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Isolation and Partial Purification of Extracellular Enzyme (1, 3)-β-D Glucanase from *Trichoderma reesei* (3929)

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Abstract: The fungus *Trichoderma reesei* produces extracellular lytic enzymes such as β-1, 4 glucanases, β-1, 6 glucanases and β-1, 3 D glucanases. In the present study the PDA medium was used for the production of β-1, 3 glucanases from the fungal strain. Extra cellular protein (β-1, 3 glucanase) was partially purified by ammonium sulphate precipitation and dialysis. The amount of reducing sugar (126 mg/100 mL) liberated by the action of enzyme was determined by using DNS method. The molecular weight of the enzyme (52-62 kDa) was determined through SDS-PAGE.

Key words: *Trichoderma reesei*, β-1, 3 D glucanases, PDA medium, SDS-PAGE

INTRODUCTION

Beta-D-glucans are non-digestable polysaccharides widely found in nature in such sources as cereal grains including oats and barley as well as in yeast, bacteria, algae and mushrooms. β-D-glucans are primarily located in the cell walls (Pallumbo *et al*., 2003). Yeast β-glucan appears to have immuno modulatory property. It can bind to various cells of the specific immune system such as macrophages and neutrophils (Nicolosi *et al*., 1999).

Members of the genus *Trichoderma* are ubiquitous soil microorganisms. They are well known for their antagonistic properties towards other fungi and of their ability to degrade cellulose (Saravanan and Jayraaj, 2004). The filamentous fungus *T. reesei* is one of the most potent cellulolytic organisms and also produces a xylanolytic system, which contains multiple enzyme activities required for the complete degradation of xylan (Nogawa *et al*., 1999). Further β-glucanases from *Trichoderma* species are important enzymes used in genetic manipulation (DNA separation, protoplast formation) and in releasing protein and pigments from cells. Glucanases are hydrolytic enzymes capable of causing lysis of cell walls. The ability of β-1, 3-glucanases to degrade the yeast cell wall seems to indicate that this enzyme may be helpful in future as a factor increasing the digestibility of protein feed of microbial origin (Maj *et al*., 2002).

β-1, 3-glucanases have been isolated from bacteria, yeast and fungi, plants and microorganisms. It has been suggested that plant 1, 3-glucanases may protect the germinating grain against pathogen attack. In yeast β-1, 3-glucanases are involved in morphogenetic events, such as cell budding, conjugation and sporulation (Phaff, 1997). The cell wall of yeast undergoes continuous rearrangement of β-glucans during growth period. The process involves making and breaking of bonds between wall polymers, manipulated by β-glucanases through controlled hydrolysis. As a consequence different glucanases are required at different stages during cell-life cycle (Kulminskaya *et al*., 2001).

The production of extracellular β-1, 3-glucanases has been reported as an important enzymatic activity in bio control of microorganisms (Zaldivar *et al*., 2001). β-1, 3-/1, 6-glucan is a powerful immune-enhancing nutritional supplement. This unique compound stimulates the immune response to help the body defend itself against foreign invaders and foreign substance. β-1, 3-/1, 6-glucan activates neutriphils and white blood cells known as macrophages, which surround, trap and engulf foreign invading substances rendering them inactive. Ingestion of β-glucans has been shown to improve the pattern of lipids in humans and experimental animals with elevated serum cholesterol. Studies indicate that β-1, 3-/1, 6-glucan may reduce the threat of cancer by slowing down the progression of tumor growth. Research also suggests that

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cancer patients undergoing chemotherapy and/or radiation treatment may benefit from β-1, 3-/1, 6-glucans (Adachi *et al*., 1994). This study describes the isolation and partial purification of extracellular β (1, 3)-D-glucanase from *T. reesei* (3929).

MATERIALS AND METHODS

Source of fungal strain: The culture of parent strain *T. reesei* (3929) was obtained from M.T.C.C, Institute of Microbial Technology, Chandigarh, India.

Maintenance of fungal strain: The stock culture of this fungus was maintained on PDA medium at 28°C and periodically sub-cultured.

Preparation of inoculum: Mycelium from stock cultures were inoculated to PDA medium in Petridish and incubated for 5 days at 28°C for obtaining inoculum.

Optimization of the media: Optimization of culture media is an important approach for improving the growth and production of extracellular glucolytic enzymes (Szengyel *et al*., 2000). This study involved using yeast extract as a carbon source to increase biomass density and product levels.

Maintenance of shake flask cultures: Duplicate shaking cultures were carried out on PDA medium enriched with yeast extract. The cultures were incubated in 250 mL flasks at 28°C for 48 h.

Collection of supernatant: Culture aliquots were centrifuged at 4°C temperature, at 5000 rpm for 20 min to remove solids. The supernatant was subjected to ammonium sulphate precipitation.

Ammonium sulphate precipitation: This was carried out at 4°C. The crude enzyme was precipitated from 100 mL of the filterate, obtained from the induction medium with ammonium sulphate (60-70% saturation) and was dissolved in 10 mL of 0.01 M sodium phosphate buffer (pH 7.2).

Partial purification of enzyme: Chilled acetone (stored at -20°C) was slowly added to 100 mL of the culture filtrate (70% saturation) on a magnetic stirrer. After standing overnight at 4°C and centrifugation (10,000 x g, 30 min at 4°C), the precipitate was resuspended in 15 mL of 0.1 M sodium phosphate buffer (pH 7.2) and dialyzed against the same buffer. The available flat width of dialysis membrane (Himedia) used was 29.31 mm, diameter 17.5 mm and capacity approximately 2.41 mL cm^{-1}. This enzyme solution was used for characterization studies.

Assay of enzyme activity: Glucanase activity was quantified by measuring the amount of glucose liberated from laminarin substrate using Glucose Oxidase Peroxidase method specific for glucose (Ramasamy, 1984). The reaction mixture contains 0.5 mL of dialysed sample, to that 0.5 mL of 0.05 M laminarin substrate (pH 4.8) was added and incubated at 35°C for 40 min. The reaction was terminated by the addition of 2 mL 6 N HCl. Formation of red colour indicated the presence of glucanases in the dialyzed sample.

Estimation of protein: The total protein content was estimated by following the method of Lowry *et al*. (1951).

Estimation of reducing sugar: The reducing substance (sugar) obtained due to the enzymatic reaction was determined by DNS method (Miller *et al*., 1959).

Determination of molecular weight of the protein: Molecular weight of the β-glucanase enzyme produced by *T. reesei* (3929) was confirmed by SDS-PAGE.

RESULTS AND DISCUSSION

A successful growth of *T. reesei* had seen in a potato dextrose slant as a green colour lawn after 5 days of incubation of the culture at the temperature range of 25 to 28°C. The selection of a suitable carbon and energy source has particular importance in the process of extra cellular production of hydrolyses by filamentous fungi. To achieve the maximum enzyme production, yeast extract was added to the shake flask culture medium at a concentration of 4%.

Separation of glucanases: Since the glucanases are extra cellular enzymes, the fungus releases the enzyme in to the culture medium. The glucanase separated from the culture medium by centrifugation of the culture at 5000 rpm for 20 min under cold condition was collected in the supernatant.

Ammonium sulphate precipitation and dialysis: Different proteins were gradually precipitated out from the aqueous solution by the addition of ammonium sulphate. As the concentration of the salts increased, they compete with proteins from water molecules and lead to their gradual precipitation (Lejeune and Baron, 1995).

In the present study the proteins were precipitated from the culture supernatant by treating it with 60-70% saturated ammonium sulphate and the precipitated proteins were dialyzed and used for further study.

Assay of enzyme activity: Enzyme activity was estimated by the process of glucose oxidase peroxidase method. Formation of red colour was observed.

In the present study the enzyme activity was estimated by using laminarin substrate, a linear β-(1, 3) glucan. Glucanases hydrolyzes the glucan molecules, leads to the production of glucose residues. Glucose oxidase catalyses the oxidation of glucose to gluconic acid with the formation of hydrogen peroxidase. The oxygen liberated from the hydrogen peroxide by action of peroxidase, reacts with the O-dianisidine and oxidizes it to a red chromophore product. Formation of red colour indicated the presence of glucanases in the dialyzed sample.

Estimation of protein: The total amount of the protein present in 100 mL of dialysed sample was found to be 140 mg (Fig. 1).

Estimation of reducing sugars: One hundred milliliter of the enzyme was found to be releasing 126 mg of reducing sugar (glucose) (Fig. 2).

Determination of the molecular weight of the protein: In the present study, BSA was used as the standard protein marker, since it has more or less equal molecular weight as

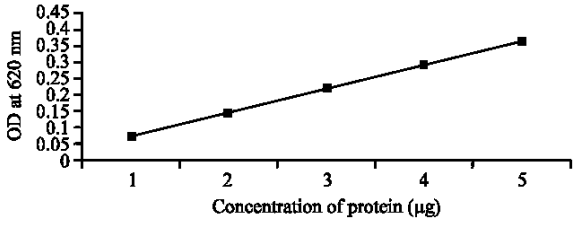


Fig. 1: Estimation of proteins

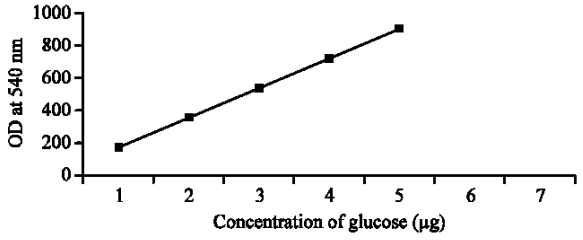
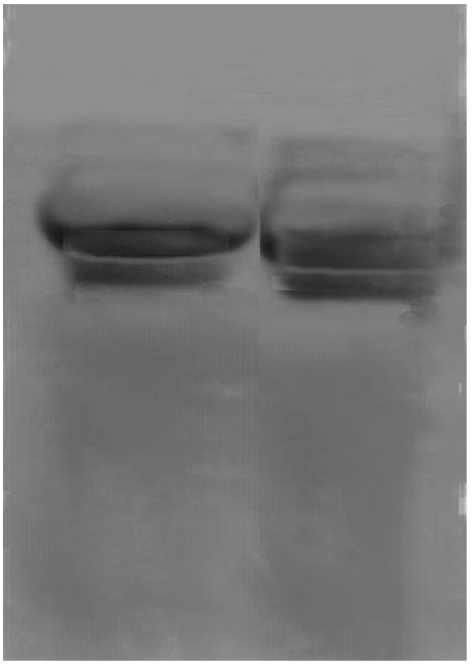


Fig. 2: Estimation of reducing sugar



Lane 1: Sample Lane 2: Marker

Fig. 3: SDS-PAGE

that of the glucanases. This observation is in the line with the reports of Emert *et al.* (1974) who has recorded the molecular weight of glucanase as 52 to 62 kDa (Fig. 3). Brown (1972) found that β-glucanases behaved as acidic proteins, which is thought that β-glucanases as typical extra cellular enzymes might be considered as glycoproteins.

In general, enzyme properties such as molecular weight and sedimentation co-efficient are in the range reported by other authors (Bull, 1967; Kitamura *et al.*, 1974). It seems clear that the purified enzyme is unspecific one, which may hydrolyse both (1,3) -β- and (1,6)-β-linkages. It is noteworthy that this enzyme shows affinity against laminarin than on (1,3)-β-D-glucan. Present results shows that the mode of action of the enzyme corresponds to an exospliting mechanism when it acts on laminarin. This study can be extended for purification and kinetic studies can reveal the stability of the enzymes to a wide variety of environmental condition like pH and temperature, which can be exploited for engineering enzymes.

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