concentration. On the other hand, reduction in enzyme production at high moisture may be due to the reduction in substrate porosity, changes in the structure of substrate particles (Baysal *et al.*, 2003).

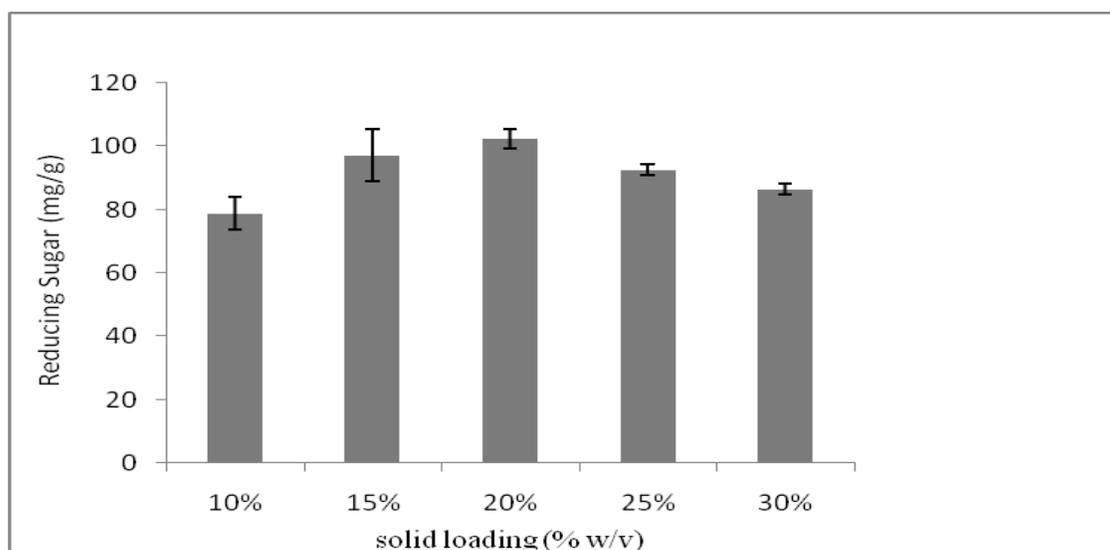


Fig. 4. Effect of solid loading on the yield of reducing sugars of *Cyperus* sp. Values held constant: enz. titre : 20 IU/mL; pH : 5; temp.: 50° C; incubation time: 8 h

Effects of temperature and pH

Subsequently, the effects of temperature and pH were also measured. The optimum pH for the maximum release of sugar was 5, and a temperature of 50 °C resulted in maximum hydrolysis of the enzyme treated substrate. (Fig. 5 and 6). The optimum pH for saccharification was the optimum pH for synthesis of cellulolytic enzyme by fungus (Baig et al., 2003). Similarly temperature for the synthesis of the enzyme was the optimum for the saccharification of *Cyperus* sp. An increase in temperature speeds up enzyme-mediated reactions; when heated beyond an optimum temperature leads to denaturation.

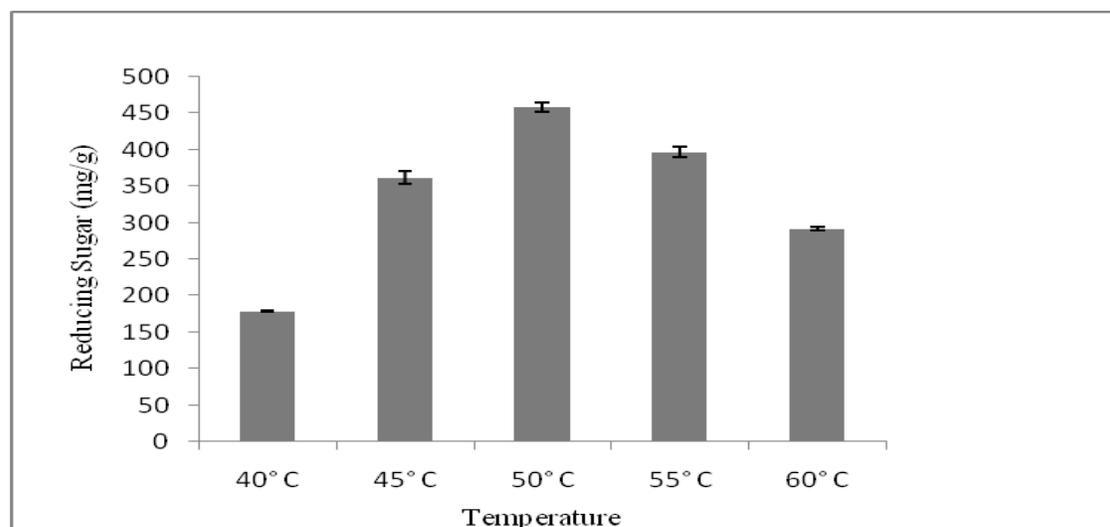


Fig. 5. Effect of temperature on the yield of reducing sugars of *Cyperus* sp. Values held constant: enz. titre : 20 IU/mL; solid loading : 20 %; pH : 5; incubation time: 8 h

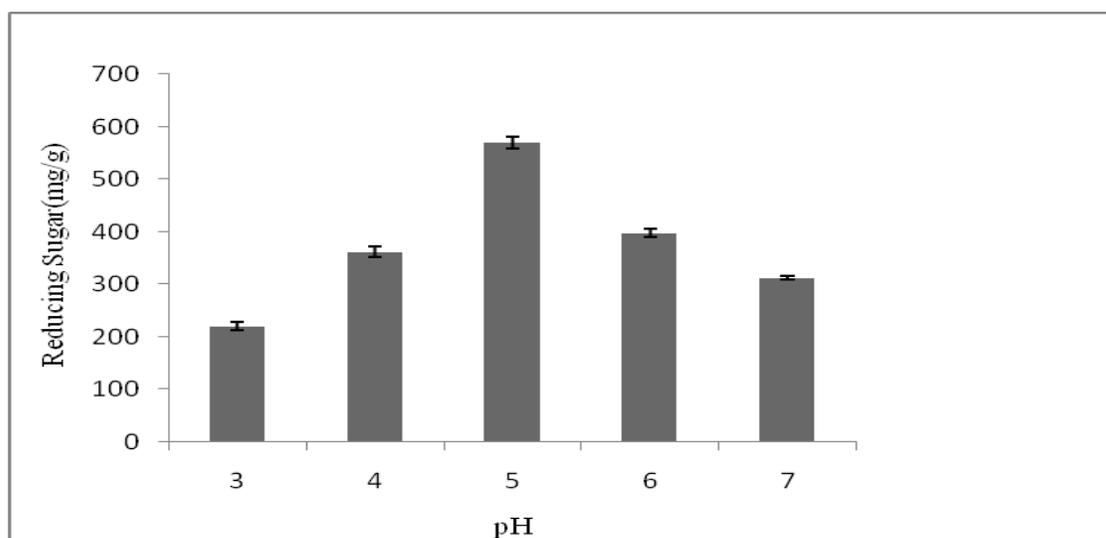


Fig. 6. Effect of pH on the yield of reducing sugars of *Cyperus* sp. Values held constant: enz. titre : 20 IU/mL; solid loading : 20 %; temp.: 50° C; incubation time: 8 h

Fermentation of saccharified cyperus sp.

Ethanol estimated through the dichromate method was found to be 6.49 g/L after 36 h of incubation which is significantly higher as compared to other study by Kumar et al. (2013), who have reported 4.4 % ethanol in *Cyperus rotundus*. The yeast strain produces small amount of acetic acid and succinic acid as byproducts along with the ethanol. Similar bioethanol production was reported for dilute acid pretreated rice straw (Karimi et al., 2006).

Saccharification efficiency

A saccharification efficiency of 74.2 % was calculated. Enzymes are optimally active at a specific pH and temperature. The effect of pH of the medium revealed that the optimum value for enzyme production was obtained at pH 4.8. Optimal pH is very important for growth of the microorganism and its metabolic activities. As the metabolic activities of the microorganism are very sensitive to changes in pH, cellulase production by *T. reesei* was affected by varying pH of the medium. The present findings are comparable to previously reported results from literature. Cellulases from *T. reesei* work better in more acidic pH (4.5 to 5.0), (Gomes et al., 2006; Yang et al., 2004). This corroborates the results of Tenborg *et al.* (2001), where the optimum glucose yields were obtained at pH 4.8. pH is among most important factors for any saccharification and fermentation process and depends upon microorganisms because each microorganism possesses a pH range for its growth and activity (Lonsane et al., 1985). Increase and decrease in pH on either side of the optimum value resulted in decrease in sugar yields. The rate and extent of saccharification depend on the nature and pretreatment of the substrate, enzyme and substrate concentration, product inhibition, and enzyme stability (Fan et al., 1982; Saddler et al., 1993). All of these interact to cause the rate of hydrolysis to fall rapidly with time. As the more susceptible portions of the cellulose are hydrolyzed, the residue is rendered increasingly crystalline and resistant, products accumulate, and

they competitively inhibit the enzymes. Since cellulose simultaneously decreases, the inhibitor/substrate ratio rises (Converse, 1993).

The difference in the amount of total reducing sugars released can be attributed to the differences in the cellulose structure for the amorphous cellulose after pretreatment and largely crystalline untreated cellulose. Cellodextrins (β -1,4-glucose oligomers) with a degree of polymerization from 2 to 6 are soluble in water (Klemm et al., 1998; Pereira et al., 1988; Zhang and Lynd, 2005) and cellodextrins with DP from 7 to 13 are slightly soluble in hot water (Schmid et al., 1988; Zhang and Lynd, 2003, 2004). The pretreated amorphous cellulose substrate hydrolyzes rapidly to short glucose oligomers, mitigating mass transfer limitations on overall reaction rates. Pretreated amorphous cellulose is hypothesized to have a higher fraction of β -glucosidic bonds accessible to cellulase due to the decreased crystallinity and potentially increased surface area obtained from the pretreatment process. Endoglucanases act at random sites in the internal accessible regions of cellulose polysaccharide chains, resulting in a rapid decrease in degree of polymerization of the substrate due to the formation of smaller cellulose chains of varying lengths (Zhang and Lynd, 2004). Exoglucanase or cellobiohydrolase acts on the chain ends liberated by the action of endoglucanase, producing primarily cellobiose (Lynd et al., 2002; Teeri, 1997). In native cellulose, the low surface area limits the endoglucanases accessibility to β -glucosidase bonds and hence the decrease in degree of polymerization is not as rapid as in the regenerated cellulose and the native cellulose remains insoluble. Relatively less work has been done in this species of weed. This study emphasizes the use of *Cyperus* sp. as an alternative source, for the production of bioethanol.

CONCLUSIONS

1. From the experiments for saccharification, pH 5, temperature 50 °C, solid to liquid ratio 1:5, enzyme concentration 20 IU/ml and incubation time 6 h were found to be most effective for the saccharification of *Cyperus* sp. yielding 456 mg/g of reducing sugars and saccharification efficiency of 74.23 %.
2. 6.49 g/L ethanol was estimated after 36 h of fermentation *S. cerevisiae* strain. The results obtained in the present investigation are indicative of improved conversion efficiency for prospective production of bioethanol.

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