Media Optimization for Hyper-production of Carboxymethyl Cellulase using proximally analyzed agro-industrial residue with *Trichoderma harzianum* under SSF

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Abstract

Now a day’s enzyme production is a growing field of biotechnology especially related to industrially important enzymes. The present study was carried out to investigate the potential of a filamentous fungus, *Trichoderma harzianum* for hyper-production of third most demanded industrial enzyme carboxymethyl cellulase using cheap and easily available agro-industrial residue wheat straw as growth supporting substrate under still culture solid state fermentation technique. Production of carboxymethyl cellulase was substantially enhanced through media optimization process. To promote carboxymethyl cellulase production, we evaluated the effect of several kinetic parameters like pretreatment, substrate concentration, initial moisture content, pH, incubation temperature and inoculum size on carboxymethyl cellulase production. Samples were harvested after every 24 hrs to study the profile of cellulase enzyme produced by the fungus on proximally analyzed wheat straw. By optimizing the SSF medium containing 2% HCl pretreated wheat straw; maximum carboxymethyl cellulase activity (480±4.22 µM/mL/min) was recorded after 7th day of incubation at pH 5.5; temperature, 35°C; moisture, 40% and inoculum size, 10%, using optimum substrate concentration (3%). Outcome of the research will be supportive in the improvement of low cost system for hyper-production of carboxymethyl cellulose for industrial application.

Keywords: Carboxymethyl cellulase, lignocellulosic residue, media optimization, proximal analysis, SSF, *Trichoderma harzianum*

Introduction:

Cellulose, hemicelluloses and lignin are the major components of plant cell walls, with cellulose being the most abundant component of plant biomass among all of them, which comprises on average 35 to 50% of plant biomass, however, the cellulose fibers are embedded in a matrix of other structural biopolymers, primarily hemicelluloses and lignin, which comprise 20 to 35% and 5 to 30% of plant dry weight respectively. Approximately 30 individual cellulose molecules are assembled into larger units known as proto fibrils, which are crammed into larger units called micro fibrils, and these are in turn assembled into the familiar cellulose fibers.

There is a wide spectrum of micro-organisms which can produce the variety of enzymes like ligninases, cellulases, hemicellulases, pectinases, esterases, oxidoreductases and proteases under appropriate culture conditions and among them *Trichoderma, Aspergillus, Penicillium*, and *Fusarium* genera are some of them and *Trichoderma harzianum* is one of the most efficient cellulases producer which has been extensively studied for the production of cellulose degrading enzymes from various agro-industrial waste materials and their by-products such as wood, wheat bran, rice straw, corncobs, corn stover, wheat straw, rice husk, and bagasse; these wastes are not been discarded properly in developing countries and become a major origin of ecological pollution and the use of such agro based waste materials makes them environmental friendly.

Cellulose degrading enzymes system is a complex of three major types of enzymes that exhibit higher collective activity and degrade cellulose, a phenomenon known as synergism. Cellulolytic enzyme system composed of: (i) carboxymethyl cellulase (CMCase) also called as endoglucanase (endo-1,4-β-D-glucanase, EG, EC 3.2.1.4), (ii) Exoglucanases, including 1, 4-D-glucan glucanohydrolases also known as celledextrinases (EC 3.2.1.74) and 1, 4-D-glucancellulbiohydrodrolases (cellbiohydrodrolases) (EC 3.2.1.91), and
(iii) glucosidases or glucoside glucohydrolases (EC 3.2.1.21) 5,6,35. CMCases (endoglucanase) converts polymeric appearance of cellulose into oligosaccharide outline, and then exoglucanase separates cellobiose into glucose units 16.

Being an agricultural land, Pakistan produced million of tones of agriculture waste annually in the form of crop residues and wheat is one of the most important agricultural crops. Wheat straw contains 29-35% cellulose in it. Cellulases have wide range of applications and are being used in the field of cotton processing, paper recycling, in juice extraction, as detergent and animal feed additives 35 brewery, textile and laundry industries, animal feed industry, wine, agriculture as well as in the field of research and development 25,37.

This paper work reports the remarkable carboxymethyl cellulase production potential of Trichoderma harzianum from agro-industrial residue wheat straw, which has sufficient amount of cellulose (39.4 %), used as an alternative energy source for microorganism.

Materials and Methods

Chemicals and lignocellulosic Substrate: All the chemicals used were of analytical grade unless otherwise stated and purchased from Fluka (France), Merk (Germany), Scharlau (Spain) and Sigma Chemical Co., USA. Lignocellulosic agro-industrial waste, wheat straw was obtained from Student research Farms, University of Agriculture, Faisalabad (UAF), Pakistan. The substrate was sun and oven dried (60°C) and ground to 40 mm mesh size and stored in air tight polyethylene bags to keep it moisture free.

Fungal Culture and Inoculum development: The pure culture of Trichoderma harzianum was available in Molecular Biotechnology Laboratory; UAF was used as cellulase producer. An inoculum was prepared by growing the fungus in inoculum medium. Inoculum medium was the Vogel’s nutrient medium [39] supplemented with (1%) Millipore filtered sterile glucose as an additional energy source and trace element solution containing Citric acid.H2O, 5g; ZnSO4.7H2O, 5 g; Fe (NH4)2(SO4)2.6H2O, 1 g; CuSO4.5H2O, 250 mg; MnSO4.H2O, 50 mg; H3BO3, 50mg; Na2MoO4.2H2O, 50 mg and H2O up to 100 mL was added for optimal growth [5]. The medium was sterilized (121°C) in laboratory scale autoclave (Sanyo, Japan) for 15 minutes. After cooling to room temperature, loopful spores of Trichoderma harzianum from PDA slant were transferred into the broth under sterilized conditions in laminar air flow (Dalton, Japan). The inoculated flasks were shaken (180rpm) at 30°C for 5 days in an orbital shaker (Sanyo-Gallemkemp, UK) to get homogenous fungal spore suspension.

Pretreatment of wheat straw: Lignocellulosic wheat straw (10g) was carefully pretreated with 1 to 5% HCl in an Erlenmeyer flask (250 mL) kept at room temperature up to 2 hrs, autoclaved at 121°C and 15 lb/inch² pressure for 15min. After this slurry of substrate was filtered through four layers of muslin cloth, residues were washed 4 to 5 times with distilled water and used for production of carboxymethyl cellulase and further proximate analysis; while, filtrate was used for analysis of total sugars and reducing sugars.

Fermentation Protocol: Vogel’s nutrient fermentation media was used to moist the pretreated substrate (10g) in an Erlenmeyer flask (250 mL) for carboxymethyl cellulase production [4]. The initial pH value of the medium was adjusted to 6 before sterilization at 121 °C and 15.0 lbs/inch² pressure for 15 min. The autoclaved medium was inoculated with 5 mL of freshly prepared fungal spore suspension and incubated at 30±1°C in a temperature controlled incubator (Sanyo-MIR-254, Japan) for stipulated time period under still culture conditions.

Proximate Analysis: Lignocellulosic substrate wheat straw was proximally analyzed under standard experimental conditions for determination of % moisture, ash, fat, lignin and cellulose content before acidic pretreatment, to investigate chemical composition of substrate.

Analytical Methods:
**Total sugar Estimation:** To measure the total sugar in the medium, Phenol sulfuric acid method⁰¹ was used and optical density was checked at 470 nm to measure color intensity.

**Reducing Sugar Estimation:** Reducing sugar was measured using 3, 5-dinitrosalicylic acid (DNS) by the method of Miller, ²⁴ and optical density was checked at 550 nm to measure color intensity.

**Protein Estimation:** Protein in the medium was determined by the method of Lowery, ²¹ with bovine serum albumen as standard.

**Enzyme extraction:** At the end of stipulated fermentation time, Citrate buffer 0.05 M of pH 4.8 was added in 1:10 (w/v) ratio to the fermented biomass and the flasks were shaken at 120 rpm for 30 minutes ¹⁹. The contents were filtered through muslin cloth and washed thrice with citrate buffer. The filtrates were centrifuged at 10,000 rpm (4°C) for 10 minutes and carefully collected supernatants were used for enzyme activity determinations.

**Enzyme activity:** Enzyme activity of supernatants collected at the end of each optimization step were determined using spectrophotometer (T60, PG Instruments, UK) by the method of Ghose, ¹⁵. The reaction mixture contained 0.5 mL of carboxymethyl cellulose as substrate in 0.05 M Na–citrate buffer of pH 4.8 and finally 0.5 mL of diluted crude enzyme and incubated at 50 °C for 30 min. An appropriate control which contained 0.5 mL of distilled water instead of crude enzyme extract was also run along with the test. At the end of the incubation period, tubes were removed from the water bath, and the reaction was stopped by addition of 3 mL of 3, 5-dinitrosalicilcic acid reagent per tube. The tubes were incubated for 5 min in a boiling water bath for color development and were cooled rapidly. The activity of reaction mixture was measured against a reagent blank at 540 nm. The concentration of glucose released by enzyme was determined by comparing against a standard curve constructed similarly with known concentrations of glucose. **Unit enzyme activity** was defined as the amount of enzyme required for liberating 1µM of glucose per milliliter per minute and was expressed as µM/mL/min.

**Statistical analysis:** All experiments and enzyme assays were performed in triplicate, statistically evaluated ³⁶ and results have been presented as mean ± S.E. (standard error). The S.E values have been displayed as Y-error bars in figures.

**Results and Discussion**

1. **Chemical Composition of Substrate:** The suitability of agro-industrial waste residue wheat straw for carboxymethyl cellulase production from *Trichoderma harzianum* under solid state fermentation condition was investigated. For this purpose chemical composition of wheat straw was determined through proximal analysis under standard experimental conditions. Results of proximal analysis showed moisture, ash, fat, cellulose, lignin and protein content of wheat straw were 5.074 %, 7.30 %, 1.854 %, 39.4 %, 6.4 % and 3.50 % respectively while, the amount of total sugar and reducing sugar were 0.552 % and 0.535 % respectively (Table 1). Almost similar amount of moisture, ash and fat contents were also reported by Oluremi *et al.* ²⁸. In structural and chemical composition wise, lignocellulosic wheat straw contained almost 29-35% cellulose ²³. Agro-industrial and agricultural residue contained Cellulose, hemicellulose and lignin content in % of 33–40, 20–25 and 15–20 respectively ³⁰.

2. **Effect of Pretreatment on Cellulase Production:** To study the effect of pretreatment of substrate on cellulose production, acidic pretreatment was given to wheat straw with HCl at different concentrations ranging from 1 to 5 % in an Erlenmeyer flask (250 mL) to ensure proficient deprivation of lignocellulosic contents of wheat straw to get optimum cellulase production. It was observed that pretreatment cause the removal of lignocellulosic contents including lignin and hemicelluloses successfully, while at the same time pretreatment also cause the loosening in structure of lignin and decrease the crystallinity of cellulose that improves the porosity characteristic of substrate. A significant difference in enzyme activity was observed when pretreated substrate was inoculated with freshly prepared inoculum of *Trichoderma*
harzianum with the change in acid concentration and became maximum 175±2.78 µM/mL/min at 2% while, further increase in concentration did not enhance the enzyme activity (Figure 1). The acid catalyzed pretreatment significantly effective for hemicellulose removal from the biomass and this removal in turn displays the cellulose to enzymatic attack 7. The rate of β-glucosidase and CM-cellulase production by Aspergillus fumigatus in H2SO4 and HCl pretreated wheat straw mineral medium reached maximum 9. Ojumu et al. 27 reported high cellulase activity from 3% pretreated saw dust, bagasse and corn cob as substrate respectively.

3. Effect of Substrate Concentration on Cellulase Production: 2 % HCl pretreated wheat straw was used to investigate the effect of substrate concentration on carboxymethyl cellulase production. As shown in figure 2 different concentrations of substrate (wheat straw) ranging from 1-5% were used and maximum cellulase activity 278±1.66 µM/mL/min was obtained at 3% substrate concentration. A dynamic influencing feature that affects the yield and initial hydrolysis rate of cellulose is substrate concentration 32. Low substrate concentration results in an increase in yield and reaction rate of the hydrolysis while, high substrate concentration can cause substrate inhibition, which substantially lowers enzyme formation 20,34.

4. Effect of Moisture Content on Cellulase Production: In the solid state fermentation moisture content is a key factor for enzyme production. In order to optimize moisture content, different %ages of moisture were employed ranging from 10-50% at 2 % acidic pretreated lignocellulosic substrate wheat straw. The present study indicates that maximum carboxymethyl cellulase production was observed at 40% moisture level (Figure 3) while, further increase in moisture influenced the enzyme production negatively. These results indicated a positive relationship between cellulase production and moisture content. Higher and lower water contents adversely affect the primary metabolic activities of microbes leading to slower enzyme synthesis 13. Optimal water fractions in the solid substrate appear to be 40 to 60 % (by mass) under solid-state fermentation 26,34 reported that optimization of incubation temperature and initial moisture content of the medium resulted in a 6.2 fold increase in production from 0.605 to 3.8 U/gds of cellulase.

5. Effect of initial pH on cellulase production: 2% acidic pretreated wheat straw was moistened (40%) using Vogel’s nutrient fermentation media of varying pH (3-7). The effect of initial pH on enzyme production was determined and maximum carboxymethyl cellulase activity (392±3.33µM/mL/min) was recovered at pH 5.5 (Figure 4). Further increase or decrease in pH from this level retarded the enzyme activity. It was reported that the optimal pH for a cellulase varies from species to species and has a broad range between 3 and 9 2, 3. Similar results are also reported by Sami et al. 33 and Pushalkar et al. 31 they found that the CMCase and β-glucosidase were more active on substrate at pH 5.8 and 4.0-5.5 respectively.

6. Effect of Incubation Temperature on Cellulase Production: To reveal the effect of different temperatures on cellulase production from Trichoderma harzianum from pretreated wheat straw by solid-state fermentation experiments were conducted at various temperatures between (25-45ºC). It was observed that enzyme activity was increased with increase in temperature up to 35ºC with maximum activity of 422±3.21 µM /mL/min (Figure 5). The temperature of the fermentation medium is one of the vital factors that have deep influence on the end product 3. Our results are highly correlated with Gori and Malana, 16 who reported maximum CMCase activity at 35ºC as an optimum temperature while, Acharya et al. 2 reported maximum cellulase production at 28ºC. Such different results may become visible because of differences within the same genus of the same fungus.

7. Effect of Inoculum size on Cellulase Production: Effect of different inoculum sizes 5-25% (v/v) were studied for the production of carboxymethyl cellulase enzyme from Trichoderma harzianum on pretreated wheat straw in SSF under temperature controlled still culture condition. Maximal cellulase activity (480±4.22 µM /mL/min) was noted at 10% inoculum size while, further increase in an inoculum’s size showed a decline in enzyme activity which became least (322±3.67 µM /mL/min) at 25% inoculum size.
(Figure 6). Hence 10% inoculum size was found optimum for the production of cellulase enzymes. Optimum spore density (number of spores per unit weight of substrate) is important for SSF process. Fadel, 12 reported maximum enzyme activity (216.2 IU/g) at 10% inoculum size with wheat straw as substrate. Omojasola and Jilani, 29 reported maximum cellulase activity with 8% inoculum size. Lower inoculum size shortens the early lag phase whereas; larger inoculum size increased the moisture content to considerable extent and cause lower enzyme formation. The free overloaded liquid prevents further obstruction with that imposed by the solid nature of the substrate and leads to a decrease in growth and enzyme production 1.

Table 1 Chemical composition of wheat straw*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Constituent</th>
<th>Value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Moisture</td>
<td>5.07 ± 0.24</td>
</tr>
<tr>
<td>2</td>
<td>Ash</td>
<td>7.30 ± 0.42</td>
</tr>
<tr>
<td>3</td>
<td>Fat</td>
<td>1.85 ± 0.02</td>
</tr>
<tr>
<td>4</td>
<td>Cellulose</td>
<td>39.40 ± 0.72</td>
</tr>
<tr>
<td>5</td>
<td>Lignin</td>
<td>6.40 ± 0.19</td>
</tr>
<tr>
<td>6</td>
<td>Protein</td>
<td>3.50 ± 0.13</td>
</tr>
<tr>
<td>7</td>
<td>Total sugar</td>
<td>0.552 ± 0.015</td>
</tr>
<tr>
<td>8</td>
<td>Reducing sugar</td>
<td>0.535 ± 0.009</td>
</tr>
</tbody>
</table>

*Proximal Analysis

Figure 1 Effect of varying HCl concentrations on carboxymethyl cellulase production by *Trichoderma harzianum* in SSF of wheat straw
Figure 2 Effect of varying substrate concentrations on carboxymethyl cellulase production by *Trichoderma harzianum* in SSF of wheat straw

Figure 3 Effect of varying moisture levels on carboxymethyl cellulase production by *Trichoderma harzianum* in SSF of wheat straw

Figure 4 Effect of varying pH values on carboxymethyl cellulase production by *Trichoderma harzianum* in SSF of wheat straw
This paper work reports a superior way out for proper waste management for agro based waste material, through their bio-utilization with a filamentous fungus *Trichoderma harzianum* for carboxymethyl cellulase production that could be used in the industrial applications such as bioethanol production. The results of this present study indicate the remarkable carboxymethyl cellulase production potential of *Trichoderma harzianum* from agro-industrial residue wheat straw. Classical method of optimization was used to evaluate the physico-chemical factors for hyper-production of carboxymethyl cellulase. However, the suitability of the enzymes for industrial applications can be investigated through kinetic characterization of the purified enzymes as thermo-stability is a desired characteristic of an enzyme for its possible use in industry.

**References**


