Effective utilization of an aquatic weed Salvinia Molesta as a substrate for the production of Cellulase Enzyme – Eradication through utilization

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ABSTRACT

Most of the freshwater systems in our country are infested with one kind of aquatic weed or the other causing serious environmental problems. All efforts to control the growth and spread of these weeds have failed miserably and hence the concept of eradication through utilization is being adopted by many researchers. Cellulases are a group of hydrolytic enzymes capable of hydrolyzing the most abundant organic polymer i.e. cellulose to smaller sugar components including glucose subunits. The high cost of production of cellulase enzymes has hindered the industrial application of cellulose bioconversion. Production cost of Cellulases may be brought down by multifaceted approaches which include the use of cheap lignocellulosic substrates for fermentation production of the enzyme, the use of cost-efficient fermentation strategies and the optimization of the key factors of the fermentation process. This study is conducted to produce cellulase by wild type Pseudomonas strain, using Salvinia Molesta an aquatic weed as substrate. About twenty five bacteria were isolated from decayed Salvinia waste. It was found that three isolates showed significant positive results with clear zone around the cultures. According to the morphological studies and staining techniques two isolates were found to be the genus of Bacillus and Pseudomonas. The highest crude enzyme production was observed at pH 7 and temperature of 50°C in a medium that was supplemented with Salvinia molesta as carbon source using Pseudomonas strain.

Keywords: Cellulase production, Salvinia molesta, wild type Pseudomonas stain, enzyme purification, enzyme characterization.

1. Introduction

Increasing demand, rising cost of fossil fuels and global climatic changes have shifted global efforts to utilize renewable resources for the production of alternative energy. Cellulose being an abundant and renewable resource is a potential raw material for the microbial production of food, fuel, and chemicals (Ojumu et.al., 2003). The bioconversion of cellululosic materials has been receiving attention in recent years. The high cost of production of Cellulase enzymes has hindered the industrial application of cellulose bioconversion (Narasimha.G.et.al 2006). Production cost of Cellulase may be brought down by multifaceted approaches which include the use of cheap lignocellulosic substrates for fermentation production of the enzyme, the use of cost-efficient fermentation strategies and the optimization of the key factors of the fermentation process (Fan et al., 1987).

Cellulase could be produced by many lignocellulosic feed stocks such as straws, bagasse, wheat bran, corn stover, corncob, etc. Large quantities of lignocellulosic wastes are generated...
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everyday which remain unutilized and accumulate as wastes in the environment thereby causing pollution problem (Immanuel, G et.al., 2006). Salvinia molesta was known as one of the fastest growing plants and a kind of unwanted species in India. The large-scale outbreak of it leads to many environmental problems and causes huge economic loss by impeding water flow, accelerating water evaporation, increasing mosquito breeding. In order to resolve these problems, the concept of eradication through utilization is being adopted by many researchers. (Nagendra Prabhu, 2001)

The approach of utilizing Salvinia molesta for cellulase production was expected to serve the twin function of removal of nuisance weeds as well as reduction of enzyme cost. However; no data about cellulase production from Salvinia molesta has been reported at present. Salvinia molesta have high content of hemicellulose, cellulose and protein which can provide enough nutrients for Cellulase producing microorganisms. In this study, Salvinia molesta was used first time as the main substrate for Cellulase production by the microorganisms. The present investigation was conducted to isolate native microorganisms from the decayed Salvinia molesta and to screen them for their ability to utilize cellulose from Salvinia molesta in a cost-effective and ecofriendly way.

2. Materials and methods

2.1 Isolation and identification of cellulolytic microbes

The decayed Salvinia molesta wastes were collected in the sterile container and were shaken for 1hr on a rotary shaker at 250 rpm to disperse the samples. These samples were diluted and spread plated on a nutrient agar and the plates were incubated for 48hrs at 37°C for bacteria.

2.2 Screening of cellulase producing microorganisms

Carboxymethyl cellulose (CMC) agar medium with 1% of cellulose was prepared and sterilized (autoclave at 121°C for 15 min). This medium was inoculated with loopful of culture at the centre of agar plate. Three replicates were used for the each isolates. The inoculated plates were incubated at 37°C for 4 days. After incubation, the zone was identified around the culture by treating with Congo red and NaCl. The cellulase positive colonies were identified by Gram staining methods and biochemical test and prepared pure culture of these microorganisms.

2.3 Cellulase production by submerged fermentation using salvinia molesta as substrate

Production of cellulase enzyme under submerged fermentation using Salvinia molesta as the substrate was carried out using standard techniques. The strain, for submerged fermentation was cultivated in 100 ml Erlenmeyer flasks containing 10 g of Salvinia molesta with 50 ml of minimal media composed of (g/250ml): (NH4)2SO4 0.35, KH2PO4 0.51, CaCl2 0.075, MgSO4.7H2O 0.075, Citric acid 0.0625, Tween 80 -0.5 ml, yeast 0.25 and Trace metal stock solution 1 ml. The trace metal stock solution composed of (g/500 ml): FeSO4 2.55, MnSO4. H2O 0.93, ZnSO4. H2O 1.78, C(NO3)2.6H2O1.25, and Conc. HCl 5 ml. The production medium was incubated for 48 hrs on a rotary shaker 120rpm at 37°C. The culture was centrifuged aseptically as extracellular cellulase preparation.

2.4 Protein purification by ion exchange chromatography
Ion-exchange chromatography was used for purification of cellulase. Cellulase purification was conducted using ion exchange column by Amersham Pharmacia Biotech. Running buffer used was 50 mM phosphate buffer (pH 7.0) and elution buffer was 1M NaCl in 50mM phosphate buffer pH 7.0.

2.5 Measurement of enzyme activity

Filter paper activity (FPase) for total cellulase activity in the culture filtrate was determined according to the standard method. 0.5ml of culture filtrate as enzyme source was added to whatman no. 1 filter paper strip (1 x 6 cm; 50 mg) immersed in 1ml of 0.5 M Sodium citrate buffer of pH 5.0. After incubation at 50 °C for 1h, the reducing sugar released was estimated by dinitrosalicylic acid (DNS) method. One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar from filter paper per ml per min. CMCase activity was measured using a reaction mixture containing 1 ml of 1% carboxymethyl cellulose (CMC) in 0.5 M citrate acetate buffer of pH 5.0 and 0.5ml of enzyme supernatant filtrate. The reaction mixture was incubated at 50°C for 30 min and the reducing sugar produced was determined by DNS method.

2.6 Reducing sugars content

Reducing sugars analysis was conducted by using 2 ml of culture filtrate which was added to 3 ml of DNS and boiled for 15 min. After cooling, 1 ml of Rochelle salt was added. The absorbance was recorded at 575 nm using a spectrophotometer against the blank of distilled water.

2.7 Effect of incubation period in enzyme production

Fermentation period is important parameter for enzyme production by Pseudomonas sp. Enzyme production medium was inoculated with Pseudomonas sp and incubated on shaker at 120 rpm at 37°C for 7 day’s period. At the intervals of 24 hr, the supernatant was filtered and centrifuged at 11000 x g for 10 min and the supernatant was used as crude enzyme solution.

2.8 Effect of temperature and pH on enzyme production

The most suitable pH of the fermentation medium was determined by adjusting the pH of the culture medium in the range of pH 3 to 9 using different buffers. In order to determine the effective temperature for cellulase production by the Pseudomonas sp. fermentation was carried out at 10 0 C intervals in the range of 20 to 50 0 C for 2 days.

2.9 Partial characterization of enzyme

The temperature profile for cellulase activity was determined by varying the incubation temperature between 20°C to 60°C at pH 7. In the same way, cellulase activity was determined in the pH range of 3.0–9.0 using sodium citrate for pH 3.0–6.0, sodium phosphate for pH 6.0–8.0, Tris–HCl for pH 8.0–9.0.

3. Results and discussion

3.1 Isolation, identification and screening of cellulase producing microorganisms from lignocellulosic waste
Twenty Five strains of cellulose-degrading microorganisms were isolated from decayed Salvinia molesta. All strains of bacteria at 37°C and fungi at room temperature grew well on CMC-containing medium under aerobic condition. The five cultures were tentatively identified by observing colony morphology and staining techniques. Bacteria were identified by Gram staining and biochemical tests. All strains were a Gram positive except fourth culture which is Gram negative and rod-shaped bacterium. Fungus identification was done by morphological and lacto phenol cotton blue staining under microscope (Table1). Screening of cellulase enzyme production was carried out by Congo red test. The five microbial cultures showed significant clear zone around the colony in the plate confirming that all five microbial colonies were capable of degrading cellulose efficiently and utilize it as carbon source. (Table2). Interestingly, strains of Pseudomonas sp. found to be effective secretors of cellulolytic enzymes and render a promising, industrially relevant alternative to fungal systems because of its high productivity. So that further studies carried out with Pseudomonas sp

Table 1: Identification of Cellulase producing bacteria by morphological and biochemical tests

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Organisms</th>
<th>Staining</th>
<th>Morphology</th>
<th>Indole</th>
<th>Methyl red</th>
<th>Voges–Proskauer</th>
<th>Citrate</th>
<th>Catalase</th>
<th>Amylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bacillus sp.1</td>
<td>Positive</td>
<td>Rod</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Bacillus sp.2</td>
<td>positive</td>
<td>Rod</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Pseudomonas sp</td>
<td>negative</td>
<td>Rod</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2: Screening of Cellulase producing microbes by Congo red test

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Organisms</th>
<th>Clear Zone diameter in mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bacillus sp.1</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>Bacillus sp.2</td>
<td>23</td>
</tr>
<tr>
<td>3</td>
<td>Pseudomonas sp</td>
<td>28</td>
</tr>
</tbody>
</table>

3.2 Cellulase production by submerged fermentation

Production of Cellulase using Salvinia molesta as substrate had been carried out with Pseudomonas strain. The results showed (Table 3) that Salvinia molesta was the best carbon substrate for Cellulase production by Pseudomonas sp. The enzyme production from 10 g of substrate was 1.53U/ml for CMCase activity and 0.968 U/ml for FPase activity. These results were confirmed by the results of Abo State et al.(2010).

Table 3: Production of cellulase enzyme from Salvinia molesta by Pseudomonas sp

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme activity (U/ml)</th>
<th>Reducing sugar(µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CMCase</td>
<td>FPase</td>
</tr>
<tr>
<td>Salvinia</td>
<td>1.53</td>
<td>0.968</td>
</tr>
</tbody>
</table>

3.3 Effect of incubation period in enzyme production
The incubation period is directly related with the production of enzyme and other metabolic process up to a certain extent. The incubation period to achieve peak cellulase activity by the isolate Pseudomonas sp. was 2\textsuperscript{nd} day (Figure 1). It might be due to the depletion of nutrients in the medium which stressed the bacterial physiology resulting in the inactivation of secretary machinery of the enzymes.

![Figure 1: Effect of incubation time on enzyme production](image1)

3.4 Effect of temperature and pH on enzyme production

Temperature is also an important factor that influences the cellulase yield. Maximum enzyme production using Salvinia molesta as substrate by Pseudomonas sp. was found to be between 40 to 50\textdegree{}C (Figure 2). Many workers have reported different temperatures for maximum cellulase production either in flask or in fermentor studies using Trichoderma sp. suggesting that the optimal temperature for cellulase production also depends on the strain variation of the microorganism (Murao et al., 1988, Lu et al., 2003).

![Figure 2: Effect of Temperature on enzyme production by pseudomonas sp using Salvinia as substrate](image2)

Like temperature, Cellulase yield by Pseudomonas sp. appear to depend on pH value. Cellulase production, gradually increased as the pH value increased from 6 to 8 and reached
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its maximum at pH of 7. It was also noted that the enzyme activity was stable at pH range of 6.0 to 8.0.

### 3.5 Partial Characterization of Enzyme

The effect of the pH on the crude cellulase activity of *Pseudomonas* sp. was examined at various pH ranging from pH 3.0 to 9.0 (Figure 3). The enzyme has a broad range of pH activity (pH 6-8) with optimal at 7 which was close to the optimal pH value of most bacterial cellulases.

![Figure 3: Effect of pH on the Cellulase enzyme activity](image)

The effect of temperature on crude Cellulase’s activity was determined at various temperatures ranging from 20 to 60°C at pH 7.0 (Figure 4.). The enzyme showed a good activity between 35 to 50°C with maximum activity (1.3 U/ml) at 50°C, the optimum temperature for cellulase activity found at 50°C.

![Figure 4: Effect of temperature on the Cellulase enzyme activity](image)

### 4. Conclusion

Cellulase enzyme production accounts for 40% of cost in bio ethanol production. To reduce the cost of Cellulase production, lignocellulosic substrate is used instead of synthetic cellulose due to their reasonable cost, high enzyme production capacity etc. The reduction in
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cost paves an economically easy way of ethanol production. It is an important issue to deal with the residue both the comprehensive utilization of lignocellulosic resources and for the prevention of environmental pollution. Large amount of money is wasted by many countries for the removal of the aquatic weeds. Due to their ability for quick vegetative reproduction and production of dormant seeds, the cleared areas are again infested. The only way of successful eradication is by popularizing the concept of utilization. Most of the aquatic weeds can be changed into many other useful products, as bio fertilizer, processed food, biogas, for waste water treatment, for the production of paper and fiber etc. The microorganism Pseudomonas sp used in this study was able to grow and produce Cellulase using Salvinia molesta as sources of carbon. The optimum of pH and temperature of enzyme production were of 7.0 and 50°C, respectively, and the Cellulase retained 50% of activity at 50°C. The results herein obtained make this strain and low cost substrate, worthy of further investigation, and potentially feasible for biotechnological applications in different area.

5. References


