ISSN 0975 - 6299 Vol 2 / Issue 3 / Jul - Sept 2011

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE

BIOTECHNOLOGY

ENDOGLUCANASE PRODUCTION BY *Bacillus amyloliquefaciens* USING COFFEE PULP AS SUBSTRATE IN SOLID STATE FERMENTATION

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ABSTRACT

In this study, endoglucanase was produced from coffee pulp waste by *Bacillus amyloliquefaciens* MTCC610. The effects of the fermentation parameters such as pH, temperature, and the carbon source sucrose with different percentages (2% -10%) were used in coffee pulp substrate. The endoglucanase production was assayed using carboxymethyl cellulose (CMC) as the carbon source. *B. amyloliquefaciens* MTCC610 was maximally produced endoglucanase at 45°C, at pH 7.0 with 6% of sucrose as carbon source in addition with coffee pulp waste. The enzyme activity recorded during the fermentation was 780 U/ml after 72 hours of incubation. The productivity of the enzyme increased the substrate with sucrose (6%) in the fermentation medium. The 60 to 80 % of ammonium salt purification was improved upto 5.4 fold than the crude enzyme and further improved upto 9.0 fold by DEAE column chromatography. From this findings coffee pulp waste is an abundant solid waste at coffee processing industries, it has the potential substrate for endoglucanase production.

KEY WORDS

Endoglucanase, *Bacillus amyloliquefaciens*, Coffee pulp waste, DEAE column chromatography.

INTRODUCTION

Cellulose, a major polysaccharide constituent of plant cell walls and one of the most abundant organic compounds in the biosphere, consists of long chains of β-1,4-linked glucose units, which in turn form higher-order febrile structures. It is present to a large extent in plant materials in a crystalline, water-insoluble form and cannot be utilised directly by most organisms (Clarke, 1997). *Bacillus* sp. are efficient producers of cellulolytic enzymes, which comprise three types of enzymes acting synergistically Endo1,4-β-glucanase (E.C 3.2.1.4) is characterized by its activity toward substituted cellulose derivatives such as Carboxy methyl cellulose (CMC), and exo-1,4-β-glucanase hydro lyses microcrystalline cellulose, producing cello oligosaccharides and cellobiose (Parry *et al*., 2002). Endoglucanase preferentially break the internal glycosidic bonds of cellulose chains and synergistically act with cellobio-hydrolase and β- glucosidase during the solubilization of crystalline cellulose.

Enormous amounts of agricultural, industrial and municipal cellulosic wastes have been accumulating or are used inefficiently due to the high cost of their utilisation processes (Kim, Yoo, Oh, & Kim, 2003). Therefore, it has become of considerable economic interest to develop processes for the effective treatment and utilisation of cellulosic wastes as inexpensive carbon sources. Cellulases provide a key opportunity for achieving tremendous benefits of biomass utilisation (Wen, Liao, & Chen, 2005). They are also used for improvement of the manufacture of recycled paper (Stork et al., 1995), biopolishing of textiles, additives in washing powder and animal feed, pulping, processing of fruit juices and beverages, baking and in bioethanol production (Tolan & Foody, 1999; Zaldivar, Nielsen, & Olsson, 2001). Endoglucanase play a vital role in increasing the yield of fruit juices, beer filtration, and oil extraction. It improves the nutritive quality of bakery products and animal feed, and also enhancing the brightness, smoothness, and improves over all quality of cellulosic garments (Parry *et al.,* 2002).

Coffee pulp as a waste is usually disposed off with-out any treatment and left to degrade naturally in heaps, with the uncontrolled liberation of noxious odor and nutrient load leaches out as a consequence. It may take six to eight months to achieve stabilization of the organic matter and moreover the nitrogen in the residue does not contain more than 2% of the dry weight **(**Gloria *et al*., 1999). Coffee is the one of the most important agricultural commodity in the world. *Coffea arabica* and *Coffea robesta* are the two principal verities of the genus cultivated all over the world for commercial production. Coffee has traditionally been grown under the canopy of the flowering forest or inter-cropped fruit trees.

In modern years, the researchers have been an increasing trend towards efficient utilization of agricultural by products such as coffee pulp, coffee husk, etc. (Pandey *et al*., 1998). Diverse methods have been developed that utilize these raw materials for the production of value added fine products such as, industrial enzymes, ethanol, and amino acids, organic acids, secondary metabolites, etc. (Pandey *et al*., 1992).

Solid state fermentation (SSF) has been considered as a useful tool for biomass energy conservation, solid waste treatment and production of value-added molecules such as enzymes, organic acids etc. (Pandey *et al.,*

1999). Modern industrial biotechnology has potential opportunities for economic utilization of agro-industrial residues such as coffee pulp and coffee husk. Coffee pulp is a fibrous mucilaginous material derived during the processing of coffee cherries by wet processing (Pandey, 2000).

Application of coffee pulp in bio processes on one hand provides alternative substrates and on the other hand helps solving environmental issues, which otherwise their disposal may cause (Pandey *et al*., 2000). The advantage of biotechnological innovation mainly in the field of enzyme and fermentation technology, many new avenues have opened for their utilization. In this work, SSF of coffee pulp with different concentrations of sucrose added was assessed for production of endoglucanase at different parameters such as temperature, pH and the fermentation time, the sucrose at different percentage by *Bacillus amyloliquefaciens* MTCC610. The enzyme also subjected to purified by ammonium salt purification and DEAE- cellulose chromatography.

MATERIALS AND METHODS

***Organism & Growth condition**:*

The *Bacillus amyloliquefaciens* strain MITCC No.610 used in the current investigation was obtained from the Institute of Microbial technology (IMTECH), Chandigarh, India. It was maintained on nutrient agar media (Hi-media) (g/l): Peptone 10.0; NaCl.5; Beef extract 3; and Agar 20 at 4°C.

Processing of Coffee pulp:

The coffee pulp waste of *Coffea arabica* was collected from the MSP plantation coffee seed processing Industry at Yercaud, India and air dried in Laboratory (Selvankumar *et al.,* 2010).

Preparation of inoculum:

The *Bacillus amycoliquefaciens* was cultured in 100ml Erlenmeyer flasks containing 50ml of nutrient broth inoculated with 5 ml of 24 hrs culture. The flasks were incubated at 37°C for 12 hrs and the inoculum thus obtained was used for the inoculation of the solid substrate medium.

Total viable cell count (TVC) of the bacterial cells in nutrient broth was determined by colony count method using colony counter (Sabu *et al*., 2006). One milliliter of the cell suspension was serially taken from each diluted sample and was poured on to sterile Petri dishes containing Nutrient agar medium and then spread uniformly. The plates were then incubated at 37°C for 48 hrs and the colonies were counted. It was found that there was a cell density of $8X10^8$ cells/ml.

Preparation of substrates:

The 50 gm of coffee pulp substrates and sucrose in the various proportions (2 - 10%) were prepared into a 500 ml Erlenmeyer flask. The solid substrates were moistened with acetate buffer (pH 7.0, 0.1M). The content at the flasks were mixed thoroughly and autoclaved at 121˚C for 15 min.

Solid state fermentation:

After sterilization the flasks were cooled and inoculated 5 ml of the 24hrs old culture and incubated at 37˚C for 120 hrs. The moisture content of 60-80% was maintained during the experimental study. During incubation, the fermented substrates from the flasks were harvested and assayed at 24 hrs intervals.

Endoglucanase Assay:

Endoglucanse activity was measured by using a reactive mixture containing 0.5ml of 1% (w/v) carboxy methyl cellulose (CMC) in 0.1M citrate buffer (pH 6.8) and 0.5ml of culture supernatant (Mendels *et al*., 1976).

The liberated reducing sugar was estimated with DNS reagent after incubating the reactive mixture for 30 min at 40˚C (Miller *et al*., 1959). One unit of enzyme activity expressed as the amount of enzyme required

to release one µ mole reducing sugar / mL under the standard assay condition.

Protein Assay:

Protein content in the supernatant was estimated by using egg albumin fraction as standard (Lowry *et al*., 1951).

Purification of Endoglucanase:

Enzyme preparation obtained after the cultivation of the organism in production medium containing coffee pulp with sucrose as carbon source was subjected to ammonium salt precipitation of protein at different saturation levels (60-80%). The precipitate was dissolved in a small quantity of the buffer (Tris buffer, 50 mM, pH 9) and dialyzed against the same buffer (buffer outside the dialysis bag was replaced with the fresh one after every 6 hours). The enzyme obtained after dialysis was analyzed for endoglucanase activity and total protein content. Partially purified enzyme (using ammonium sulphate precipitation) was subjected to DEAE--cellulose chromatography for further purification (Oyekola.O.O, 2007). The concentrated cell-free enzyme extract (6 ml) was loaded on to a DEAE-cellulose ion exchanger column (1 cm×25 cm) equilibrated with sodium phosphate buffer (0.05 M, pH 6.0). The column was washed with the same buffer until the absorbance at 280 nm (*A*280 nm) of the eluate reached base line. Bound endoglucanases were eluted from the column by a stepwise increase in NaCl (0–1 M)in sodium phosphate buffer (0.05 M, pH6.0) at a flow rate of 1ml/min. Fractions containing endoglucanase activity were pooled, concentrated and subjected to studied enzyme activity.

RESULTS & DISCUSSIONS

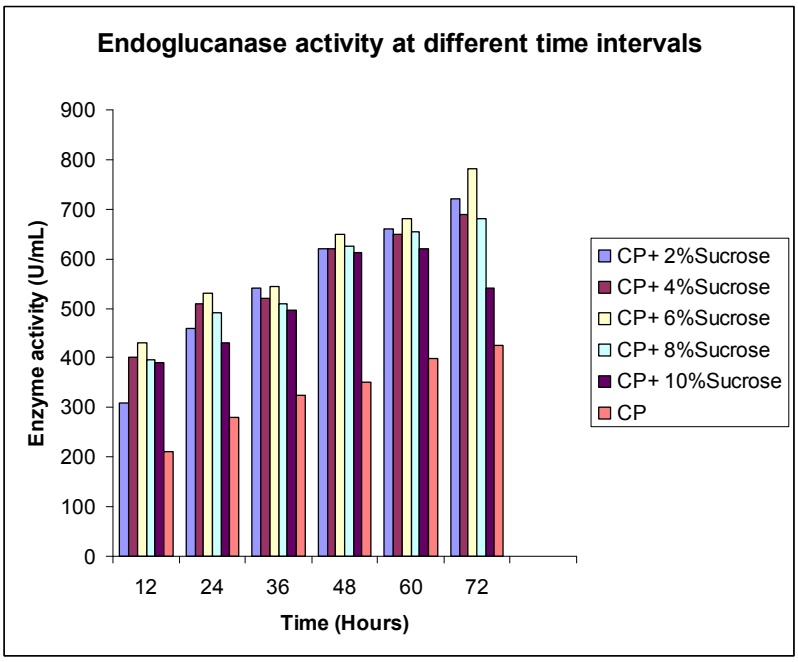
In SSF, the selection of a suitable solid substrate for a fermentation process is a critical factor and thus involves the screening of a number of agro-industrial residues for microbial growth and product yield. Here, the substrates supported growth and enzyme formation by the culture, while coffee pulp proved as superior substrate in combination with sugar based agro-residues. A higher titer of endoglucanase activity was obtained in a medium containing coffee pulp with the sucrose as the substrate.

Different solid substrates were found to effect the production of Amylase enzymes. It was previously reported that coffee pulp was found to be one of the best source for alpha amylase enzyme production by *B.amyloliquefaciens* (Selvankumar *et al*.,2010) .

Time course of endoglucanase production:

Endoglucanase production by *Bacillus amycoliquefaciens* was studied in the production medium containing coffee pulp as the substrate in solid state fermentation. The enzyme production was reached maximum after 60h of fermentation. The highest activity of endoglucanase was observed after 72 hrs (780U/mL) in coffee pulp with 6% of sucrose (Fig. 1).

Figure 1

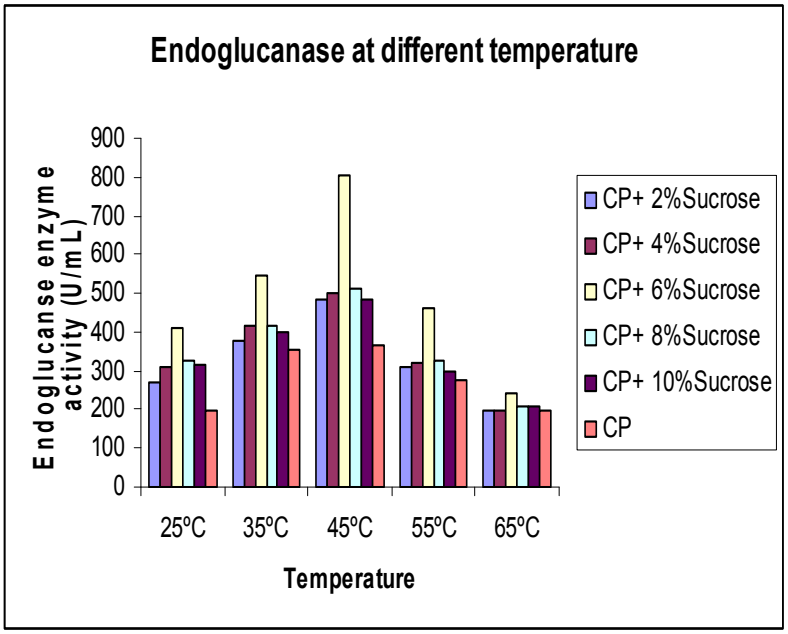


Enzyme activity at different Temperature & pH:

Effect of temperature on endoglucanase activity

The temperature and pH are the most important factors, which directly influence enzyme activity. Partially purified enzymes preparation form the substrates showed activity over a broad range of temperature (25-65˚C) with the maximum activity at 45˚C (802 U/mL) (fig.2). This result also correlated with previous works (Bijender *et al.,* 2009). It indicates that enzymes may be of great commercial value on its thermo stability of the enzyme.

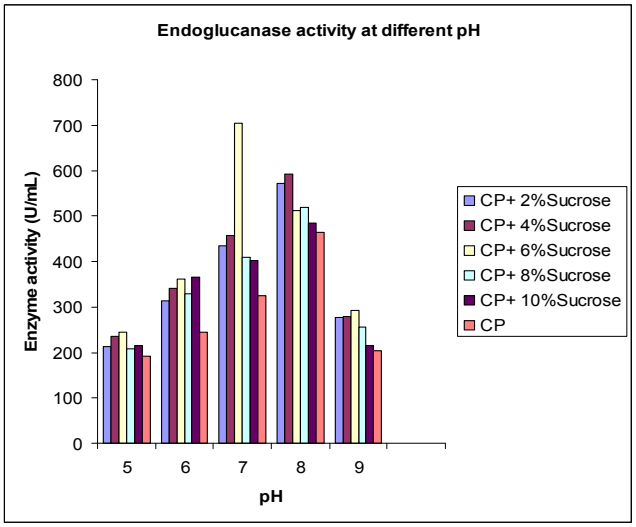
Figure 2.



Effect of pH on endoglucanase activity

Endoglucanase assay was done in reaction mixture at varying pH by using suitable buffers. It was reported that the enzyme has neutral range of activity at pH 7 (fig 3) and produced 704 U/ ml. Maximum stability of the enzyme was found at pH 7.0 but the enzyme was stable at pH 5-6.5. Most endoglucanases are optimally active till the pH 6.5 (Immanuel *et al.,*2007) and (Baker *et al.*2005,).

Figure 3



Enzyme activity of crude and partially purified enzyme*:*

The maximum enzyme activity of partially purified enzyme at the 70% of fraction with the high yield of 98.2% and specific activity of 5.3 was shown on table 1.

Table 1

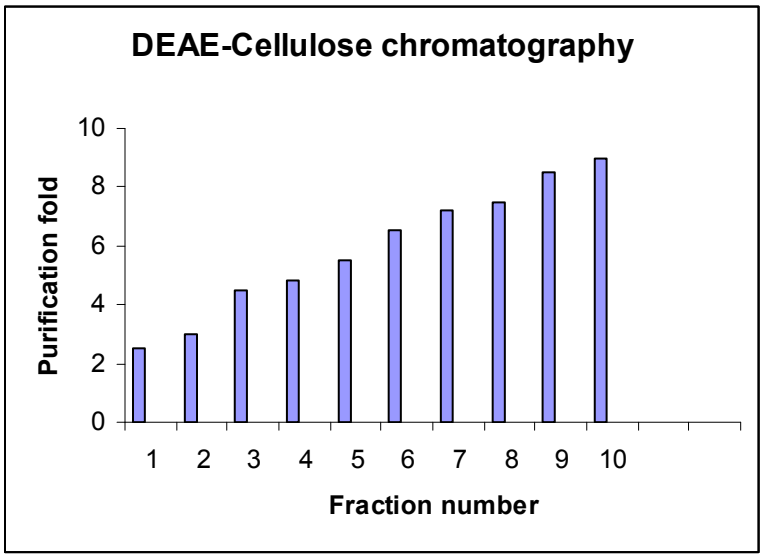
Enzyme activity of crude and partially purified enzyme

	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	% Yield	Purification fold
Supernatent	5040	282	17.9	100	1.0
Ammoniumsulphate precipitation60-80%	2553	26.3	97.1	50.7	5.4

DEAE- Cellulose chromatography:

Partially purified enzyme preparation (using ammonium sulphate precipitation was subjected to DEAE-cellulose chromatography for further purification. DEAE cellulose chromatography reported in purification fold of.2.5 to 9.0(fig4).

Figure 4



From this reports *Bacillus amyloliquefaciens* MTCC610, a moderately thermo tolerant organism, is capable of successfully growing at neutral pH and moderately elevated temperature (60-70˚C). Further more the organism successfully utilized crude carbon sources of coffee pulp, the enzyme production much higher titer when mixed sucrose with the substrate. Carbon source plays an important role in cellulase production. This is because the enzyme is induced based on the type of carbon source used. High cost of substrates can be a limiting factor in enzyme production. For example, the cost of cellulase contributes to 80% of the total saccharification cost (Zacchi, G., *et al.,*1988) in industries. Attempts have been made to produce low-cost cellulase. Among the substrates that have been tested are corn cobs, wheat straw, sugar cane bagasse, aspen wood, and waste from newspaper industry (Liming, X. and Xueliang, S. 2003). Lignocellulosic materials are being highlighted as the next substrates in endoglucanse production. From these results we concluded that the higher yield of endoglucanase production was achieved by using coffee pulp waste with sugar 6% as a substrate under SSF. This substrate is easily available and also causes pollution in the environment. So the biotechnological approach towards this process can be further improved for large scale production of valuable enzymes from the coffee pulp wastes.

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