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OPTIMIZATION OF FERMENTATION PARAMETERS FOR THE PRODUCTION OF EXTRACELLULAR ENDOGLUCANASE, β -GLUCOSIDASE AND ENDOXYLANASE BY A CHROMIUM RESISTANT STRAIN OF *TRICHODERMA PSEUDOKONINGII*

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ABSTRACT

Trichoderma pseudokoningii, a chromate reducing fungal strain, was isolated from the tannery-effluents. The present Cr (VI) resistant strain was found to produce good amount of various extracellular enzymes that included cellulases (endoglucanase and β -glucosidase) and hemicellulase (endoxyylanase) in submerged fermentation (SmF). The titre of β -glucosidase was found to be higher than that of endoglucanase. Cellulases were best induced in presence of 1% of respective substrates whereas only 0.5% xylan could induce endoxyylanase production in this strain. Although the optimum temperature for all three enzymes was found to be 27°C, the pH optimum of cellulases (pH 5) were different from that of endoxyylanase (pH 6). Under optimized conditions, maximum of production of all these enzymes was achieved within 48 hours of cultivation. Among nitrogen sources tested, potassium nitrate was found to be the most effective followed by gelatin.

Keywords: β -glucosidase, endoxyylanase, endoglucanase, *Trichoderma pseudokoningii*, optimization, submerged fermentation

INTRODUCTION

Lignocellulosic materials are ubiquitous and consist mainly of cellulose (40–60%) with lesser, but significant, amounts of hemicelluloses (20–30%) and lignin (15–30%) (Dekker, 1983). Hence, lignocellulosic pool acts a major carbon sink in the forest ecosystems and accounts for roughly 20% of the terrestrial feed stock carbon storage, offering an enormous, renewable source of feedstock for bio fuel production (Binder et al., 2009). Cellulose and hemicellulose, the primary components of lignocellulosic biomass, are the most abundant organic compounds on earth and have the potential to be a renewable source for energy and chemicals (Kaushal et al., 2012) the bioconversion of which typically involves the action of cellulase and xylanase to fermentable sugars (Berlin et al., 2006). Majority of glucose in lignocellulose is locked into highly crystalline cellulose polymers which are sequentially hydrolysed by endoglucanases (β -1,4-D-glucan-4-glucanohydrolase, EC 3.2.1.4, carboxymethyl cellulase), exoglucanases (β -1,4-D-glucan-4-glucohydrolase, EC 3.2.1.91, cellobiohydrolase, CBH), and β -glucosidase (cellobiase, β -Dglucoside glucohydrolase, EC 3.2.1.21, β -1,4-D-glucosidase) (Siddiqui et al., 2000). Another most important enzyme endoxyylanase (EC 3.2.1.8) is responsible for degradation of xylan which is a major constituent of hemicellulose fraction of biomass.

Although *Trichoderma inhamatum* (Morales-Barrera and Cristiani-Urbina, 2008) and *T. viride* (Levinskaite, 2002; Singh et al., 2012) were reported to reduce hexavalent chromium and a number of cellulolytic and xylanolytic strains of *Trichoderma* (Moosavi-Nasab and Majdi-Nasab 2007; Liu and Yang, 2007; Deshpande et al., 2008; Ahmed et al., 2012; Mohan et al., 2011) were known, so far the literature study is concerned, no report on production of both cellulase and xylanase by a chromate tolerant strain of *Trichoderma* is available. Therefore the objective of the present study was optimization of different parameters for the production of extracellular cellulases and xylanase from a chromium tolerant fungal strain.

MATERIAL AND METHODS

Microorganism

The working strain was isolated from the soil sample collected from tannery-effluents of Leather Complex, Kolkata, India and was screened by serial dilution for 3-4 times. The strain was identified as *Trichoderma pseudokoningii* by

Agharkar Research Institute, Pune, India and it was repeatedly sub cultured on cellulose-agar plates and maintained at 4°C for further studies.

Chemicals

All chemicals used were of analytical grade and were purchased from Sigma chemicals Co. (St. Louis, MO, USA), Merck, Germany and Himedia, India.

Cultivation of the Strain

The strain was cultivated in 100 ml Erlenmeyer flasks each containing 10 ml Basal Medium (BM) composed of (g/L⁻¹): peptone 0.9; (NH₄)₂HPO₄ 0.4; KCl 0.1; MgSO₄·7H₂O 0.1; K₂Cr₂O₇ trace and with any of the carbon source (pure carboxymethyl cellulose, cellobiose or xylan) 10 (pH 7) for 48 hours.

Enzyme Assay

The grown culture was centrifuged at 10,000 rpm for 10 min and the supernatant was used as the crude enzyme. To measure the activity of each type of enzyme, the assay mixture (1 ml) containing an equal volume of enzyme and 1% (w/v) of respective substrate dissolved in 0.1(M) phosphate buffer (pH 7) was incubated at 27°C for 10 min. For assaying the activity of β -glucosidase, endoglucanase and endoxyylanase, the respective substrates used were cellobiose, CMC (carboxymethyl cellulose) and birchwood xylan. The reducing sugar released in each case, was measured by the dinitrosalicylic acid method (Bernfeld, 1955). Blanks were prepared with inactivated enzymes. One unit of the enzyme activity was defined as the amount of enzyme producing one micromole of product per minute under the assay conditions. β -glucosidase and endoglucanase activity of the culture supernatant was determined as described by Karmakar and Ray, 2011. Endoxyylanase activity in the cultural filtrate was determined according to the method of Kundu and Ray, 2011. Similarly, the activities of exoglucanase (avicelase) and β -xylosidase were measured using avicel (Mukherjee et al., 2011) and β -D-xylopyranoside (Panbangred et al., 1983) respectively.

Optimization of production parameters

The concentrations of cellobiose, CMC and xylan (birchwood) used as sole carbon source, were varied from 0.5% - 3% to optimize the substrate concentration of submerged culture of *Trichoderma pseudokoningii*. The

optimum pH for enzyme production was determined by adjusting the initial pH of the fermentation media at a range from 4.0-9.0 using citrate, phosphate and Tris glycine buffers. Most favourable production temperature was studied by incubating the cultivation medium at different temperatures (7°C, 27°C, 37°C, and 55°C). Similarly, the effects of various nitrogen sources namely peptone, gelatin, ammonium sulphate, tryptone, potassium nitrate, and urea 0.9% (w/v) were tested. The time course of growth and enzyme production by the strain under optimized culture conditions was studied by checking the enzyme production kinetics for 0 to 120 hours at 27°C.

Photo micrographic study

The cotton blue-lacto phenol stained mycelia were visualized under Axioscop-40 (Zeiss) microscope at 100X.

All experiments were done in triplicate and the values were averaged.

RESULTS AND DISCUSSION

The strain with small conidia was identified by their basic morphology and biochemical properties as *Trichoderma pseudokoningii* (Figure 1) and was found to grow in chromium (VI) supplemented medium. It was found to produce many extracellular lignocellulose digesting enzymes of which β -glucosidase production was highest followed by endoxylanase and endoglucanase (Figure 2). This in turn indicated that the strain had the ability to utilise both glucose and xylose as carbon source, which could be further confirmed by the relative good growth rate in presence of cellobiose and xylan (data not presented). However, no extracellular exoglucanase and β -xylosidase could be detected.

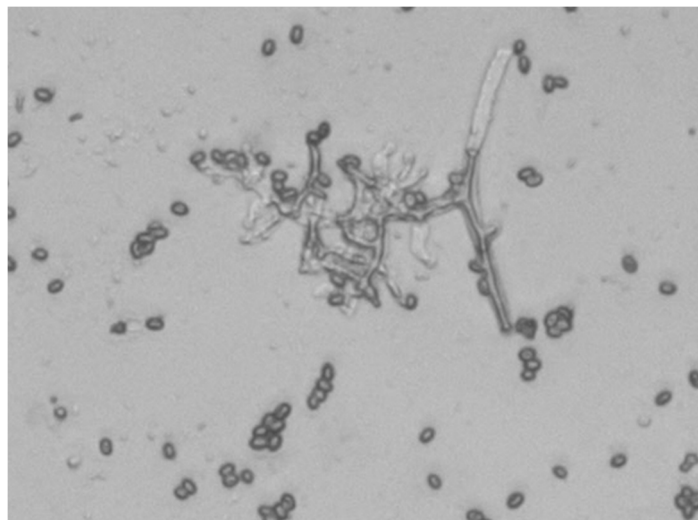


Figure 1 *Trichoderma pseudokoningii* from a chromium supplemented dextrose medium.

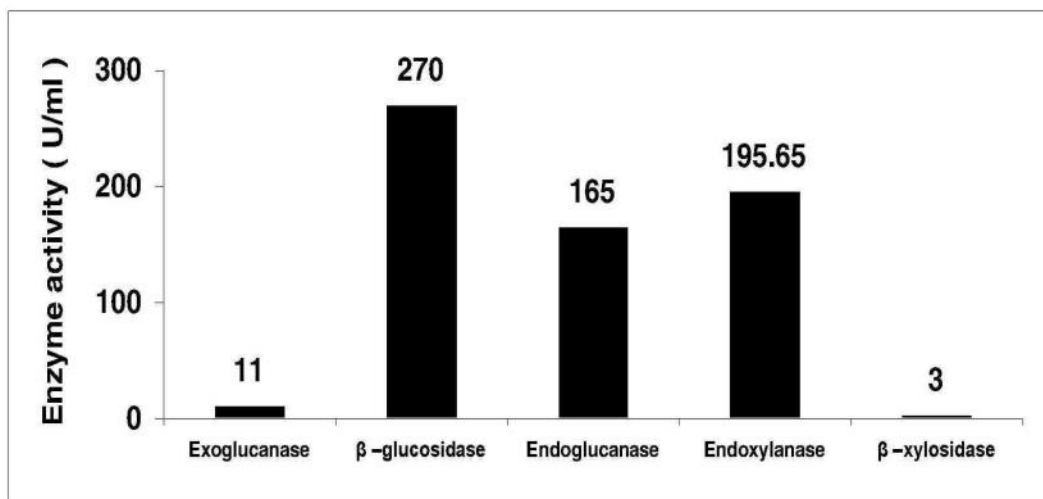


Figure 2 Effect of various substrates as inducer of cellulases and hemicellulase production by *Trichoderma pseudokoningii* (Cellobiose, Carboxy methyl cellulose and Birchwood xylan were used as the respective substrates for assaying β -Glucosidase, Endoglucanase and Endoxylanase respectively).

Effect of substrate concentration

Although β -glucosidase and endoglucanase synthesis was found to increase gradually with increase in substrate concentration and highest production was achieved at 1.0% (w/v) of pure cellobiose and CMC respectively, but the former dropped suddenly with further substrate increase. On the other hand endoxylanase showed its optimum activity in presence of only 0.5% xylan. Probably due to overload of nutrient in the cultivation media, in all cases enzyme synthesis decreased drastically with further increase in substrate concentration. (Figure 3). Similar type of observation was found by **Kundu and Ray, 2012** and **Karmakar and Ray, 2010**.

Effect of temperature

Most favourable production temperature for β -glucosidase, endoglucanase, endoxylanase production by *Trichoderma pseudokoningii* was found to be 27°C (Figure 4), slightly lower temperature than 37°C for *Bacillus subtilis* β -glucosidase (**Agarwal et al., 2013**), 32-33°C for both endoglucanase and β -glucosidase production by *Rhizopus oryzae* (**Karmakar and Ray, 2011**), 40-45°C for exoglucanase and endoglucanase synthesis by *A. niger* (**Gautam et al., 2011**). Further increase in temperature above the optimum level seriously affected the growth and consequent enzyme synthesis by the strain probably due to the thermo inactivation of the metabolic enzymes.

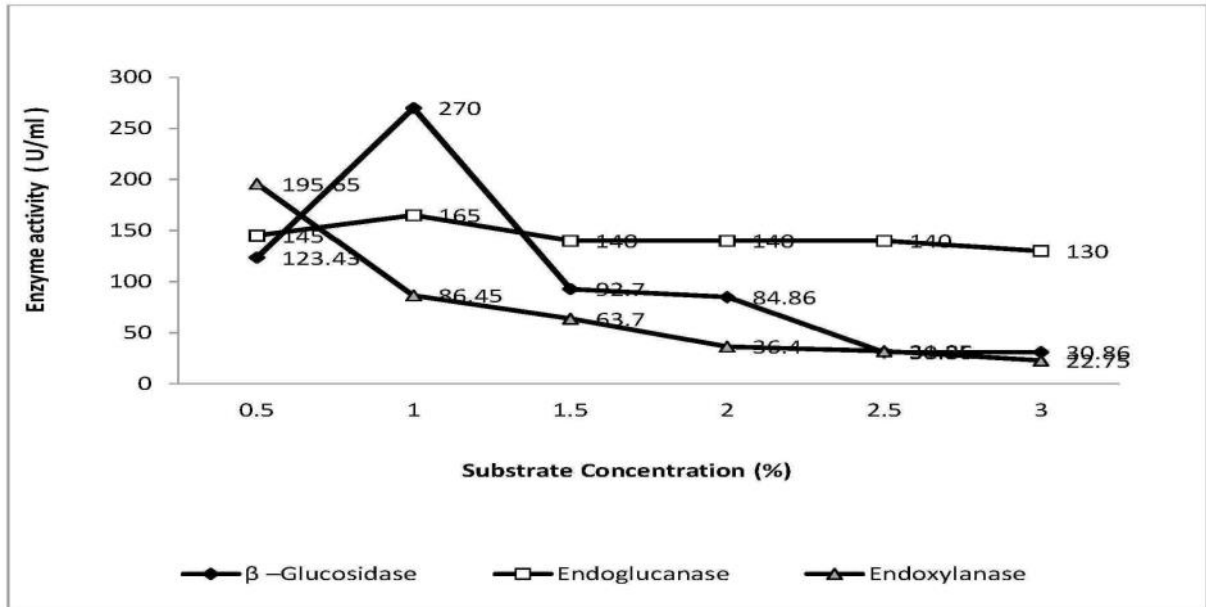


Figure 3 Effect of various substrates as inducer of cellulases and hemicellulase production by *Trichoderma pseudokoningii* (Cellobiose, Carboxy methyl cellulose and Birchwood xylan were used as the respective substrates for assaying β -Glucosidase, Endoglucanase and Endoxylanase respectively).

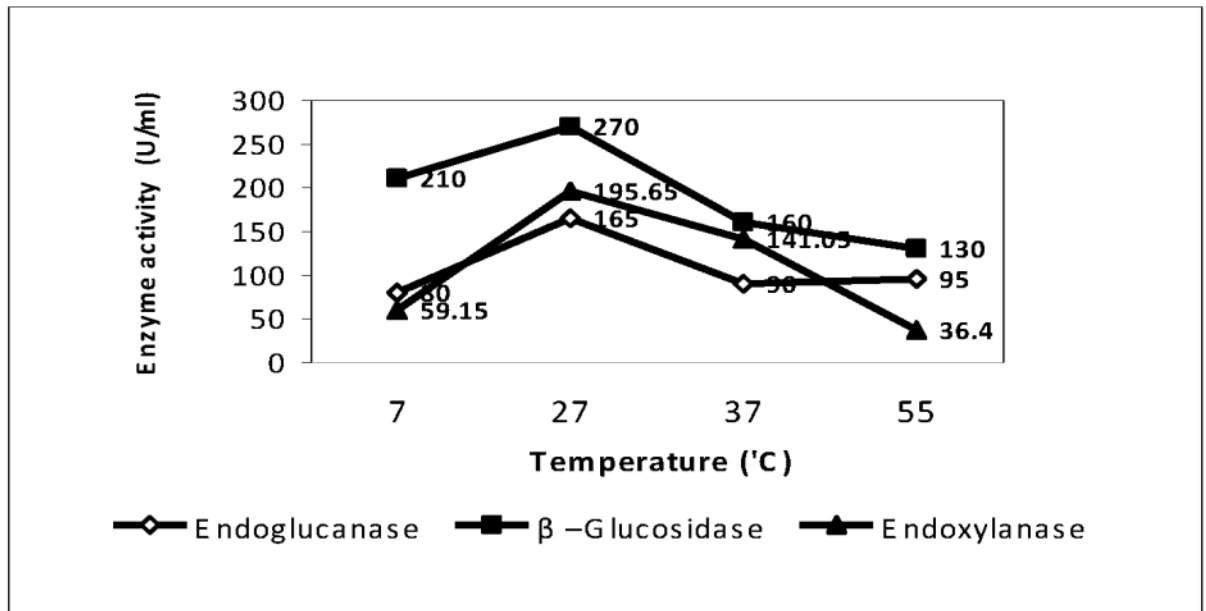


Figure 4: Effect of temperature on cellulases and hemicellulase production by *Trichoderma pseudokoningii* (Cellobiose, Carboxy methyl cellulose and Birchwood xylan were used as the respective substrates for assaying β -Glucosidase, Endoglucanase and Endoxylanase respectively)

Effect of pH

A slight variation in pH preference was found for cellulase and xylanase as the former group showed affinity towards more acidic pH (pH 5), whereas the later had the maximum production at pH 6 (Figure 5). But endoglucanase showed

more susceptibility to alkalinity as depicted by the sharp reduction of production after pH 6. In *Trichoderma* sp., the pH optimum for all types of cellulase were found between 5-6 (Gautam et al., 2011). For xylanase production by *Trichoderma* spp. (Mohan et al., 2011) and by *Trichoderma harzianum* (Ahmed et al., 2012) the pH optimum was found at 5.

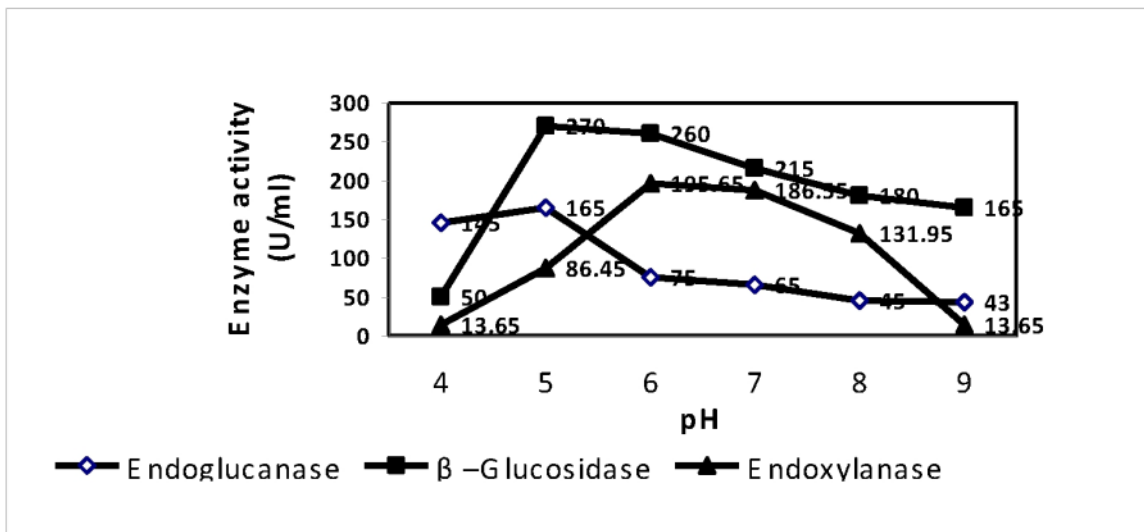


Figure 5 Effect of pH on cellulases and hemicellulase production by *Trichoderma pseudokoningii*

Effect of cultivation time

Maximum amount of enzyme production of *Trichoderma pseudokoningii* could be achieved within only 48 hours of growth (Figure 6) after which due to exhaustion of nutrients in the culture medium the enzyme synthesis gradually dropped down. On the contrary, in *Trichoderma resei*, (Deshpande et al., 2008) and in *Trichoderma harzianum* (Seys et al., 2005), maximum xylanase production could be reached at a much longer cultivation time of 9th and 7th day

respectively. In *Trichoderma koningii* highest endoglucanase production was obtained within 84 hours of incubation (Liu and Yaung, 2007).

Effect of nitrogen source

Although various inorganic nitrogen sources have been optimized by different workers for hemicellulase production (Sherief et al., 2010), in the present strain, only gelatin and potassium nitrate could bring about a noteworthy increase in endoxylanase production (Figure 7).

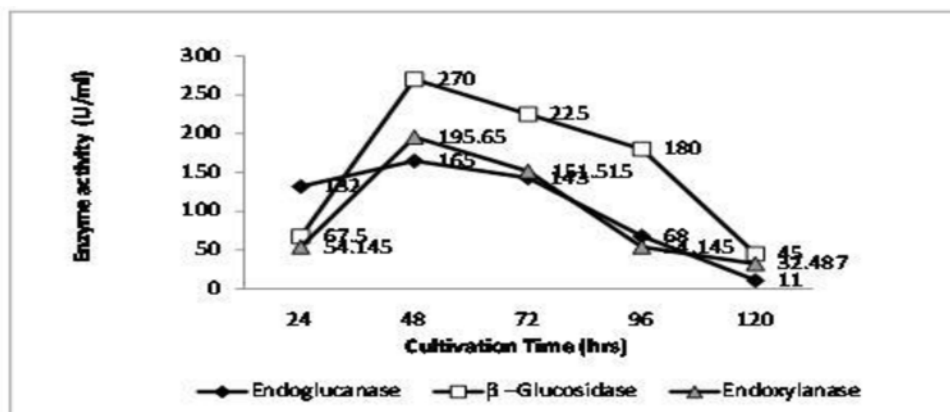


Figure 6 Effect of cultivation time on cellulases and hemicellulase production by *Trichoderma pseudokoningii*

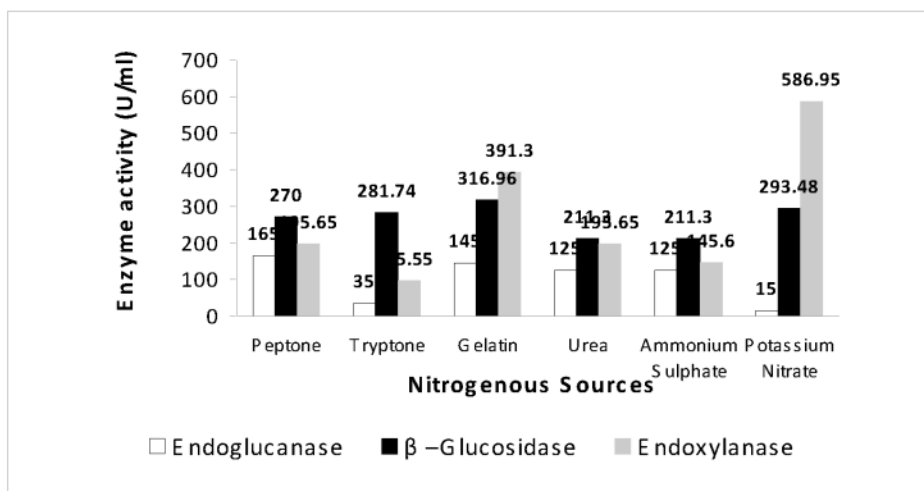


Figure 7 Effect of various nitrogen sources on cellulases and hemicellulase production by *Trichoderma pseudokoningii*

CONCLUSION

The working strain being a rapid and relatively hyper producer of cellulases and xylanase might be used for the commercial production of the enzyme. Moreover, its chromium tolerance would add some advantage in application of this strain in bioremediation and biodegradation of hemicellulosic biomass usually discarded as wastes.

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