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Influence of Nutritional Conditions for Endoglucanase Production by *Trichoderma viride* in SSF

Muhammad Irfan, Muhammad Nadeem and Quratualain Syed

Food & Biotechnology Research Center, PCSIR Labs Complex, Ferozpure road Lahore, Pakistan

Abstract: The present study dealt with the production of endoglucanase by *Trichoderma viride* in solid state fermentation in 250ml Erlenmeyer flask at 40°C for seven days of fermentation period. Various substrates like corn cobs, rice husk, rice polish, soybean meal, sunflower meal, wheat bran and sugarcane bagasse were evaluated for endoglucanase production. Of all these substrates, sugarcane bagasse treated with 1% H_2SO_4 gave the better yield of enzyme production using Vogel's medium. CMC and peptone at concentration of 0.5% as additional carbon and nitrogen sources enhance the titer of endoglucanase. Further supplementation 0.01% tween-80 as a surfactant significantly improves the enzyme production by Trichoderma viride in solid state fermentation.

Key words: Endoglucanase · *Trichoderma* Sp. · Agricultural Wastes · SSF

INTRODUCTION

Today cellulases are of great attention due to the large industrial applications. These cellulase complex are of three different types an endo-1,4-β-glucanase [Carboxymethyl cellulase (EC 3.2.1.4)], a 1,4-β-cellobiohydrolase [Exoglucanase (EC 3.2.1.91) and a 1,4-β-glucosidase [Cellobiase (EC 3.2.1.21)] which are produced by filamentous fungi. Among the cellultyic filamentous fungi, *Trichoderma* spp and *Aspergillus* spp are widely studied due to its high cellulose degrading ability [1]. There are some reports on the cellulose production by filamentous fungi in solid state fermentation by using agricultural wastes [2, 3].

Trichoderma spp has been used for long time for industrial enzyme production and the cellulase production has been tried in both liquid and solid state fermentation [4, 5]. There are various reports on the production of cellulase by *Trichoderma* species in solid state fermentation by using different strains and mutants different culture conditions using different substrates [6-10]. The cellulase enzyme produced by *Trichoderma* species are biochemically characterized and widely used in starch processing, pulp and paper industry, extraction of fruit and vegetable juices, malting and brewing, animal feed production and alcohol fermentation [11-13].

In whole fermentation process, cost is one of the most important factor which affects the feasibility of the process at industrial scale. Main problem in cellulase production by fermentation is the utilization of expansive substrates. Production cost may also be reduced by using low cost carbon and nitrogen sources in the formulation of the fermentation medium [14, 15]. This cost of the substrates may be reduced by possible modifications of cellulosic materials using high cellulase producer microorganisms [16]. Our objective of the study was to enhance production of cellulase (endoglucanase) by selecting the appropriate substrate, pretreatment of the substrate and addition of suitable carbon, nitrogen and surfactants to the fermentation medium.

MATERIALS AND METHODS

Lignocellulosic Substrates: Sugarcane bagasse, wheat bran, rice husk, rice polish, sunflower meal, soybean meal and corn cobs were procured from local market of Lahore city, Pakistan which was used as a substrate for the production of endoglucanase (CMCase). The substrate was washed and oven dried at 65°C and then ground to powder form (2mm) by hammer beater mill.

Corresponding Author: Muhammad Irfan, Food & Biotechnology Research Center, PCSIR Labs Complex, Ferozpure road Lahore, Pakistan.

Pretreatment of Substrate

Chemical Pretreatment: Chemical pretreatment of the substrate was done as previously described [17]. Sugar cane bagasse samples (10g) were soaked in 2.5% (w/v) KOH 1% (v/v) H_2SO_4, 3% (v/v) H_2O_2 solutions at the ratio of 1: 10 (solid: liquid) for 2hr at room temperature. After then the samples were autoclaved at 121°C for 15 min. Then samples were filtered and solid residues were washed up to neutrality.

Biological Pretreatment: Twenty grams of substrate was taken in 500ml conical flask and moistened with 30 ml of tab water and autoclaved at 121°C for 15 min. After autoclaving, the contents of the flask were allowed to cool at room temperature. After cooling the flask was inoculated with 10ml spore suspension of *Trichoderma viride* and incubated at 40°C for seven days. The contents of the flask were mixed each day during incubation. After seven days of incubation the substrate was washed, dried and used as a biologically treated sample source for enzyme production.

Microorganism: *Trichoderm viride* was obtained from Fermentation Biotechnology Laboratory, PCSIR Labs. Complex Ferozpure road Lahore and was used for the production of CMCase. It was maintained on PDA slants and revived biweekly.

Preparation of Conidial Suspension: Inoculum was prepared by adding sterilized distilled water into the 8-day old slant. With the help of inoculating loop the mycelia was mixed and one ml (2×10^5) of spore suspension was used as inoculum. Inoculum size was measured with heamacytometer as described earlier [18].

Fermentation Technique: Solid state fermentation was carried out for CMCase production using *Trichoderma viride*. In 250ml conical flask 5g of ground pretreated wheat straw was moistened with diluent (g/l, ammonium sulphate 10. Calcium chloride 0.5, Magnecium sulphate 0.5, Potassium dihydrogen phosphate 4) and then autoclaved at 121°C for 15 min. After sterilization the media was inoculated with 1ml of spore suspension and incubated at 40±1 °C.

Effect of Carbon and Nitrogen Sources on Endoglucanase Production: Various carbon sources like glucose, starch, xylose, fructose, maltose, carboxymethyl cellulose, arabinose, galactose, filter paper and sucrose were tested for optimized production of endoglucanase. Different nitrogen sources such as peptone, trypton, urea, yeast extract, casein, skim milk, lab lamco powder, $NaNO_3$, NH_4Cl, $(NH_4)_2SO_4$ and NH_4NO_3 were optimized for CMCase production using *Trichoderma viride* in solid state fermentation.

Enzyme Recovery: CMCase from the fermented mash was extracted by simple contact method as reported by Krishna and Chandrasekaran [19]. In 5g substrate of flask 50ml of distilled water (1:10 solid to liquid ratio) was mixed and placed on shaker at the agitation speed of 150 rpm for 2hrs. After complete mixing it was filtered through muslin cloth and the residues was discarded and the filtrate was used for further analysis.

Estimation of Endoglucanase (CMCase): Endoglucanase activity was determined as described earlier [17]. Reaction mixture containing 0.5 ml of crude enzyme sample with 0.5 ml of 1% CMC (0.05M Citrate buffer pH 5) was incubated at 50°C for 30 min. After incubation, the reaction was stopped by the addition of 1.5 ml of DNS and then boiled for 10 min in boiling water bath. The reaction mixture was allowed to cool and the reducing sugars released were estimated by Miller's method [20].

Estimation of Total Proteins: Total proteins in the culture filtrate were estimated by Lowery method [21] using bovine serum albumin as standard protein.

Statistical Analysis: All the data was statistically analyzed by using Microsoft Excel program and data represented the means of the replicates.

RESULTS AND DISCUSSION

Screening of Substrates for Endoglucanase Production: Different substrates such as sugarcane bagasse, wheat bran, rice husk, rice polish, sunflower meal, soybean meal and corn cobs were screened for endoglucanase production by *Trichoderma viride* in solid state fermentation. Of all these sugarcane bagasse exhibited highest production of endoglucanase followed by corn cobs and rice husk respectively. Sunflower meal gave the lowest production among all the substrates. Highest enzyme production in sugarcane bagasse was due to the presence of more cellulosic contents. This production variation in each substrate was due to the difference in the compositional analysis like cellulose, hemicellulose and lignin. Fawzi and Hamdy [22] evaluated different substrates like barly straw, corn cobs, rice straw, soy stalk, sugarcane bagasse and wheat straw for CMCase production and reported sugarcane bagasse was best

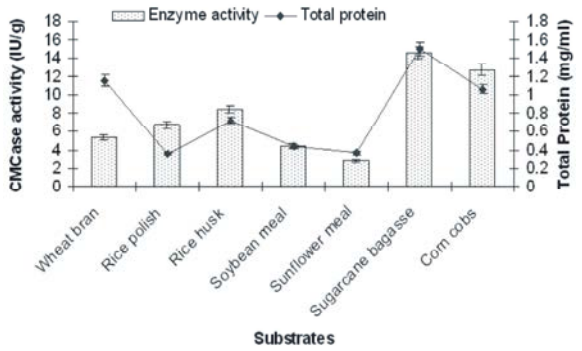

Fig. 1: Screening of substrates for endoglucanase production by *Trichoderma viride* in SSF at 40°C for seven days of fermentation period. Error bars indicates the SD among replicates.

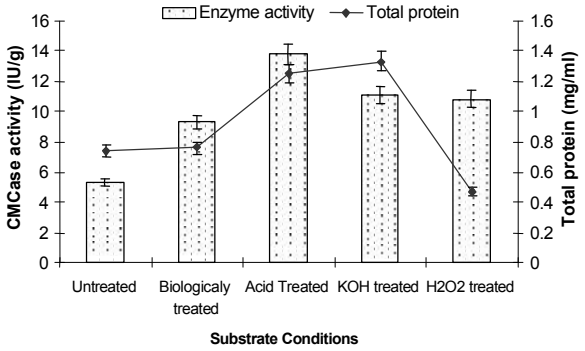

Fig. 2: Effect of pretreatment of selected substrates for endoglucanase production by *Trichoderma viride* in SSF. Error bars indicates the SD among replicates.

substrate for endoglucanase production by *Chaetomium cellulyticum* NRRL 18756 in solid state fermentation. In previous studies different substrates were reported by different workers for CMCase production by various fungal strains like, wheat straw by *Trichoderma viride* FBL-1 [23], empty fruit bunches by *Trichoderma reesei* RUT C-30 [24], oil palm empty fruit bunch by *Aspergillus terrus* [25], apple pomece by *Trichoderma* sp. [12] are the best substrates for CMCase production.

Effect of Pretreatment on Endoglucanase Production: The substrate which gave highest yield of endoglucanase production was further selected for subsequent studies. To enhance the production yield, the substrate was chemically treated with 1% H_2SO_4, 2.5%KOH, 3% (v/v) H_2O_2 and biologically treated with *Trichoderma viride* was used for endoglucanase production in SSF. Results indicated (Fig. 2) that acid treated sugarcane bagasse (13.8±0.73 U/gds) produced more enzyme production as compared to untreated sugarcane

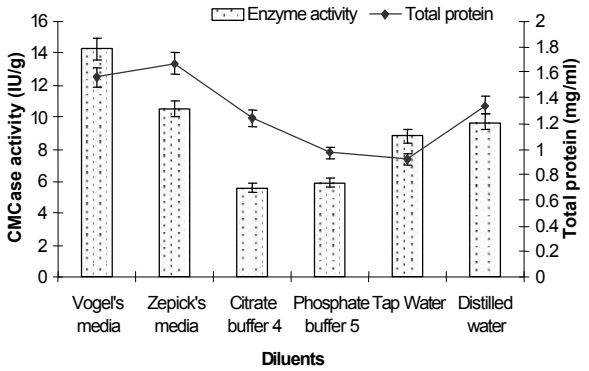

Fig. 3: Effect of different diluents for endoglucanase (CMCase) production by *Trichoderma viride* in SSF. Error bars indicates the SD among replicates.

bagasse (5.3±0.31 U/gds). 2.5% KOH treated (11.1±0.54 U/gds) and 3% H_2O_2 treated sugarcane bagasse (10.8±0.48 U/gds) also yield better in enzyme production as compared to untreated, but comparatively less than acid treated sugarcane bagasse. Some workers reported that treated sugarcane bagasse enhance the CMCase production by *Humicola insolens* [26] and *Trichoderma reesei* [27]. The main action of the pretreatment is to cause destruction in the structure which causes perforations in the substrates thus providing more surface area for microbial attack [28].

Selection of Diluent: Nutrition is the most important factor for the cultivation of any microorganism. Various diluents like Vogel's media, Zepick's media, citrate buffer pH 4, phosphate buffer pH 5, tab water and distilled water were used to optimize the endoglucanase production by *Trichoderma viride* in SSF. The best diluent was observed was Vogel's media which gave better titer of endoglucanase (14.3±0.65 U/gds) in SSF as shown in Figure 3. Zepick's media (10.5±0.42 U/gds) also produced endoglucanase but less as compared to Vogel's media. Vogel's medium is widely used in fungal growth for the production of cellulases [29-32]. *Trichoderma reesei* has the ability to produced cellulase in non buffered media as compared to buffered media [33].

Supplementation of Nitrogen and Additional Carbon Sources: Carbon and nitrogen are the main components of an organism. These form the basic sketch in the living organism. In this experiment various additional carbon sources were evaluated for maximum endoglucanase production by *Trichoderma viride* in SSF. Results (Table 1) indicated that supplementation of CMC at the concentration of 0.5% to the medium significantly enhance the enzyme production (12.7±0.87 U/gds) as

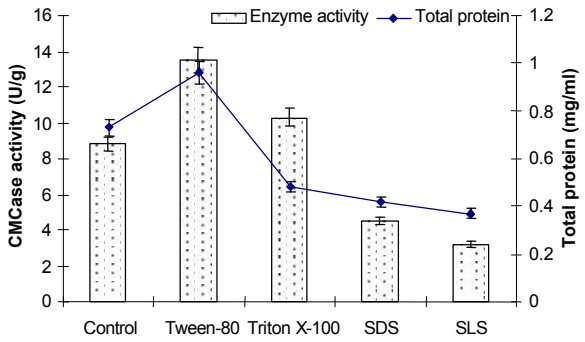

Fig. 4: Effect of different surfactants on endoglucanase production by *Trichoderma viride* in SSF. Error bars indicates the SD among replicates.

Table 1: Supplementation of nitrogen and carbon sources for CMCase production by *Trichoderma viride* in SSF

Sr. #	Nitrogen Sources	Enzyme activity (U/gds)	Carbon sources	Enzyme activity (U/gds)
1	Control	4.7±0.17	Control	3.8±0.11
2	Yeast extract	9.7±0.43	Glucose	8.9±0.51
3	Peptone	13.2±0.92	Starch	4.5±0.22
4	Tryptone	7.5±0.52	Xylose	4.9±0.26
5	Urea	2.5±0.11	Maltose	5.8±0.30
6	Casein	6.9±0.33	CMC	12.7±0.87
7	Skim milk	6.3±0.42	Filter paper	10.6±0.76
8	Lab lamco powder	5.3±0.41	Sucrose	8.7±0.46
9	$NaNO_3$	7.4±0.47	Arabinose	6.4±0.37
10	NH_4Cl	6.3±0.39	Galactose	5.6±0.31
11	$(NH_4)_2SO_4$	9.7±0.51	Fructose	6.3±0.32
12	NH_4NO_3	5.8±0.44		

compared to control (3.8±0.11 U/gds). Some workers reported lactose [12], sucrose [34] and glucose [35] as an additional carbon source for CMCase production by *Trichoderma* sp and *Aspergillus* sp.

When different nitrogen sources were tested for endoglucanase production, it was observed (Table 1) that peptone as organic nitrogen source was suitable for maximum endoglucanase production by *Trichoderma viride* in SSF. Inorganic nitrogen sources did not significantly enhance the endoglucanase yield. Peptone is the best nitrogen source for CMCase production by *Chaetomium cellulyticun* NRRL 18756 in solid state fermentation [22]. Inorganic nitrogen sources showed less enzyme production as compared to organic nitrogen sources [36] and reported that maximum CMCase enzyme production was found in medium containing yeast extract [25] and corn- steep solid [12].

Effect of Surfactants on Endoglucanase Production: Figure 4 demonstrated the effect of various surfactants such as tween-80, triton X-100, sodium dodecyl sulphate (SDS) and sodium lauryl sulphate (SLS) on endoglucanase production in SSF. Results indicated that tween-80 (13.8±0.53 U/gds) enhanced the enzyme production as compared to control (8.8±0.25 U/gds). SLS yield lowest amount of enzyme production (3.2±0.07 U/gds) among all the tested surfactants. Similar findings were also reported by Fawzi and Hamdy [22] who reported that addition of tween-80 is most effective in enzyme production. Shankar and Isaiarasu [37] suggested that addition of tween-20 to the medium enhanced the cellulase production. The use of tween-80 for enzyme production is very beneficial because it does not denature the enzymes [25].

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