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Production and bioapplication of cellulase from *Aspergillus niger*

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ABSTRACT

In an energy deficient world, cellulase plays a major role for the production of alternative energy resources. Cellulase is an important enzyme which can be obtained from cheap agrowaste, as well as cellulose substrate used in submerged fermentation and solid state fermentation. Cellulose can be degraded by cellulase enzyme produced by cellulolytic fungi (*Aspergillus niger*). Cellulases are a group of hydrolytic enzymes, which are responsible for the release of sugar, and they are being produced by the microorganisms grown over cellulosic matter. This enzyme is used to extract carotenoids from vegetable source (sweet potato peel) and it was one of the bioapplications of cellulase enzyme. Carotenoids are a group of pigments naturally present in vegetable raw materials that have biological properties.

Introduction

Enzymes are the most important products obtained for human needs from microbial sources. Many industries in areas of environmental and food technology utilizes enzymes at some or other stages. Current research in biotechnology is being carried out to new applications for enzymes (Ashok *et al.,* 1999). Cellulase is a synergetic enzyme which is accustomed to spilt cellulose into glucose and/or different oligosaccharide compounds (Chllapandi and Jani 2008). Cellulase enzymes may be divided into three types: Endoglucanase (endo-1,4-beta-D-glucanase, Endoglucanase (EG), Enzyme Commission number (EC) 3.2.1.4); cellobiohydrolase (CBH) or Exoglucanase (exo-1,4-beta-D-glucanase, Cellobiohydro lase (CBH), Enzyme Commission number (EC) 3.2.1.91) and Beta-glycosidase (1,4-beta-D-glucosidase, Beta-galactosidase (BG), Enzyme Commission number 3.2.1.11) (Gao *et al.,* 2008). Whereas Endoglucanase (EG) being the foremost economical enzyme (Nutt *et al.,* 1999). The cellulase complex is used in simultaneous saccharification and fermentation systems (SSFS) (Debing *et al.*, 2007). Many fungal cellulases have a two domain structure with one catalytic domain and one cellulose binding domain, which are connected by a flexible linker (Bignell De *et al.*, 2011).

The enzymatic hydrolysis of cellulosic materials involves synergistic actions of the three components of cellulase enzyme complex (Lynd *et al.,* 2002). Even though these enzymes are produced by several microorganisms like bacteria, fungi, and actinomycetes, fungi are known for secretion of cellulase in copious amount.

The fungi *Aspergillus niger* is used mostly for the production of enzymes in large amounts. *Aspergillus niger produces* highly active cellulase when grown in liquid media by both surface and submerged culture methods and recently by solid state fermentation (Ikram-ul-Haq *et al.,* 2005). *Aspergillus* species are highly aerobic and are found in almost all oxygen-rich environments, when they commonly grow on molds on the surface of a substrate (Varga *et al.,* 2007).

Cellulase has a major bioapplication on producing carotenoids. All colored fruits and vegetables are good carotenoids sources but because they are fat soluble substances, the absorption largely depends on the preparation with oil or fats. A form of carotene provitamin A are found in dark green and yellow-orange color. Darker colors are associated with higher levels of pro vitamins (Ambrassio *et al*., 2006).

Carotenoids are a generic term used to designate the majority of pigments naturally found in animal and plant kingdom. All photosynthetic organisms (including plant algae and cyanobacteria) and some non photosynthetic bacteria and fungi synthesize the carotenoids. The two classes of carotenoids are found in nature (a) the carotenes such as beta-carotene, which consists of linear hydrocarbons that can be cyclized at one end or both ends of the molecules, and (b) the oxygenated derivatives

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of carotenes such as lutein, violaxanthin, neoxanthin and zeaxanthin known as xanthophylls (Botella-pavin *et al.*, 2006).

The system of conjugated double bonds gives these pigments high chemical reactivity that can be easily isomerized and oxidized (Oliver *et al.,* 2000). Due to the coloring properties of carotenoids, they are often used in food, pharmaceutical, cosmetics, and animal feed industries. Beta-carotene is a thermos labile orange pigment, light, and oxygen sensitive, and it is also protects against heart diseases and cancer (Gale *et al.*, 2001). The common application of Cellulase enzymes is used in bioremediation, wastewater treatment and also for single cell protein production. It has importance in food sciences like food processing, drying of Branson coffee, efficient purification of juices, paper and pulp industry and as a supplement in animal feed industry. The main objective of this work was to examine, the production of cellulase by *Aspergillus niger* and carotenoid concentration from sweet potato.

Materials and Methods

Chemicals

Potato dextrose agar was obtained from Himedia Laboratories Pvt.Ltd, Mumbai, India. Carboxy methyl-cellulose (CMC) and magnesium sulphate ($MgSO_4.7H_2O$ potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), Calcium chloride ($CaCl_2$), yeast extract, peptone, glucose, Sodium nitrate ($NaNO_3$), Dipotassium hydrogen phosphate (K_2HPO_4), ferrous sulphate ($FeSO_4.7H_2O$), distilled water,Folling reagent, Lowry reagent, Ammonium sulphate, sodium acetate buffer were obtained from reputed companies.

Screening test

Carboxymethyl cellulose (CMC) acts as a good indicator of celluloytic ability. The fungal strain isolated, was primarily screened by zone activity technique by using Congo red dye. Congo red dye is a metachromatic dye that can react with cellulose faster. The Congo red dye was poured on the plate. After treatment of CMC with cellulase enzyme, the remaining dye was removed gently by washing with NaCl (1%) and an observation was made to note the substrate utilization zone around the colony. The unstained region indicates that the CMC has broken down into beta-1, 4 glycans, which contain a fewer glucose residues and it is shown in the Figure 1. Screening test is the proper and cheap method to identify the zone.

Cellulase production from fungal strains

At first the sporulation medium was prepared for 50 ml in Erlenmeyer flask containing potato starch (1 g), dextrose (1 g), Agar (1 g), magnesium sulphate ($MgSO_4.7H_2O$, 0.025 g), potassium chloride (KCl, 0.025 g), potassium dihydrogen phosphate (KH_2PH_4, 0.004 g) and distilled water (50 ml), and it was sterilized for 25 minutes, after cooling it was poured on a petri plate. After solidifying, the fungal strains were streaked on the plate and kept for 24 hours incubation for the growth of fungus *(Aspergillus niger),* which is shown in the Figure 2. After growing, the fungus was inoculated into the inoculation medium, which contains Ammonium sulphate ($(NH_4)_2SO_4$ 2.8 g), potassium dihydrogen phosphate (KH_2PO_4, 4 g), calcium chloride ($CaCl_2$ 0. 6g), Magnesium sulphate ($MgSO_4$, 0.6 g), yeast (0.5 g), peptone (1.5 g), glucose (4 g), distilled water and pH was adjusted to 5.5 and then it was sterilized for 25 minutes and after cooling it was incubated for three days for the proper growth of fungus (*Aspergillus niger),* which was shown in the Figure 3. After growth, it was then inoculated into the fermentation medium or the production medium prepared for 250ml and it contains yeast extract (0.5 g), CMC (1.25 g), $NaNO_3$ (0.125 g), dipotassium hydrogen phosphate (K_2HPO_4, 0.25 g), magnesium sulphate ($MgSO_4.7H_2O$, 0.125 g), ferrous sulphate ($FeSO_4.7H_2O$, 0.025 g) , potassium chloride (KCl, 0.5 g) and distilled water (250 ml). It was then sterilized for 25 minutes and incubated for five days to produce the enzyme and it is shown in the Figure 4.

Partial purification of enzyme

After five days, the production medium (250 ml) was centrifuged at 10,000 rpm for 10 mins, where the supernatant (150 ml) was collected and the pellet was removed and to the supernatant (150 ml), 70% (105 g) of Ammonium sulphate was added and incubated at 4° C for one night to get precipitate. From the precipitated medium, supernatant was collected and centrifuged at 10,000 rpm for ten minutes to get the pellet, and which is a crude enzyme.

Dialysis

Dialysis was done by preparing a dialysis buffer [sodium acetate buffer (0.1M)] for 200 ml of pH 5 which contains sodium acetate trihydrate (2.72 ml), acetic acid (1.143 ml) and distilled water (198.857 ml). Pellet was mixed to the buffer in small amount and then the dialysis membrane was used to get a clear enzyme. Before subjecting for this process, the membrane was pretreated in the buffer for 10 mins. After pretreatment, the pellet was injected to the membrane through the needle. Both the ends of the membrane were tightly tied in order to prevent leakage and now membrane with pellet was inserted into the beaker containing buffer and the beaker was closed with a glass plate. This setup was kept for two days, and the buffer was changed every 24 hours and it is shown in the Figure 5. During dialysis, the pure enzyme will get attached to the membrane and the other waste particles are removed. After two days, the pure cellulase enzyme can be obtained.

Results and Discussion

From this study, it has been shown that cellulase was produced by *Aspergillus niger.* And its characterization (protein estimation, enzymatic activity, application(carotenoid extraction), SDS-PAGE) was estimated.

Protein estimation

In this, protein concentration was estimated by using the protocol of Lowry method (1951). The protein concentration was estimated by adding, (100 µl of sample (enzyme) and 900 µl of water and 1 ml of Lowry reagent) and, these are all said to be a reaction mixture. Then the reaction mixture was incubated for 10 mins. After incubation, 100 µl of follin reagent was added, which is shown in the Figure 6, then the OD was taken at 620 nm in a spectrophotometer. A similar procedure was carried out without enzyme and with enzyme for the estimation of protein concentration. Finally it was observed that the protein concentration was higher in the enzyme treated mixture than the non-enzyme treated mixture, which is shown in the Table 1.

Enzyme activity

Cellulase activity was measured by the DNS (3, 5- dinitro salicylic acid) method. This method determines the amount of reducing sugar liberation. The enzyme activity was determined by taking (900 µl of water, 100 µl of sample (enzyme) and 1 ml of 1% (0.1 g) CMC) and added together, and then the mixture is incubated for 20 mins at 60° C.

Figure - 1: The fungal culture produced zone of hydrolysis

Figure - 2: Fungal growth on Sporulation plate

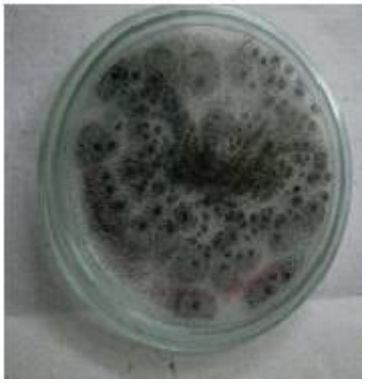

Figure - 3: Fungal growth on Inoculation medium

Figure - 4: Fungal growth on Production medium

Figure - 5: Dialysis process

Figure - 6: Lowry's test

Table 1: Concentration of protein

Sl. No.	Test	Protein concentration (µl/ml)
1	Enzyme treated	2.24
2	Enzyme untreated	1.22

Table 2: Measurement of enzyme activity

Sl. No.	Test	Enzyme activity (Units/ml)
1	Enzyme treated	1.99
2	Enzyme untreated	1.05

Figure - 7: SDS-PAGE

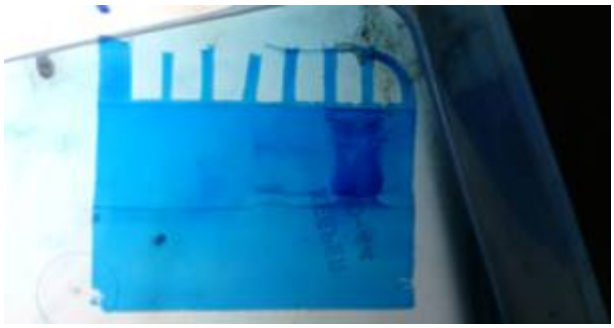

Table 3: Estimation of Absorbance value

Sl. No.	Test	Absorbance value (450 nm)
1	Enzyme treated	2.278
2	Enzyme untreated	1.876

Table 4: Estimation of Carotenoids

Sl. No.	Test	Carotenoids extracted (µg)
1	Enzyme treated	1.423
2	Enzyme untreated	1.172

After the period of incubation, 2 ml of DNS reagent was added to stop the reaction. And then the reaction mixture was incubated for 10 mins at 25° C. The same procedure was carried out without enzyme to determine the enzyme activity. The OD value was recovered at 550 nm in spectrophotometer. It was finally observed, that the enzyme treated mixture produced good result than the non-enzyme treated mixture, and it is shown in the Table 2.

SDS-PAGE

SDS- PAGE was performed to determine the molecular weight of the enzyme. The sample was mixed with the loading buffer. In the first well, the protein marker was loaded, where in the other wells the samples were loaded and then the entire setup was connected to a power supply at 70V for 1 hour 20 minutes. This setup was made to run for 1 hour and then the gel was removed and DNA bands were observed using destainer and it is shown in figure -7.

Application

Cellulase enzyme was used to extract the carotenoid content from vegetable source (sweet potato peel) and the result was compared by treating the sample with enzyme and without enzyme. It was finally observed that the enzyme treated mixture produced good result than the non-enzyme treated mixture.

Sweet potato peels were collected at Koyembedu, Chennai. 1 g of sweet potato peel was homogenized with 25 ml sodium acetate buffer, (pH 5), 0.3 ml produced cellulase enzyme was added to this mixture. Samples were stirred on a magnetic stirrer at medium speed for 24 hours at 37° C. After 24 hrs the enzyme was treated with the mixture of methanol/ethyl acetate/ petroleum ether (1:1:1, v/v/v) containing BHT (Butylated hydroxytoluene) as antioxidant was added to the enzymatic mixture. After 2 hrs of extraction at room temperature, the extract was washed two times with diethyl ether in a separate funnel. The organic layer containing carotenoid was dried over anhydrous sodium sulfate and evaporated to dryness. The extraction of Carotenoid without the enzyme was carried out by using the same procedure. The samples were kept under nitrogen at -20° C until the further utilization. The carotenoid content was estimated spectrophotometrically, at 450 nm and the value was shown in Table 3. The total Carotenoids content from the sweet potato peel was calculated according to the formula in Table 4.

X (mg carotenoids) = (A x V x 1000) / (2500 x 1 x 100)
Where
A = the sample absorbance at lambda max = 450 nm
V = sample volume (ml)
2500 = molar absorption coefficient for carotenoid (E1%)
1 = 1 cm the length of spectrophotometer curve

Conclusion

From this study, it can be concluded that *Aspergillus niger* possesses great celluloytic activity. Further, a small amount of cellulase enzyme was produced by *Aspergillus niger* on a pilot scale. Partial purification was carried out by precipitation (Ammonium sulphate) method followed by dialysis and then its activity, protein concentration, etc. were estimated. And the carotenoid is extracted from the vegetable (sweet potato) by treating with the cellulase enzyme produced, and its containing the higher concentration of carotenoid (1.423 µg) compared to the enzyme untreated.

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