

Optimization of Physical and Nutritional Factors for Induced Production of Cellulase by Co-Culture Solid-State Bio-Processing of Corn Stover

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Abstract: - Bio-conversion of cellulosic based biomass materials for cellulase production is one among the major increasing demand for various biotechnological applications. Therefore, the purpose of the present research work was to study the production and physical and nutritional parameters optimization for the synthesis of cellulase from co-culture of *Trichoderma viride* and *Ganoderma lucidum* in solid state fermentation (SSF) using agro-industrial material corn stover as fungal growth supported substrates. Analysis profile showed that when the conditions of the SSF medium containing 15 g corn stover substrate (50% w/w moisture) inoculated with 6 mL of inoculum were optimal, the maximum productions of cellulase (466 ± 5.6 U/mL) were recorded after 6 days of incubation at pH 6 and 35 °C. In conclusion, the present research findings will be supportive in the improvement of low cost system for hyper-production of carboxymethyl cellulase for industrial application.

Key-Words: - Cellulase, Agro-industrial residue, Optimization, Co-culture, *T. viride*, *G. lucidum*

1 Introduction

A broad spectrum of micro-organisms especially most of the fungal species including *Trichoderma*, *Aspergillus*, *Penicillium*, and *Fusarium* have the ability to produce industrially important enzymes [1, 2]. *Trichoderma* is one among the most efficient cellulose-degrading enzymes producers from various agro-industrial waste materials and their by-products, such as citrus peel, corn stover, rice straw, wheat straw, banana waste, bagasse, and many others has been extensively studied [3-5].

The major components of plant cell walls are cellulose, hemicellulose and lignin, with cellulose being the most abundant component. Cellulose is a homologous polymer of glucose, composed of repeating D-glucose units linked by β -1, 4-glucosidic bonds [6]. Cellulases and hemicellulases are two important classes of enzymes produced by filamentous fungi and secreted into the cultivation medium [1]. Cellulases from *Trichoderma* and *Aspergillus* species have good potential to be used in cellulase production and β -glucosidase, for complete hydrolysis of cellulose [7].

Currently, cellulase is being used in many industrial applications, especially in the fields of cotton processing, paper recycling, and agriculture,

as animal feed additives, and in research and development [1-5, 8, 9]. The most important biotechnological applications is the conversion of agricultural wastes into products of commercial interest such as ethanol, bio-based products and bio-energy to replace the diminishing fossil fuels [10]. By keeping in mind the extensive industrial applications of cellulases, the purpose of the present research work was to study the production and physical and nutritional parameters optimization for the synthesis of cellulase from co-culture of *Trichoderma viride* and *Ganoderma lucidum* in solid state fermentation (SSF) using agro-industrial material i.e., corn stover, in order to introduce its potential applications.

2 Materials and Methods

2.1 Chemicals and Substrate

All the chemicals used were of analytical laboratory grade. The agro-industrial waste, i.e. corn stover was obtained from a local fruit market in Gujrat, Pakistan. Corn stover was crushed into pieces, dried and ground to powder size, and stored in plastic jars.

2.2 Cultures and Inoculum Development

The pure cultures of *T. viride* and *G. lucidum* were obtained from the Department of Biochemistry, University of Gujrat, Pakistan. To develop homogeneous inoculums, spores of the both cultures were cultivated at 30 °C for 5-7 days using 250 mL capacity Erlenmeyer flask containing 30 mL of Potato Dextrose broth. This was then incubated for the development of the fungal spore suspension.

2.3 Pre-treatment of Corn stover

Corn stover was pre-treated with 2% HCl in an Erlenmeyer flask at room temperature for 2 h, then autoclaved at 121 °C and 15 lb/in² pressure for 15 min. The slurry of corn stover was filtered through Watman No 1 filter paper; both the filtrates and the residues were saved and used for the production of cellulase enzymes and further analysis.

2.4 Cellulase Production Protocol

10 g of pre-treated corn stover was moistened with nutrient salt media in an Erlenmeyer flask (250 mL) for cellulase production. The initial pH of the fermentation medium was adjusted to 5 before sterilization, inoculated with 5 mL of freshly prepared fungal co-culture spore suspension, and incubated at 30°C in an incubator for stipulated fermentation time period.

2.5 Optimization of Physical and Nutritional Parameters

2.5.1 Optimization of time period

To optimize the fermentation time period, each flask containing pre-treated corn stover was sterilized, inoculated, and fermented at 30°C for ten days, in a still culture temperature controlled incubator. Flasks were harvested after every 24 h of fermentation time and analyzed for cellulase activity.

2.5.2 Optimization of pH

Fermentation media containing pre-treated hydrolyzate was adjusted to varying pH levels (3.5-7) before inoculation with 5 mL of fresh spore suspension and allowed to ferment for 6 days fermentation time period.

2.5.3 Optimization of temperature

To obtain the maximum enzyme production, duplicate flasks containing pre-treated corn stover were adjusted to pH 6, inoculated, and subjected to fermentation at varying temperatures ranging from 20 to 45 °C.

2.5.4 Optimization of substrate level

To investigate the optimum substrate level, varying levels of pre-treated substrate (5, 10, 15, 20, and 25 g/100 mL) were used. Duplicate flasks inoculated with fungal co-cultures were subjected to fermentation for 6 days at pH 6 and 35 °C.

2.5.5 Optimization of inoculum size

To determine the optimum inoculum level, the duplicate flasks were inoculated with varying volumes (2 to 10 mL) of freshly prepared co-cultures inoculums and then processed for 6 days at optimum pH and temperature.

2.6 Extraction of Cellulase Complex

After 6 days fermentation time period, the cellulase was extracted from the fermented corn stover biomass via the addition of a citrate buffer, 0.05 M of pH 4.8 in a 1:10 (w/v) ratio, and then the flasks were shaken at 120 rpm for 30 min [1]. The contents were filtered through Wattman No 1 filter paper and washed twice with the same buffer. The filtrates were centrifuged at 10,000×g (4 °C) for 10 min, and the collected supernatants were used as a crude enzyme extract to determine the activity.

2.7 Cellulase Assays

The quantity of cellulase was assayed according to the methodology described earlier by Iqbal *et al.* [3].

3 Results and Discussion

The optimization of various physical and nutritional growth parameters caused an increase in the enzyme activities; therefore, in this study the aim was to investigate the effects of such growth parameters in order to achieve optimized enzyme production.

3.1 Optimization of fermentation time period

The maximal enzyme activity (215 U/mL), occurred after the 6th day of inoculation with fresh spores of *T. viride* and *G. lucidum* (Fig. 1), while beyond this fermentation time period inhibition of enzyme was observed. In an earlier study, Quiroz-Castañeda *et al.* [7] has achieved maximum activity of the cellulases after 8 days of inoculation using wheat straw as a growth substrate. In present study, co-culture of *T. viride* and *G. lucidum* produced higher titers of cellulase without any additional supplements in comparison to previously studied different fungi which produced maximum enzymes after 6-8 days of fermentation [7].

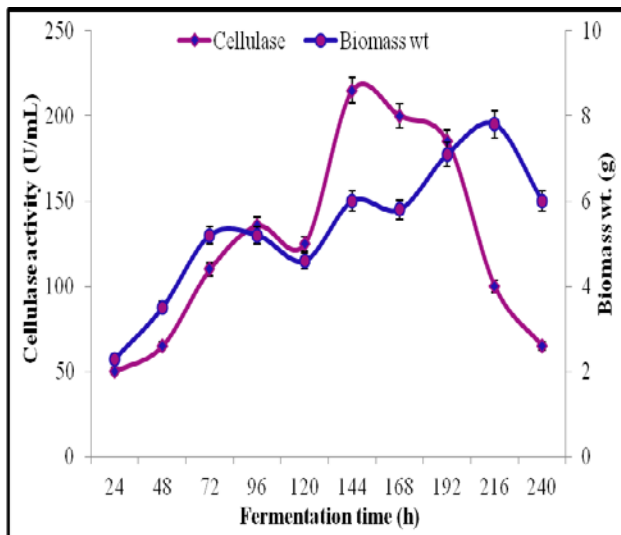


Fig.1. Cellulases activity under different incubation time period

3.2 Optimization of Initial pH

The maximum cellulase enzyme (262 U/mL) activity was recovered at pH 6 (Fig. 2). It has been reported that the optimal pH for fungal cellulases varies from species to species; though in most cases the optimum pH ranges from 3.0 to 9.0 [11]. Similar results are also reported by Pushalkar *et al.* [12], who found that β -glucosidase was more active on the substrate between pH 4.0 and 5.5. Similar results are also reported by Sami *et al.* [13] and found that the CMCase was more active on substrate in the pH 5.8.

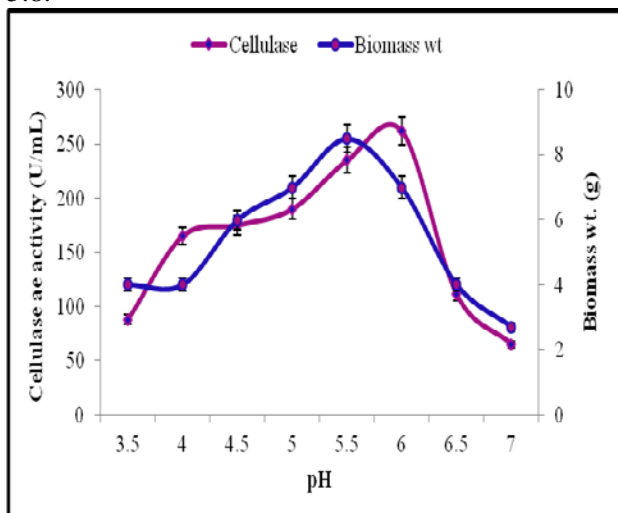


Fig.2. Cellulases activity at different pH levels

3.3 Optimization of temperature

The temperature of the fermentation medium is one of the important factors that have a deep influence on the product of interest. Figure 3 illustrates that the enzyme activity increased with the initial increase in temperature and maximal peak activity

was at 35 °C, while further increase in temperature showed decreasing trend in enzyme activity. The same phenomenon of incubation temperature has also been reported by Omojasola and Jilani [14]. Similar to our findings, an incubation temperature of 30 °C was optimal for the production of cellulase (CMCase) from *Trichoderma harzianum* [1], whereas the maximum production of CMCase by *Trichoderma* sp. was recorded on apple pomace at 32 °C by Sun *et al.* [15].

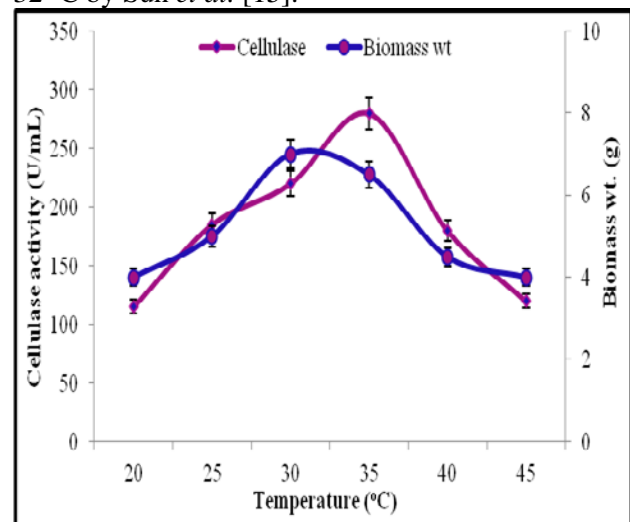


Fig.3. Cellulases activity at varying temperatures

3.4 Optimization of substrate concentration

The maximum cellulase activity (380 U/mL) activities were obtained at 15 g substrate concentration (Fig. 4). Earlier it has been reported that substrate concentration is a dynamic influencing factor that affects the product yield and the initial hydrolysis rate of cellulose [1]. A high substrate concentration can cause substrate inhibition, which substantially cause the reduction in enzyme formation [5].

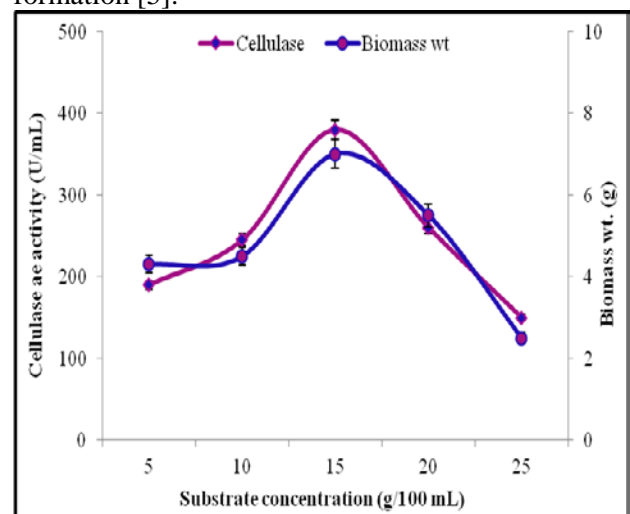


Fig.4. Cellulases activity on different substrate levels

3.5 Optimization of inoculum size

Optimum spore density (number of spores per unit weight of substrate) is important for SSF process. The maximal enzyme activity (466 U/mL) was noted at the 6 mL inoculum size of co-culture (*T. viride* and *G. lucidum*) (Fig. 5). Lower inoculum sizes shortened the microbial lag phase stage, whereas inoculum size beyond the optimum value increased the moisture factor that caused lower levels of enzyme formation due to the overcrowding of fungal spores [3-5]. *Aspergillus niger* grown under solid state fermentation conditions gave maximum enzyme activity (216.2 IU/g) at an inoculum size of 10% using wheat straw as the substrate [16]. In an earlier study Omojasola and Jilani [14] reported the maximum cellulase activity with an inoculum size of 8%.

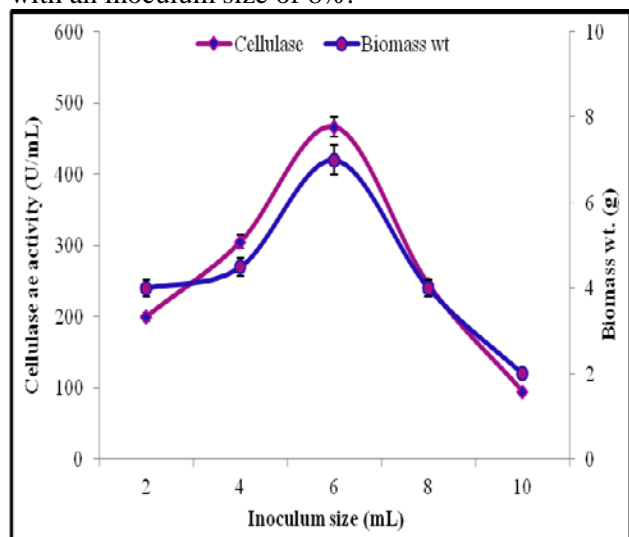


Fig.5. Cellulases activity on varying sizes of fungal inoculums

4 Conclusions

In conclusion attempt was made, to find the optimum fermentation conditions for successful cultivation of microbial co-cultures, and also towards an induced production of third most demanded industrially important enzyme cellulase. However, the suitability of the enzymes for biotechnological 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.

Acknowledgments

The authors are grateful to the Department of Biochemistry and Molecular Biology, University of

Gujrat, Pakistan for providing financial support and laboratory facilities.

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