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Cellulase production by *Aspergillus niger* under solid state fermentation using agro industrial wastes

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Cellulose,
Aspergillus niger,
agro industrial residues,
solid state fermentation.

Abstract

The aim of the present work was focused on the cellulase production by *Aspergillus niger* using different substrates (i.e) rice bran, wheat bran, black gram bran, coconut oil cake, groundnut oil cake, and gingely oil cake. Solid state fermentation holds tremendous potentials for the production of the enzyme cellulase by *Aspergillus niger*. These agro industrial residues are cheap raw materials for cellulase production. *Aspergillus niger* was identified to be the best producer of cellulase. When *A. niger* was incubated for 6 days at 37°C it showed high yield of cellulase in groundnut oil cake substrate in solid state fermentation. Sucrose and nitrogen improved the yield in the same medium.

Introduction

Enzymes of commercial or industrial importance are obtained from three main sources namely plants, animals and microorganisms. In the past, plants and animals served as main source of enzymes but today microbial sources of enzyme are becoming more popular for obvious reasons (Abu *et al.,* 2000). In order to obtain even a small quantity of plant enzymes, a large amount of plant materials has to be used and this renders large scale production of plant enzymes uneconomical, especially if the plant has some economic values or uses. Also difficulties are encountered in the extraction of the enzyme from plants (Howard *et al*, 2003). In the case of animal source, enzymes obtained from them are usually by-products of the meat industry and hence the supply can be limiting. Also other valuable products may be needed from the same organs used for enzyme production; such competition will further reduce the amount of materials from which such enzymes can be extracted. On the other hand, microbial enzymes are not subject to any of the problems of plant and animal enzymes (Emmanuel *et al*., 2007). In addition, the number of microbial enzymes is almost limitless, while the number, mode and amount to be produced at a time can be manipulated by the producers. Furthermore, enzymes of commercial values are extracellular in nature and are thus released into the cultured medium of the microorganisms and can be obtained by filtration and centrifugation rather than the vigorous methods of extraction at the end of the fermentation (Abu *et al.,* 2000).

Cellulase refers to a class of enzymes produced chiefly by fungi, bacteria and protozoan that catalyzed the cellulolysis (or hydrolysis of cellulose). Although there are also cellulose producing plants and animals, a large number of microorganism are capable of degrading cellulose, only a few of these microorganisms produce significant quantities of cell free enzyme capable of degrading cellulose *in vitro.* Fungi carry out extracellular digestion and secrete digestive enzyme into their substrates and absorb only digested food into their hyphae as such, they produce cell free enzyme. Fungi are the main cellulase producing microorganism in which *Aspergillus sp*. are known to hydrolyse both soluble and insoluble cellulose (Sridevi *et al.,* 2007).

Cellulase is used for commercial food processing in coffee. It performs hydrolysis of cellulose during drying of coffee beans. Furthermore, cellulase is widely used in textile industry and in laundry detergents. Cellulase has also been used in the pulp and paper industry for various purposes. They are even used in pharmaceutical applications. Cellulase is used in the fermentation of biomass into biofuels, although this process is relatively experimental (Okafor *et al.,* 2007). Cellulase is used as a treatment for phytobezoars, a form of cellulose bezoar found in the human stomach.

Since the production of cellulase enzyme is a major factor in hydrolysis of cellulosic material, it is important to make the process economically viable; this study therefore investigated on the bioconversion of agricultural waste like rice bran, wheat bran, black gram bran, coconut oil cake, groundnut oil cake, and gingely oil cake (which could cause pollution to the environment) into a more useful product (cellulase) using *Aspergillus niger*.

Materials and Methods

Sources of agricultural waste and pretreatment

The substrates used for this work were rice bran, wheat bran, black gram bran, coconut oil cake, groundnut oil cake, and gingely oil cake. The rice bran, wheat bran ground nut oil cake, coconut oil cake and sesame oil cake was obtained from the local market of Parangipettai. One gram each of sample was measured into separate conical flask containing 20ml of 5% NaOH solution. This was autoclaved at 121^{o}C for 1 hour to free cellulose from lignin hold. The NaOH solution was drained off by sieving through a muslin sieve. Samples were rinsed several times with distilled water, neutralized with 0.1 M HCl and finally washed with distilled water. The pretreated samples were dried in oven at 70^{o}C for 24 hrs and further made on to powder form in an electric blender (Ali *et al*., 1991).

Preparation of Inoculums

The fungus *Aspegillus niger* was isolated from Vellar estuary, in Parangipettai and maintained on Potato Dextrose Agar (PDA) slants. It was later sub-cultured on fresh sterile carboxyl methyl cellulose mineral salt agar slant; it was maintained at 37^{0}C and used throughout the experiment.

Solid state fermentation for cellulase production

The basal medium comprised of (per litre of distilled water); KH_2P0_4, 10.0g; $(NH_4)_2$ SO_4, 10.5g; $MgSO_4$. $7H_20$, 0.3g; $CaCl_2$, 0.5g; $FeSO_4.7H_2O$, 0.013g; $MnS0_4.7H_20$, 0.04g; $ZnSO_4$. $7H_20$, 0.004g; $COCl_2$. $6H_20$, 0.0067g; yeast extracts, 0.5; into separate 250 ml conical flask containing 100 ml of the basal medium was added 40g each of the treated carbon source (rice bran, wheat bran, black gram bran, coconut oil cake, groundnut oil cake, and gingely oil cake). The pH of the media was then adjusted to 5. 1% of inoculums was inoculated after sterilization and incubated at room temperature for six days. (Milala *et al*., 2005).

Enzyme Extraction:

22ml of 0.1M phosphate buffer saline (pH 7) was added to each of the inoculated substrate beds and was vigorously shaken in rotary shaker for 15 minutes at 120rpm. The mixture was filtered through cheese cloth and centrifuged at 8000rpm at 40C for 15min. The supernatant was filtered through cheesecloth and the filtrate was used as the crude enzyme preparation.

Cellulase activity

Carboxymethyl cellulose (CMCase) activity was determined according to the method of Mandels *et al*.,1976. 0.5 ml of 1% carboxymethyl cellulose (CMC) in 0.1 M citrate buffer pH 5.6 was placed in a test tube and 1.0 ml of culture filtrate was added. The test tube was incubated at 40^{0}C in a water bath with shaker for 30^{0}Cmin. The reaction was terminated by adding 2.0 ml of 3.5 –dinitrosalicylic acid (DNS) reagent to the reaction mixture , boiled for 5 min. (Miller, 1959). The absorbance of the appropriately diluted reaction mixture was read at 540nm using a spectrophotometer. One unit of cellulase was defined as the amount of enzyme that released 1µmol reducing sugar as glucose equivalent per min in the reaction mixture under the specified assay conditions. All enzyme assays were performed in triplicates.

Specific activity of the enzyme was calculated

One unit of cellulase activity is defined as the amount of enzyme, which released 1 µM of glucose per minute per milligram protein (U/ml/min).

Effect of Incubation Time

The effect of incubation period on enzyme production was investigated by checking the enzyme activity on 4th, 5th, 6th, 7th and 8th days of incubation in the different solid substrates at pH 7 and at room temperature.

Effect of pH

Solid State Fermentation investigated the effect of pH on enzyme production in different substrates by adjusting the pH of basal salt solutions to 4, 5.5, 6.5, 7, 7.5, 8 and 9. The substrates were then incubated for 6 days at room temperature.

Effect of Temperature

The effect of temperature on enzyme production was investigated by SSF in different substrates and incubated at 30^{0}C, 40^{0}C, 45^{0}C and 50^{0}C at pH 7 for 6 days.

Effect of Carbon Source

The effect of carbon sources on enzyme production was investigated by supplementing the basal salt solution, pH 7, with 2% of different carbon sources such as glucose, maltose, lactose, sucrose and starch. The substrates were then incubated for 6 days at room temperature.

Effect of Nitrogen Source

The effect of nitrogen source on enzyme production was studied by replacing the nitrogen source in basal salt solution, pH 7, with 2% of $NaNo_3$, $(NH)_4$ SO_4, NH_4 Cl, NH_4 NO_3 and KNO_3, and incubated at room temperature for 6 days.

Results

Solid State Fermentation:

Different substrates like rice bran, wheat bran, black gram bran, coconut oil cake, groundnut oil cake, and gingely oil cakewere used as solid substrates for SSF. After inoculation and incubation for six days at room temperature with pH 7, the enzyme was extracted using phosphate buffer and was estimated for the protein content and the enzyme activity. The specific activity was recorded as 15.56 U/ml/min for cellulase enzyme produced by *Aspergillus niger*, which was the highest yield in the substrate sesame oil cake.

Effect of Time of Incubation:

Aspergillus niger was inoculated into different substrates like rice bran, wheat bran, black gram bran, coconut oil cake, groundnut oil cake, and gingely oil cakeused as substrates for SSF. The moisture content of the medium was adjusted by adding basal salt solution. The cultures were incubated at room temperature for 4- 8 days. The enzyme was extracted and the specific activity of the cellulase produced at different days of incubation and in different substrates was recorded (Fig: 1). The specific activity observed was as high as 10.2 U/ml/minin the substrate gingely oil cake in 4 days of incubation. The specific activity was 31.1 U/ml/min for the enzyme at six days of incubation in groundnut oil cake substrate. The specific activity was 8.6 U/ml/min for the enzyme at five days of incubation in Black gram Bran substrate. For six days of incubation the specific activity was 31.1 U/ml/min which was higher than in any other substrates. In 7 days of incubation in Rice bran the specific activity was 6.5 U/ml/min and for eight days of incubation in coconut oil cake the specific activity was 11.2 U/ml/min. Therefore it is clear that, each substrate was utilized better in different days of incubation.

Effect of pH

Aspergillus niger was inoculated into different substrates were incubated at room temperature for four days. The enzyme was extracted and the specific activities of the cellulase produced at different pH and in different substrates were recorded (Fig: 2). The maximum yield of cellulase was in pH 5.5 and the specific activity was 1.3 U/ml/minin rice bran. This was very low when compared to other pH range. For wheat bran at pH 6.5 there was high yield (specific activity was 1.3 U/ml/min) than other substrates. There was marked increase in the yield till pH 8, and then there was very minimum activity at pH 9 in all the substrates used in SSF.

Effect of Temperature

Aspergillus niger when inoculated at different temperature 30^0C, 35^0C 40^0C, 45^0C, and 50^0C showed maximum yield of cellulase (specific activity was 15.4 U/ml/min) at 40^0C in groundnut oil cake. There was increase in yield in 40^0C in coconut oil cake medium and in rice bran medium when the temperature was 45^0C. Then a gradual decrease in yield was observed (Fig: 3)

Effect of Carbon Source

Addition of different carbon source like glucose, maltose, lactose, and starch did not show remarkable change in the yield. Lactose showed a better yield (specific activity was 31.6 U/ml/min) (Fig: 4). Notably in the groundnut oil cake medium and in coconut oil cake medium the yield was inhibited by the addition of carbon source.

Effect of Nitrogen Source

Sodium nitrate, ammonium sulphate, ammonium chloride, ammonium nitrate, and potassium nitrate were used as nitrogen source along with the solid substrates. Nitrogen source greatly increased the yield of the enzyme produced (specific activity was 25.9 U/ml/min) (Fig: 5) in groundnut oil cake medium. There was remarkable increase in the production of cellulase in ammonium nitrate supplemented black gram medium and in ammonium chloride supplemented coconut oil cake medium.

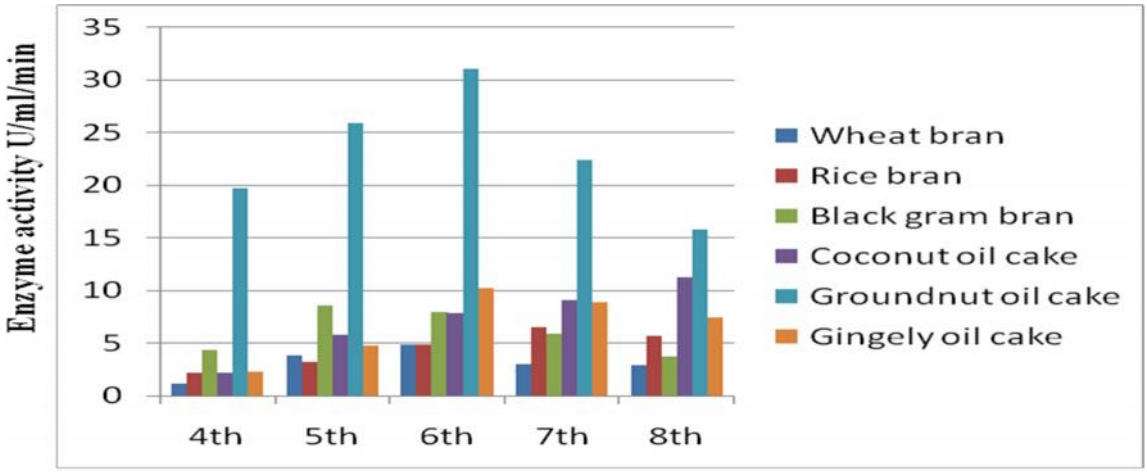

Fig 1.Effect of incubation period

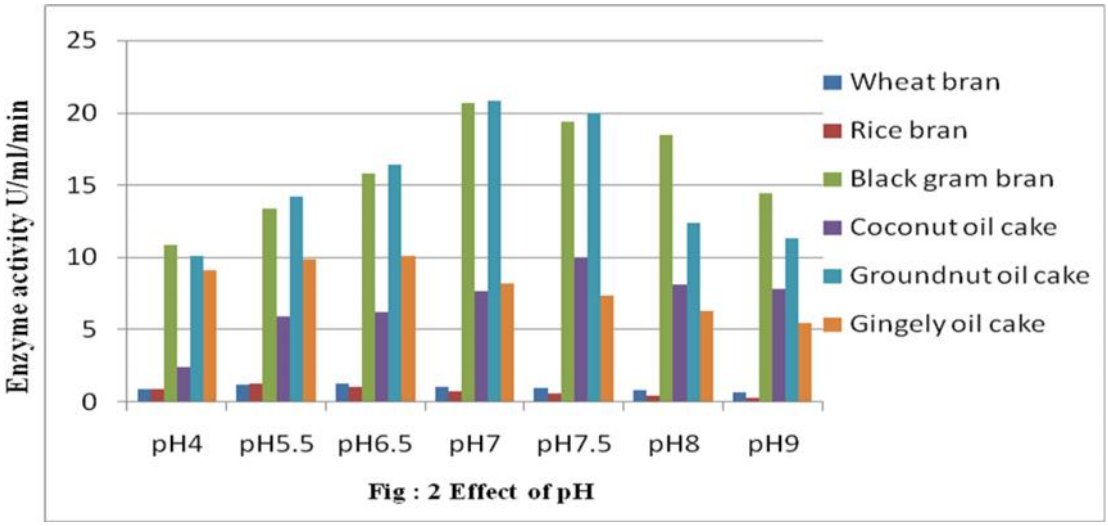

Fig : 2 Effect of pH

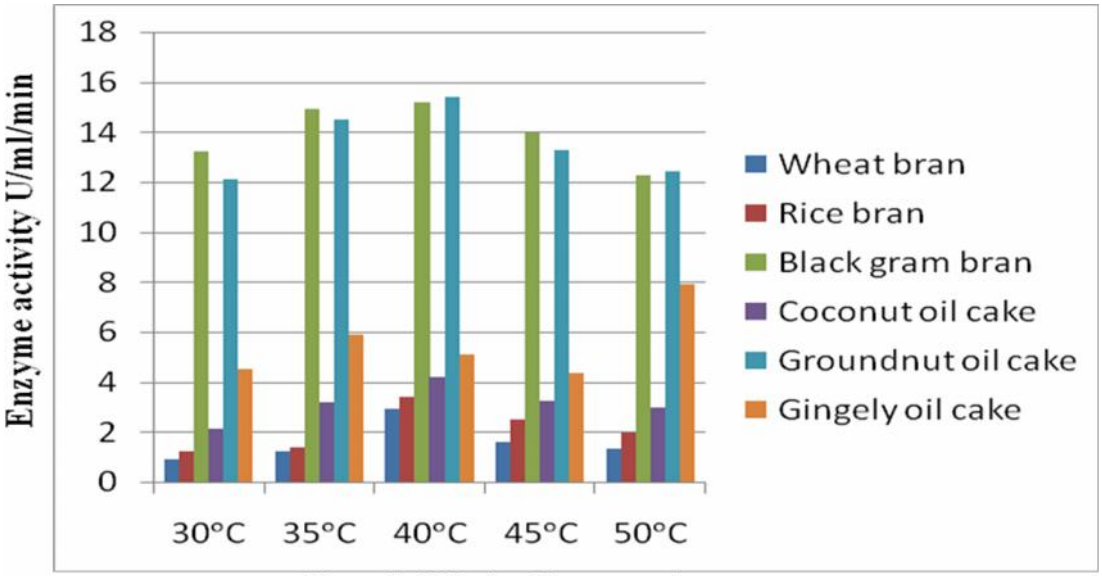

Fig : 3 Effect of temperature

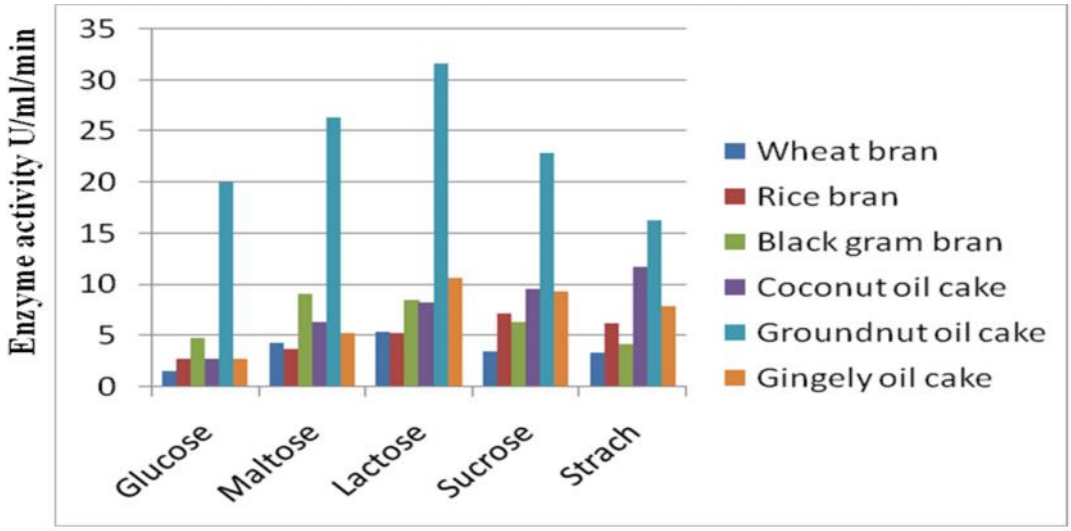

Fig : 4 Effect of Carbon source

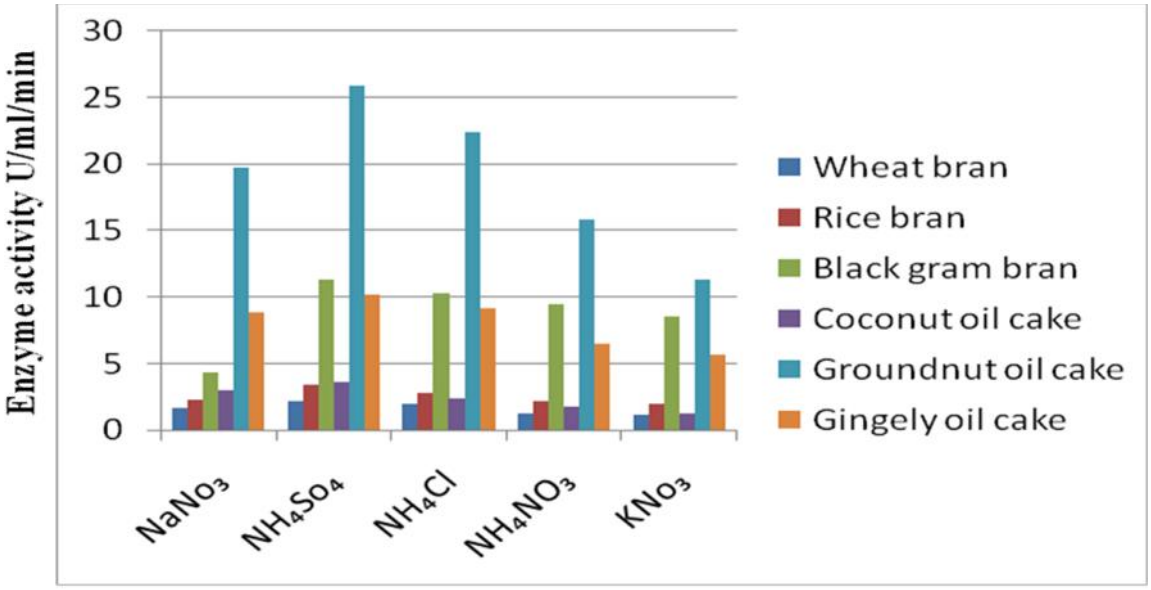

Fig : 5 Effect of Nitrogen source

Discussion

The results showed that *A. niger* produced cellulase enzyme (E.C.3.2.1.4) when cultured on mineral salt media containing rice bran, wheat bran, black gram bran, coconut oil cake, groundnut oil cake, and gingely oil cake used as carbon source. Extracellular protein with significant cellulase activity was obtained from the cultures of all the different carbon sources (Fig 1). Most members of the *A. niger* group are notable producers of extracellular enzyme such as cellulase (Solomon *et al.*, 1999).

Majority of studies on microbial production of cellulolytic enzymes were conducted with one fermentation method either submerged fermentation or solid state fermentation method but not both. Among four lignocelluloses cassava bagasse, sugar cane bagasse, rice straw and wheat bran tested in solid state fermentation by *Trichoderma reesei* NRRL 11460, sugar cane bagasse yielded maximum titers of FPase after 96 hours of growth (Singhania *et al.*, 2006). A comparative study (Sukumaran *et al.*, 2009) with two fungal cultures on wheat bran in solid state fermentation indicated that *T. reesei* Rut C-30 relatively produced higher yields of FPase and endoglucanase whereas A. niger gave relatively higher titers of glucosidase. Cultivation of Trichoderma reesei ZU02 in deep trough fermentor for 5days in solid state fermentation generated 128 U/g of FPase (Xia and Cen, 1999). Among different lignocelluoses tested in SSF for production of cellulases, wheat bran was the best substrate followed by groundnut fodder (Subhosh Chandra *et al.*, 2007). Protein content in these koji materials reached to about 5mg/gDS submerged fermentation of rice straw by *Trichoderma harzianumgave* yields of 0.13, 0.15 and 1.65 U/mL in respect of exoglucanase, endoglucanase and cellobiase under optimal conditions (Kocher *et al.*, 2007). Fusarium oxysporum produced FPase, CMCase and glucosidase to the extent of 1.34, 1.92 and 1.78 U/ mL respectively (Ramanathan *et al.*, 2010). (Victor *et al.*, 2003). Obtained 0.0743, 0.0573 and 0.0502 IU/mL of cellulase within 12 h by *Aspergillus flavus* on substrates- sawdust, bagasse and corncob, respectively. Yields of FPase and CMCase obtained with *Trichoderma viridae* in submerged fermentation were 1.5 and 1.0 U/mL (Nathan *et al.*, 2014). Cultivation of *A. niger* on saw dust in liquid culture yielded 2.42 U/mL of FPase (Narasimha *et al.*, 2006). Different yields obtained in different studies could be attributed to inherent capacities s of organism used, different cultural practices and different lignocelluloses used.

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