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Optimum Conditions for Reducing Sugar Production from Eichhornia Crassipes Biomass Using Trichoderma Virdae

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Abstract
Evaluation of optimum conditions for reducing sugar production from *Eichhornia crassipes* was carried out through hydrolysis process. Contents of reducing sugar were obtained using Dinitrosalicylic assay method. Reducing sugar contents were found maximum in treated hydrolysate in comparison to untreated hydrolysate in all conditions which was optimized. It was done due to changes in the physical and chemical structure of lignocellulosic biomass through pretreatment. In this study Water Hyacinth was used as feedstock for possible strategies by which biomass of Water Hyacinth was converted to reducing sugar. In hydrolysis, treated and untreated hydrolysate was saccharified by *Trichoderma virdae*. Saccharification was carried out at optimum comditions such as inoculation period, pH, temperature and substrate concentration. Maximum yield of reducing sugar contents were 565 µg/ml, 528 µg/ml, 475 µg/ml and 415 µg/ml in respected to optimum conditions of inoculation period 7th days, pH 5.5, temperature 30^{0}C and substrate concentration 2.0gm in untreated hydrolysate and 605 µg/ml, 591 µg/ml, 534 µg/ml and 602 µg/ml in respected to optimum conditions of inoculation period 5th days, pH 5, temperature 30^{0}C and substrate concentration 2.0gm in treated hydrolysate. In this paper compression was made between treated and untreated sample. Result showed the potential of Water hyacinth in both treated and untreated conditions for reducing sugar production.
Keywords- Hydrolysate, lignocellulosic biomass, optimization, Saccharification, Water Hyacinth, Feedstock.

Introduction

Water hyacinth (*Eichhornia crassipes*) is one of the most abundant waste materials in the world, belonging to the family *Pontederiaceae* and is native of Brazil. 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. One promising approach was to hydrolyze the cellulose to glucose with fungal enzymes and then to ferment the glucose to ethanol which could be used as a liquid fuel (Mandels *et al.,* 1974). Cellulose from various sources is all the same at the molecular level. However, they differ in the crystalline structures and bindings by other biochemicals (Nam, 1979). Freshwater biomass is aquatic weed which interfere with the use of water and constitute an irritation to the environment and human welfare (Uka *et al*., 2009). Water hyacinth is a lignocellulosic biomass and for its better utilization it can be used as a good source for the production of reducing sugars, which can be further used for production of ethanol, xylitol, organic acids and other chemicals (Xia & Sheng, 2004; Chen *et al*., 2008). This can be achieved by enzymatic hydrolysis which produces better yields than acid-catalyzed hydrolysis (Pan *et al.,* 2005). However, there are many problems obstructing the effective enzymatic hydrolysis of lignocellulosic material. Of these is the lignin seal which prevents penetration by degrading enzymes (Taniguichi *et al.,* 2005). An ideal pretreatment is needed to reduce the lignin content and crystallinity of cellulose, which slowdowns enzymatic hydrolysis (Hendriks & Zeeman, 2009). A number of chemical pretreatments of lignocellulosic sample to enhance enzymic saccharifications of cellulose have been extensively investigated. 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 (Tanaka, 1979). Water hyacinth is a lignocellulosic material that can be used as a potential source for the production of reducing sugars, which can be used for production of ethanol, xylitol, organic acid and other chemicals (Fattah and Naby 2011). Pretreatment are necessary to improve the digestibility of lignocellulosic biomass because it can break down the complex structure of biomass, decrease the lignin content and crystallinity of cellulose in order to increase accessibility of enzyme to the substrate (Nlewem and Thrash, 2010) Dilute sulfuric acid hydrolysis is one of the most promising pretreatment method because it uses short time, low temperature and pressure. Lignocellulosic materials (second generation) are gradually considered as more attractive renewable resources for fuel production owing to their easy availability and relatively low cost (Ahmad, 2013 and Bayrakci and Kocar, 2014).

Materials and method

Sampling collection

Fresh water hyacinth plants with long stem were collected from Laxmi Taal 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 H_2SO_4 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% H_2SO_4. 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 (Sun and Cheng 2002; Moiser *et al.,* 2005). 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 (60^0C). 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 Weber1969). This media contained following ingredients: Ammonium sulphate -1.4 gm, Potasium dihydrogen sulphate -2.0gm, Magnesium sulphate – 0.3 gm, Calcium chloride 0.3 gm, Ferrous sulphate -0.005 gm, Mangnese sulphate -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

Trichoderma virdae was grown on PDA slants at 27 ± 2^0C for 6 days and maintained as stock culture, then stored at 40C.inoculum was prepared using potato dextrose broth in 250 ml conical flasks. Inoculums rotated at 250 rpm at 30^0C 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 121^0C and 15 lbs for 30 min. 1 ml of spore of Inoculums of *Trichoderma virdae* 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 20^0C, 25^0C, 30^0C, 35^0C, and 40^0C 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 hydrolyate 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, 5.0ml were mixed with Mandel's media. All flasks plugged with cotton wool and then, sterilized in autoclave at 121^0C and 15 lbs for 30 min. 1 ml of spore of Inoculums of *trichoderma virdae* 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 (Miller 1959). 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 color. All test tubes were taken out from water bath and cooled then added 1ml of 1% Rochella salt. All test tubes were left at room temperature for 20 min to established red brown color. Optical density was recorded by spectrophotometer at 540nm 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 540nm (Y axis).

Results and discussion

In present study *Eichhornia* plant was used as raw material for production of reducing sugar because this is found abundantly in ponds as a waste material. It does not play an important role in agricultural food for animals and its cost is very low. Out of selected many fungal species *Trichoderma virdae,* were observed as cellulose degrading microfungi to degrade cellulose and then reducing sugar was produced.

Effect of incubation period on reducing sugar production

Reducing sugar production was determined at incubation period. It was seen that the hydrolysate of *Eichhornia crassipes* gave the higher sugar production which is 565µg/ ml in untreated samples and 602µg/ml in treated samples after 7^{th} day of incubation. And the minimum production that is 257 µg/ml in untreated samples and 372 µg/ml in treated samples were obtained after 3^{rd} day of incubation. According to Ghose, (1987), 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 (Brien and Craig, 1996). 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.

Effect of pH on reducing sugar production

Effect of pH on reducing sugar production was determined. It was observed that pH 5.5 is proved best in untreated hydrolysate and pH 5 in treated hydrolysate. 528µg/ml reducing sugar was produced at pH 5.5 in untreated and 590µg/ml reducing sugar at pH 5 in treated samples. Hydrolysate gave minimum reducing sugar at pH 3 in untreated and pH 7 in treated samples that are 310 µg/ml in untreated and 385 µg/ml in treated samples. Effect of pH on reducing production from the untreated and treated hydrolysate by *T. virdae* was shown on Table no 2. Effect of pH on reducing sugar production was shown in table no 2 which supports the findings of Lee *et al.,* (2002) in which pH optimum of β glucosidase was between 5 to 6. Microbial strains strongly depend on the extracellular pH because pH of the culture medium strongly influences many enzymatic processes and transport of various components across the cell growth and product production (Ellaiah *et. al.,* 2002).

Effect of temperature on reducing sugar production

Optimum temperature for reducing sugar production was determined. It was observed that 30^0C temperature is best at which highest reducing sugar was produced. Hydrolysate gave higher reducing sugar production at 30^0C which is 475µg/ml in untreated and 543µg/ml in treated hydrolysate. It was observed minimum at 20^0C that is 296µg/ml and 310µg/ml respectively. Results of present study showed in table no-3. Many researcher reported different temperatures for highest sugar production in flask using *Trichoderma sp*. It was suggested that the optimum temperature for sugar production also depends on micro fungi strains Suto and Tomito, (2001), Lu *et al*., (2003).

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 2.0% in both untreated and treated sample that are 415µg/ml and 602µg/ml respectively. Effect of substrate concentration for reducing sugar was shown in table no 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 polymerisation of growth medium. It is done due to specific binding of the enzyme with substrates (Gilkes, *et al*., 1984).

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Figures

Fig-1. Effect of inoculation period on reducing sugar production

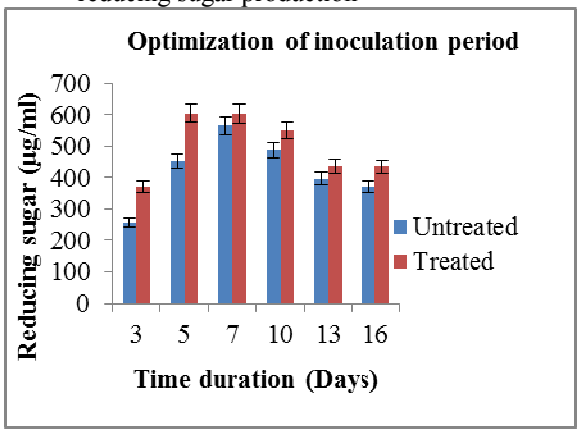


Fig-2. Effect of pH on reducing sugar production.

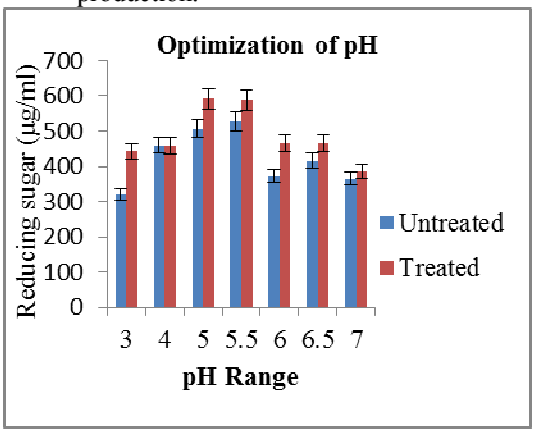


Fig-3. Effect of temperature on reducing sugar production

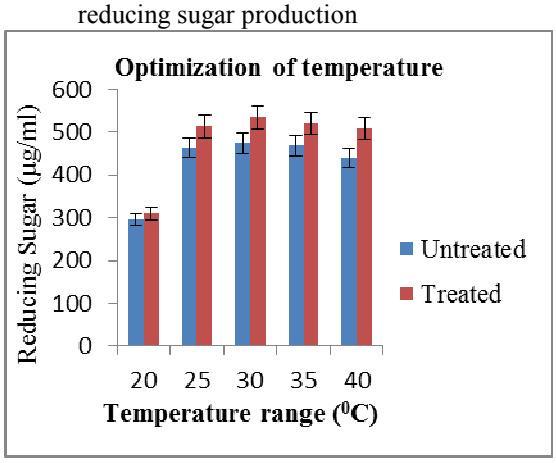
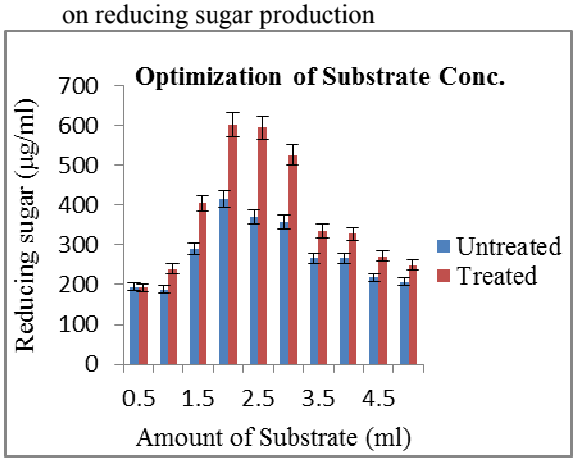


Fig-4. Effect of substrate concentration on reducing sugar production



Tables

Table -1. Effect of inoculation period for production of reducing sugar by *Trichoderma virdae.*

Inoculation period	***3rd day***	***5th day***	***7th day***	***10th day***	***13th day***	***16th day***
(Untreated) Reducing sugar (µg/ml)	257	452	565	487	397	371
(Treated) Reducing sugar (µg/ml)	372	605	602	550	436	434

Table-2. Effect of pH on reducing sugar production by *Trichoderma virdae.*

pH	***3.0***	***4.0***	***5.0***	***5.5***	***6.0***	***6.5***	**7.0**
(Untreated) Reducing sugar (µg/ml)	319	460	506	528	372	416	365
(Treated) Reducing sugar (µg/ml)	442	459	591	588	465	465	385

Table–3. Effect of Temperature on reducing sugar production of *Trichoderma virdae.*

(Tm)	***20⁰C***	***25⁰C***	***30⁰C***	***35⁰C***	***40⁰C***
(Untreated) Reducing sugar (µg/ml)	296	463	475	468	440
(Treated) Reducing sugar (µg/ml)	310	513	534	520	509

Table-4. Effect of substrate concentration on reducing sugar production by *Trichoderma virdae.*

Substrate conc.(ml)	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.0	5.0
(Untreated) Reducing sugar(µg/ml)	195	188	289	415	371	358	267	267	218	208
(Treated) Reducing sugar(µg/ml)	192	240	405	602	595	526	335	328	272	249

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