OPTIMIZATION FOR PRODUCTION OF REDUCING SUGAR FROM *Eichhornia crassipes* BIOMASS USING *Aspergillus niger*

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ABSTRACT

Water Hyacinth is used as renewable energy raw material for sugar production and then fuel production because its availability is easy and rate of propagation is very high. It is an aquatic weed, tolerates variation in pH and temperature and is unaffected by toxic substances. Determination of reducing sugar from lignocellulosic biomass of Water Hyacinth was carried out by saccharification process using micro fungi as *Aspergillus niger*. It was achieved using Dinitro alicyclic Acid (DNS) method. Reducing sugar contents were evaluated maximum at optimum period. The amount of sugar contents were maximum which were 506.0 µg/ml in untreated condition and 596.0 µg/ml in treated condition at optimum inoculation period these were found after inoculation of seventh day and 5th days respectively and 398.0 µg/ml, 545.0 µg/ml and 420.0 µg/ml in untreated condition and 665.0 µg/ml, 645.0 µg/ml and 567.0 µg/ml in treated condition at optimum conditions of pH, temperature and substrate concentration which were 6.0 or 5.5 pH, 35°C or 30°C and 2.5 ml respectively. Here the two types of samples were prepared. One was treated with 1% H₂SO₄ and other was untreated. The reducing sugar contents were obtained more in treated condition in comparison to untreated condition. The present study evaluated Water Hyacinth as raw material for possible strategies by conversion of hydrolysate to reducing sugar was maximized. For this the effect of optimum condition such as inoculation period, pH, temperature and substrate concentration on conversion of Water Hyacinth biomass for reducing sugar was studied.

Key Word: Aquatic weed, Saccharification, Hydrolysate, optimization, Dinitros alicyclic Acid, Renewable energy

INTRODUCTION

Water hyacinth plant is known as aquatic weed belonged to the monocotyledonous family Pontederiaceae. Several fungal pathogens have been reported to attack water hyacinth in various parts of the world. Lignocellulosic material of this plant contains mainly of cellulose, hemicelluloses and lignin. Lignocellulose is an interesting raw material for production of bioethanol because of its having large amount and low cost. There are two major ways of converting cellulose to glucose (1) chemical (2) enzymatic. Fungal genera like *Trichoderma, Aspergillus* and *Fusarium* are known to be cellulase producers and crude enzymes produced by these microorganisms are commercially available for agricultural use. The genus *Aspergillus* species attack cellulose producing significant amount of cell free cellulase capable of hydrolyzing cellulose into fermentable soluble sugars such as glucose; an important raw material in chemical industries. Cellulose from various sources is all the same at the molecular level. However, they differ in the crystalline structures and bindings by other biochemicals. Freshwater biomass is aquatic weed which interfere with the use of water and constitute an irritation to the environment and human welfare. Cellulose is the most abundant component of plant biomass. It is found in nature almost exclusively in plant cell walls, although it is produced by some animals e.g., tunicates and few bacteria. An ideal pretreatment is needed to reduce the lignin content and crystallinity of cellulose, which

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slowdowns enzymatic hydrolysis. The major factors that affect the efficiency of the enzymatic saccharification of water hyacinth biomass are inoculation period, pH, temperature and substrate concentration. The current study has based on maximizing yield of reducing sugars by enzymatic saccharification to enhance bioethanol production. Lignin, one of the major components of lignocellulosic biomass, is impediment to enzymatic saccharification. Cellulolytic enzymes are synthesized by a number of microorganisms. Fungi and bacteria are the main natural agents of cellulose degradation. However, fungi are well known agents of decomposition of organic matter, in general, and of cellulosic substrate in particular. Cellulase catalyzes the conversion of insoluble cellulose to simple, water soluble products. Lignocellulosic materials (second generation) are gradually considered as more attractive renewable resources for fuel production owing to their easy availability and relatively low cost. This study aims to provide better understanding of optimum condition for the production of reducing sugar through saccharification by Aspergillus niger.

MATERIAL AND METHODS

Sampling collection
Fresh water hyacinth plants with long stem were collected from LaxmiTaal of Jhansi city. Collected water hyacinth sample were washed to remove adhering dirt and chopped in small pieces. These small pieces were dried in sunlight. Dried water hyacinth biomass was pre-treated 1% v/v H₂SO₄ with soaking time of five hrs at room temperature. Pre-treated samples washed neutrality with distilled water and then dried in hot air oven and powered in grinder and stored in dry place for further use. We found finally two sample (treated and untreated) for hydrolysis and fermentation. Untreated samples meant without 1% H₂SO₄. Pre-treatment is required to alter the biomass macroscopic and microscopic size and structure as well as its sub microscopic chemical composition so that the hydrolysis of carbohydrate fraction to monomeric sugar can be achieved more rapidly and with greater yield. Hydrolyaste was prepared by mixing the dried power with 8 volume of 1%v/v sulphuric acid for 7 hours in a glass lined reactor stirred at 250 rpm on rotator shaker. The mixture was autoclaved at 121 °C, 15 lbs for 15 min and further cooled down at room temperature. The hydrolysate was filtered using Whatman filter paper No. 1 to remove the unhydrolysed material and wash with warm water (60°C). The filterate and washing were pooled together (Carvelheiro et al., 2008). This hydrolysate was detoxified harmful material by Ca(OH)₂. Then it was filtered to remove insoluble and filtrate was used for observing fermentable sugar.

Preparation of media
For reducing sugar production, Mandel’s media was used (Mandel and Weber, 1969). This media contained following ingredients: Ammonium sulphate -1.4 gm, Potassium dihydrogensulphate -2.0 gm, Magnesium sulphate – 0.3 gm, Calcium chloride 0.3 gm, Ferrous sulphate -0.005 gm, Mangnesesulphate -0.0016 gm, Peptone -1.0 gm, Urea -0.3 gm, Zinc chloride -0.0017 gm, Cobaltous chloride – 0.002 gm, CMC sodium salt -10 gm. All above chemical dissolved in distilled water and make up to 1000 ml.

Preparation of inoculums
Aspergillus niger was grown on PDA slants at 27±2°C for 6 days and maintained as stock culture, then stored at 4°C. Inoculum was prepared using potato dextrose broth in 250 ml conical flasks. Inoculums rotated at 250 rpm at 30°C for 24 hours and then used for fermentation.

Saccharification
Mandel’s media was taken in conical flask and added treated and untreated hydrolysate separately. All flasks plugged with cotton wool and then, sterilized in autoclave at 121°C and 15 lbs for 30 min. 1 ml of spore of Inoculums of Aspergillus niger was inoculated in each conical flask containing Mandel’s media after cooling.

Effect of inoculation period
All flasks were kept in incubator at 30°C for different days as 3, 5, 7, 10, 13, 16 days. All flasks were shaken twice daily. Now these samples were used for DNS test for total reducing sugar.
Effect of pH
Before autoclaving pH of Mandel’s media containing hydrolysate were adjusted at various level of pH by adding 1 N NaOH and 1 N HCl solution. pH ranges were adjusted as 3.0, 4.0, 5.0, 5.5, 6.0, 6.5, and 7.0. All flasks were kept in incubator at 30°C for 7 days. All flasks were shaken twice daily. Now these samples were used for DNS test for total reducing sugar.

Effect of temperature
All autoclaved flasks containing media with spore suspension and hydrolysate kept in incubator at different temperature ranges as 20°C, 25°C, 30°C, 35°C, and 40°C for 7 days. All flasks were shaken twice daily. Now these samples were used for DNS test for total reducing sugar.

Effect of substrate concentration
Different amount of treated and untreated hydrolysate ranging from 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml, 2.5 ml, 3.0 ml, 3.5 ml, 4.0 ml, 4.5 ml, 5.0 ml were mixed with Mandel’s media. All flasks plugged with cotton wool and then, sterilized in autoclave at 121°C and 15 lbs for 30 min. 1 ml of spore of Inoculums of *Aspergillus niger* was inoculated in each conical flask. All flasks were kept in incubator at 30°C for 7 days. All flasks were shaken twice daily. Now these samples were used for DNS test for total reducing sugar.

DNS test for reducing sugar
Reducing sugar was estimated by DNS method using DNS reagent. After applying optimum conditions, treated and untreated hydrolysate were centrifuged at 13500 rpm for 20 min and supernatants obtained from centrifugation were used as crude extract for reducing sugar. 3ml of DNS reagent was added in each sample tube containing centrifuged samples and all tubes were incubated in water bath for 10 min to develop red brown colour. All test tubes were taken out from water bath and cooled then added 1 ml of 1% Rochella salt. All test tubes were left at room temperature for 20 min to established red brown colour. Optical density was recorded by spectrophotometer at 540 nm and compared with standard curve of glucose. Absorbance was compared with the standard graph plotted by reacting known concentration of glucose (.05 to 0.1mg/ml) with DNS reagent and plotting a graph between concentration of glucose (X axis) and OD at 540 nm (Y axis).

RESULTS AND DISCUSSION

Effect of incubation period on reducing sugar production
Reducing sugar production was determined at incubation period. It was seen that the hydrolysate of *Eichhorniacrassipes* gave the higher sugar production which is 522µg/ml in untreated samples after 10th day of incubation and 596µg/ml in treated sample after 5th day of incubation. And the minimum production that is 384µg/ml in untreated sample after 16th day of incubation and 460 µg/ml in treated samples were obtained after 3 days of incubation. According to hydrolysis rates decline with time due to depletion of the more amorphous substrates, product inhibition and enzyme inactivation. After optimum condition minimum production of reducing sugar highlights sugar depletion from substrate into the medium (Sharma and Singh, 2017). Concentration of reducing sugar was found maximum in treated in compression to untreated samples in all optimized condition. So we can say acidic condition is better for reducing sugar production then non acidic condition. (Fig. 1 and Table 1)

Effect of pH on reducing sugar production
Effect of pH on reducing sugar production was determined. It was observed that pH of 6 is proved best in untreated hydrolysate and 5.5 pH in treated hydrolysate. At 6 pH, 398µg/ml was produced in untreated and at 5.5 pH, 665 µg/ml was produced in treated sample. It is maximum in both conditions. Hydrolysate gave minimum reducing sugar that is 225 µg/ml at pH 3 in untreated and, 322 µg/ml at pH 7 in treated samples. Effect of pH on reducing production from the untreated and treated hydrolysate by *Aspergillus niger* was shown on Table 2. Effect of pH on reducing
sugar production was shown in Table 2 which supports the findings of Lee et al., in which pH optimum of β glucosidase was between 5 to 6. (Fig. 2 and Table 2)

**Effect of temperature on reducing sugar production**

Optimum temperature for reducing sugar production was determined. It was observed that 30°C temperature is best at which highest reducing sugar was produced in treated sample and 35°C is best for untreated sample.\(^{25,26}\) Hydrolysate gave higher reducing sugar production which is 545 µg/ml at 35°C in untreated and 645 µg/ml at 30°C in treated hydrolysate. It was observed minimum at 20°C that is 289 µg/ml and 392 µg/ml respectively. Results of present study showed in Table 3. Many researcher reported different temperatures for highest sugar production in flask using different microfungi spp. It was suggested that the optimum temperature for sugar production also depends on microfungi strains Suto and Tomito.\(^{13,22,27}\) (Fig. 3 and Table 3)

**Effect of substrate concentration on reducing sugar production**

0.5% to 5% of substrate concentrations were considered for production of reducing sugar. It was observed that the hydrolysate produced maximum reducing sugar production at 2.5% hydrolysate concentration in both untreated and treated sample that are 420 µg/ml and 567 µg/ml respectively.\(^{28,29}\) Effect of substrate concentration for reducing sugar was shown in Table 4. This supports the finding of Haapela et al., (1995) and Jeffries, (1996) who reported that if the substrate concentration increased after optimum level for glucose production did not found in appropriate increase in glucose yield. Reducing sugar production is increased till the availability of cellulose and the optimum concentration of sugar production began to decrease by inhibitory effect of accumulated cellobiose of low degree of polymerization of growth medium.\(^{30-32}\) It is done due to specific binding of the enzyme with substrates Gilkes, et al., (Fig. 4 and Table 4).

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![Fig. 1](image1.png)

**Fig. 1** : Optimization of inoculation period for production of reducing sugar by *Aspergillus niger*

![Fig. 2](image2.png)

**Fig. 2** : Optimization of pH for production of reducing sugar by *Aspergillus niger*
Table 1: Optimization of inoculation period for production of reducing sugar by Aspergillus niger

<table>
<thead>
<tr>
<th>Inoculation period</th>
<th>3rd day</th>
<th>5th day</th>
<th>7th day</th>
<th>10th day</th>
<th>13th day</th>
<th>16th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Untreated) Reducing sugar (µg/ml)</td>
<td>397.0</td>
<td>508.0</td>
<td>506.0</td>
<td>522.0</td>
<td>385.0</td>
<td>384.0</td>
</tr>
<tr>
<td>(Treated) Reducing sugar (µg/ml)</td>
<td>460.0</td>
<td>596.0</td>
<td>593.0</td>
<td>535.0</td>
<td>526.0</td>
<td>515.0</td>
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</table>

Table 2: Optimization of pH for production of reducing sugar by Aspergillus niger

<table>
<thead>
<tr>
<th>pH</th>
<th>3.0</th>
<th>4.0</th>
<th>5.0</th>
<th>5.5</th>
<th>6.0</th>
<th>6.5</th>
<th>7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Untreated) Reducing sugar (µg/ml)</td>
<td>225.0</td>
<td>308.0</td>
<td>308.0</td>
<td>397.0</td>
<td>398.0</td>
<td>295.0</td>
<td>289.0</td>
</tr>
<tr>
<td>(Treated) Reducing sugar (µg/ml)</td>
<td>417.0</td>
<td>415.0</td>
<td>490.0</td>
<td>665.0</td>
<td>598.0</td>
<td>334.0</td>
<td>322.0</td>
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</table>

Table 3: Optimization of temperature for production of reducing sugar by Aspergillus niger

<table>
<thead>
<tr>
<th>(Tm)</th>
<th>20°C</th>
<th>25°C</th>
<th>30°C</th>
<th>35°C</th>
<th>40°C</th>
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</thead>
<tbody>
<tr>
<td>(Untreated) Reducing sugar (µg/ml)</td>
<td>289.0</td>
<td>292.0</td>
<td>520.0</td>
<td>545.0</td>
<td>332.0</td>
</tr>
<tr>
<td>(Treated) Reducing sugar (µg/ml)</td>
<td>292.0</td>
<td>324.0</td>
<td>645.0</td>
<td>596.0</td>
<td>529.0</td>
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</table>

Table 4: Optimization of substrate for production of reducing sugar by Aspergillus niger

<table>
<thead>
<tr>
<th>Substrate conc. (ml)</th>
<th>0.5</th>
<th>1.0</th>
<th>1.5</th>
<th>2.0</th>
<th>2.5</th>
<th>3.0</th>
<th>3.5</th>
<th>4.0</th>
<th>4.0</th>
<th>5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Untreated) Reducing sugar (µg/ml)</td>
<td>156.0</td>
<td>158.0</td>
<td>253.0</td>
<td>411.0</td>
<td>420.0</td>
<td>303.0</td>
<td>306.0</td>
<td>271.0</td>
<td>247.0</td>
<td>227.0</td>
</tr>
<tr>
<td>(Treated) Reducing sugar (µg/ml)</td>
<td>161.0</td>
<td>191.0</td>
<td>355.0</td>
<td>562.0</td>
<td>567.0</td>
<td>451.0</td>
<td>433.0</td>
<td>292.0</td>
<td>289.0</td>
<td>242.0</td>
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</table>
CONCLUSION

Two type of samples were prepared. One was treated with 1% H$_2$SO$_4$ whereas another was untreated. The reducing sugar contents were obtained more in treated condition. The study evaluated water hyacinth as raw material for possible strategies by conversion of hydrolysis state to reducing sugar. For this, the effect of optimum condition such as inoculation period, pH, temperature and substrate concentration on inoculation period, pH, temperature and substrate concentration on conversion of water hyacinth biomass for reducing sugar was studied.

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REFERENCES


