BIOFUEL PRODUCTION AND CELLULASE EXTRACTION FROM EICHHORNIA CRASSIPES

Madhavi M. Motankar ClafyFernandes Viena Rebello Kajal Naik Gaonkar Ayushi Amonker
Department of Biotechnology, Parvatibai Chowgule College of Arts and Science Autonomous, Gogol, Margao Goa, India.

Abstract
Cellulases are well known for industrial applications as well as for their indispensable use in the ethanol production from lignocellulosic biomass, which produces fermentable sugars that can be used to produce bioethanol. In the present investigation, cellulose producing ability of Aspergillus niger was checked by cellulose hydrolysis by the crude enzyme on Carboxy Methyl Cellulose (CMC) agar; maximum enzyme activity was found at 27˚C at a pH 5.0 by DNSA method where Eichhornia crassipes was used as a carbon source. Crude extract was purified using ammonium per sulphate at concentrations of 0-95% saturation. Filter paper assay of crude enzyme showed the activity to be 177 UmL^{-1} and there was slight decrease in the enzyme activity after purification. Bioethanol was obtained using Eichhornia crassipes after lignocelluloses hydrolysis of biomass; 6% sulphuric acid was found to be most effective. The treated biomass yielded 1.01mg of fermentable sugars per 100ml of the hydrolysate and the further fermentation with Saccharomyces cerevisae yielded 18.6% whole volume of pure bioethanol as per the Gas Chromatographic analysis done. Cellulase enzymes have a wide variety of applications in industries however, production cost is high and thus these methods can be undertaken for their production.

Keywords: Eichhornia Crassipes; Bioethanol; Cellulase; Aspergillus Niger.

1. Introduction

Eichhornia crassipes also commonly called as 'water hyacinth' is a monocotyledonous fresh aquatic plant, belonging to Family Pontederiaceae. It is recognized as the world's most predominant, persistent and troublesome aquatic weed, because of the significant ecological impacts it has on the environment (Muniappan *et al*. 2012). It contains lignocellulosic biomass, whose high hemicelluloses content can provide sugars for bioconversion to ethanol fuel (Awasthi *et al*. 2013). In the recent years, due to the world's energy demand, it has become imperative to scout alternative sources of energy which can replace conventional fossil fuel. Bioethanol is a potential replacement for fossil fuel as it is renewable and ecofriendly. There is emphasis on the use of non edible biomass as raw materials for biofuel production and water hyacinth is considered as an attractive material because of its availability in large quantities at very low cost (Babu *et al*. 2014). Additionally, *Eichhornia crassipes* can be used as a sole carbon source for production of cellulose from microorganisms. Cellulase has important commercial applications and today,this enzyme accounts for about 20% of the world enzyme market. Cellulase enzymes basically act on cellulosic materials thereby converting them into fermentable sugars, which further can be used for the production of products such as ethanol and other biochemicals (Polaina and Mac Cabe 2007).

2. Materials and Methods

2.1. Biofuel Production

Eichhornia crassipes was collected, washed thoroughly, chopped to ~2mm size and dried at 105°C for approximately eight hours. After drying, the biomass was stored in air tight containers at the room temperature. Pretreatment optimization was carried out separately in different Erlenmeyer flasks by mixing the dried biomass with different reagents viz. Conc.H_2SO_4 (2%), Conc.HCl(2%v/v), NaOH (3%) and KCl (3%v/v) respectively. The mixture was autoclaved at 121˚C, 15psi for 15min and further cooled down to room temperature. The hydrolysate was filtered using Whatman filter paper No.1. The filtrate was collected and analyzed for the reducing sugar content by DNS method (Verma *et al.* 2008). Based on the results, Concentrated sulphuric acid was found efficient in treating cellulosic material was used for further procedures. Dried biomass was treated with 2%, 4% and 6% of Conc.H_2SO_4(v/v) separately again in different flasks to check treatment which concentration will give a higher yield of bioethanol. The materials hydrolysed with different concentrations were subjected to fermentation using *Saccharomyces cerevisae.* The fermented broths were distilled and unknown alcohol estimation in each was done by preparation of standard ethanol curve. One with higher concentration of bioethanol was subjected for percentage purity analysis by Gas Chromatography.

2.2. Cellulase Extraction

Culture of *Aspergillus niger* was maintained on PDA media. Water hyacinth (*Eichhornia crassipes)* was collected, washed and few fresh leaves were blended in a mixer with equal volume of water. The macerate was used as a carbon source and as substrate. The medium for cellulose production contained $NaNO_3$(2.0gL^{-1}), KH_2PO_4 (1.0gL^{-1}), $FeSO_4$

($10gL^{-1}$), $MgSO_4.7H_2O$ ($0.5mgL^{-1}$) and water hyacinth macerate was used as carbon source. The fungi were grown in Erlenmeyer flasks containing 50ml of the basal medium supplemented with different volumes of water hyacinth macerate. Each flask was inoculated with fungal spores and incubated for 7 days at 30^0C. The crude enzyme was purified by centrifugation at 3000rpm for10min. The pellet was discarded and supernatant was collected as crude enzyme. The enzyme activity was confirmed by incubating it with cellulose followed by DNSA test (Verma *et al.* 2008) and optimum substrate concentration for maximum cellulose activity was determined by reading absorbance at 540 nm. Confirmation of cellulose activity by incubating enzymes on CMC agar plates was done by incubating crude enzyme in cellulose agar plates. These plates were then incubated at 37^0C for 48hrs. On the next day, the zone of clearance of cellulose was observed. Further the Filter Paper Assay was carried out with the crude enzyme wherein, a series of enzyme assay tubes were prepared by making dilutions of the enzyme with 0.05 M Na-Citrate buffer and incubating each dilution with Whatman filter paper. All the tubes were incubated at 50˚C for exactly 60 min. At the end of the incubation period, each assay tube was removed from the bath and the enzyme reaction was stopped by immediately adding DNSA reagent and absorbance was read at 540 nm. Similarly Glucose standard was prepared from stock solution of anhydrous glucose (10 mg/mL) (Adney and Baker 1996).

2.3. Determination of Optimal Ph, Temperature and Incubation Time for Cellulases Production from Aspergillus Niger.

The optimum pH for cellulases production was estimated at various pH values between 3.0 to 8.0 with appropriate buffer at 27˚C. For determination of optimum temperature for cellulases production, there actions were carried out at 25ºC, 26ºC, 27ºC, 28ºC, 29ºC and 30ºC at pH 5. Cellulases activities were determined by DNSA method where enzyme was incubated with cellulose at the respective pH and temperatures followed by addition of DNSA reagent and absorbance was read at 540 nm.

2.4. Purification of Cellulases

All the purification steps were performed at 4˚C. Extracted enzyme sample was purified by ammonium sulfate precipitation for 0-90% saturation (directly 90% was made) and stored for 24 hours at 4°C. Further, the precipitate obtained by centrifugation was dialyzed against 0.1M Phosphate buffer (pH 6.8) until the enzyme sample was free from ammonium sulfate salts and the enzyme activity was further tested again with DNS. Additionally, the protein content was estimated by Lowry's Method (Verma *et. al.* 2008).

3. Results and Discussions

Ethanol has attracted world wide attention due to its potential use as an alternative automotive fuel. Cellulases are well known for industrial applications and have indispensable role in ethanol production from lignocellulosic biomass, as these produce fermentable sugars that can further be used to convert to bioethanol (Uhlig1998).

3.1. Biofuel Production

Based on the pretreatment optimization hydrolysis with different reagents (Figure1), Conc. sulphuric acid was found to be effective resulting in higher yield of sugar. These results were comparable to those obtained by Awasthi *et. al.* 2013. Further investigation was carried out with different concentrations of sulphuric acid and the hydrolysed broths with each concentration was fermented with *Saccharomyces cerevisae* and distilled. This fermented product was further estimated with standard alcohol graph (Figure 4) which showed a higher percentage of alcohol in sample hydrolysed with 6% conc.sulphuric acid and GC analysis of the confirmed presence of 18.6% of ethanol in sample.

3.2. Cellulase Extraction

Cellulase production in basal medium amended with different volumes of hyacinth macerate and inoculated with spore of *Aspergillus niger* (Figure 2) showed that 8mL of macerate induced maximum cellulolytic activity. Cellulase enzyme production was confirmed by the observation of hydrolysis zones when crude enzyme suspension was incubated on CMC agar plates (Figure 3). The optimum temperature and pH for cellulose activity was found to be 27^0C and pH 5.0 respectively (Figure 5, 6). In Filter paper assay, cellulose activity measured the in terms of FPU and by calculations was 177U/mL (Figure 7) (Adney & Baker, 1996). After purification by ammonium sulphate precipitation and dialysis, the enzyme activity decreased slightly.

4. Conclusion

Bioethanol is a potential alternative for fossil fuels being ecofriendly and cost effective and has been a major part of research. In the present study, bioethanol procured was found to be 18.6% in sample through GC analysis. From the results,

we can infer that these methods are moderately efficient and further enhancement of the enzymatic hydrolysis protocols for bioethanol production can be employed. Also, cellulose enzyme was extracted from *Aspergillus niger* using water hyacinth as carbon source. Cellulase enzymes have a wide variety of applications in industries and production cost is quite expensive, so these methods can be taken into account for further research in their production.

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Conflicts of Interest
The authors declare no conflict of interest.

References

1. Adney B. And Baker J. (1996) Measurement of Cellulase Activities, Battelle: Midwest Research Institute.
2. Awasthi M., Kaur J.and Rana S. (2013) Bioethanol Production through Water Hyacinth, *Eichhornia crassipes* via Optimization of the Pretreatment Conditions. *International Journal of Emerging Technology and Advanced Engineering*, 42-46.
3. Babu V., Thapiyal A. And Patel K.G. (2014) Biofuel Production. Canada: John Wiley & Sons.
4. Muniappan R., Reddy G.V. and Raman A. (2012) Biological Control of tropical weeds using Arthropods. New York: Cambridge university press.
5. Polaina J. And Mac Cabe A. P. (2007) Industrial Enzymes: Structure, Function and Applications. Netherlands: Springer.
6. Uhlig H. (1998) Industrial Enzymes and their Applications.Canada: John Wiley & Sons, Inc.
7. Verma A., Das S. And Singh A. (2008) Laboratory Manual for Biotechnology New Delhi: S. Chand and Company Pvt Ltd.

Figure 1. Pretreatment of WHB with Different Reagents **Figure 2. Basal Medium with Various Concentrations of Substrates**

Figure 3. Appearance of Zones of Hydrolysison CMC Agar Plate

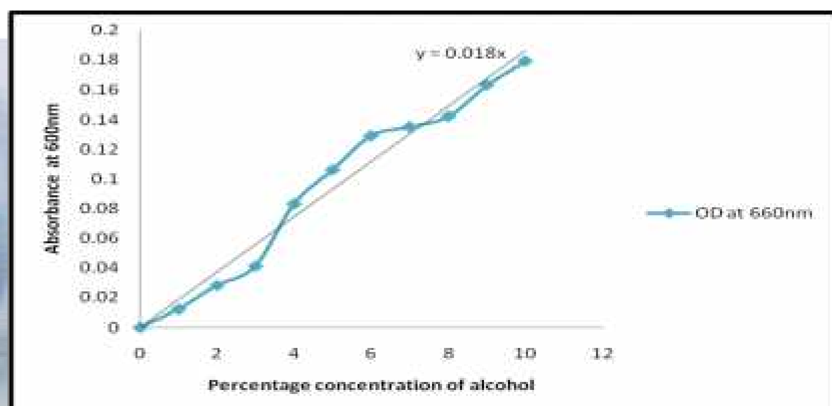

Figure 4. Alcohol Estimation Graph

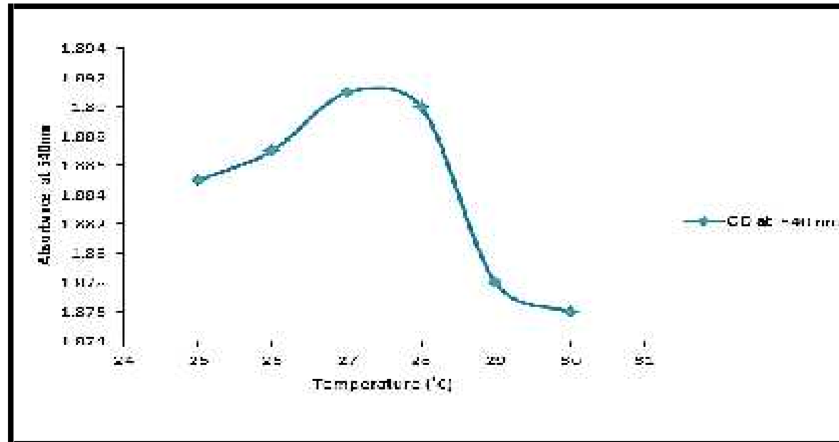

Figure 5. Graph of Temperature Optimization for Enzyme Activity

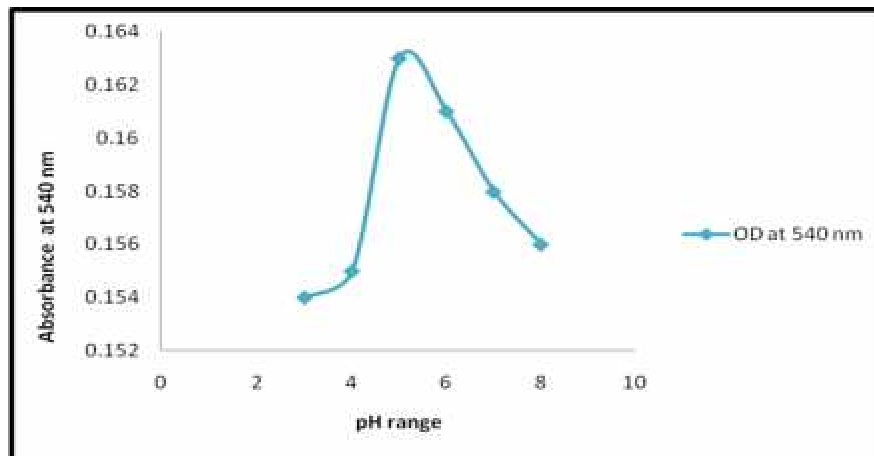

Figure 6. Graph of Ph Optimization for Enzyme Activity

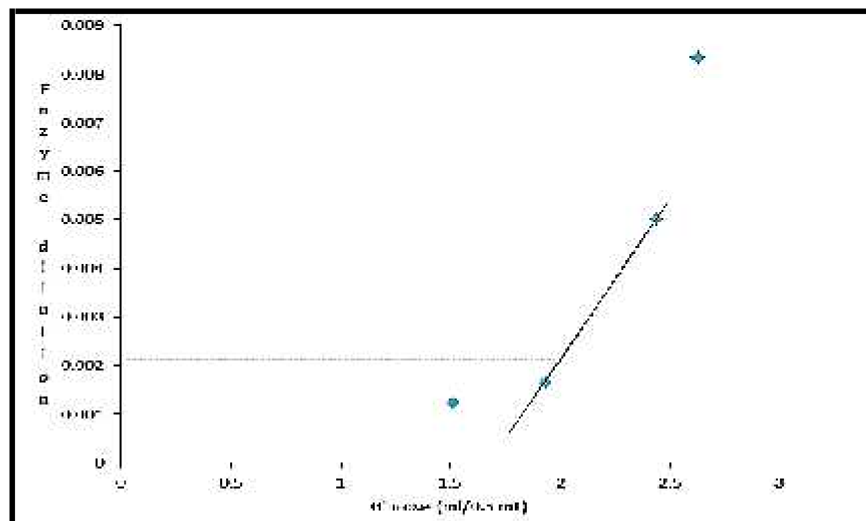

Figure 7. Graph Showing Concentration of Enzyme which has Released Exactly 2.0 Mg of Glucose