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RESEARCH **P**APER

Production and optimization of cellulase enzyme using cheap substrates by A*spergillus niger isolated* from soil

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Studies were conducted on the production and optimization of cellulase enzyme using cheap substrates namely saw dust, sugarcane waste, coconut coir waste and newspaper waste by *Aspergillus niger*. *A. niger* was isolated from soil, cultured and sub-cultured in the laboratory to obtain pure culture. Cellulase production was done by solid state fermentation using four substrates separately. The isolate *Aspergillus niger* was studied for its growth kinetics, cellulose enzyme production, optimum pH and temperature, and time profile. Growth kinetics of *Aspergillus niger* showed that the stationary phase reached between day 4 and 6. *A. niger* reported growth and enzyme production in all the four substrates but among the four, coconut coir showed maximum cellulase enzyme activity (3.0±0.10 U/g) followed by sugarcane waste (2.8±0.12 U/g), newspaper waste (2.4±0.15 U/g) and saw dust (2.0±0.12 U/g). The optimal pH and temperature for the maximum biosynthesis of cellulase by *A. niger* were reported as 6.2±0.15 and 28±0.5ºC, respectively. The production of cellulase was noticed after 96 h of incubation but maximum production of cellulose enzyme was reported in 120 hours.

Key words : Cellulase, *Aspergillus niger*, Saw dust, Sugarcane waste, Coconut coir waste, Newspaper waste

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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. 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. 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. On the other hand, microbial enzymes are not subject to any of the problems of plant and animal enzyme. Furthermore, enzymes of commercial values are extracellular in nature and are, thus, released into the cultured medium of the micro-organisms and can be obtained by filtration and centrifugation rather than the vigorous methods of extraction at the end of the fermentation (Abubakar and Oloyede, 2013).

Aspergillus niger is a member of the genus *Aspergillus* which includes a set of fungi that are generally considered asexual, although perfect forms have been found. It is ubiquitous in nature and commonly found as a saprophyte growing on dead leaves, stored grain, compost piles, and other decaying vegetation. The primary uses of *A. niger* are the production of enzymes and organic acids by fermentation. It is the most commonly used fungus for citric acid production due to the high

yield and relatively high tolerance to acid accumulation (Pandey *et al.*, 2013). It has been used by many researchers and in many studies, mainly in solid-state fermentation (SSF), for its ability to live and grow in an environment similar to its natural habitat. In the last 3 decades, SSF has gained great interest from researchers and industries as an alternative technique to the traditional submerged fermentation (SMF). The unique characteristics of SSF, using solid materials, stimulated researchers to use waste such as agro-residual and agro-industrial wastes, fruit waste as an alternative to raw materials for the production of citric acid. Several advantages of solid-state fermentation over submerged fermentation have encouraged researchers to study and develop it, such as lower energy requirements, less risk of bacterial contamination, and fewer environmental concerns regarding the disposal of solid waste (Pandey *et al*., 2013).

Cellulases are enzymes produced chiefly by fungi, bacteria and protozoans that catalyze hydrolysis of cellulose. In the natural world and in the area of industry cellulases are highly essential enzymes since they have a significant activity in the carbon cycle globally which are able to break down cellulose. Cellulases have been divided into endocellulase, exocellulase, cellobiase or beta glucosidase, oxidative cellulases and cellulose phosphorylase based on their mode of hydrolysis. Endocellulase breaks internal bonds of cellulose to disrupt the crystalline structure and expose individual cellulose polysaccharide chain. Exocellulase cleaves two to four units from the ends of the exposed chains produced by endocellulase resulting in the tetrasaccharides or disaccharides such as cellobiose. Cellobiase or beta glucosidase hydrolyses the exocellulase product into individual monosaccharides. Oxidative cellulases depolymerize cellulose by radical reactions. Cellulose phosphorylase depolymerizes cellulose using phosphates instead of water (Chouhan, 2014). The production of this enzyme has increased significantly and today its production is around 20% of all the enzymes produced in the world. Large demand of cellulases has increased their prices to a large extent and the major reason is the cost of substrate and fermentation procedure. It is the need of the time to search for cheaper substrates and reduced fermentation cost so that the production cost can be reduced to a large extent (Reddy *et al.*, 2015). Hence, in the present study, *Aspergillus niger* isolated from soil rhizosphere region was used for the production of cellulase enzyme from cheap substrates namely saw dust, sugarcane waste, coconut coir waste and newspaper waste by SSF.

RESEARCH METHODOLOGY

The soil samples were aseptically collected from rhizosphere region. Serially diluted sample was prepared and spread on the surface of potato dextrose agar (PDA) and incubated for seven days at 30ºC. Colonies were picked and sub-cultured to obtain pure culture. Stock cultures were maintained on potato dextrose agar at 4ºC. The isolated strains were identified by studying the morphological characteristics which include colour of the colony and growth pattern studies. The vegetative and reproductive structures were observed under the microscope using lactophenol cotten blue stain. The fungal isolate was screened for its cellulase producing abilities on carboxymethyl cellulose (CMC) agar plates in which the isolate was streaked centrally on CMC agar media and incubated at 28°C for 48hours. After the completion of incubation period the plates were flooded with 0.1% Congo red solution and washed with 1 M NaCl for 15–20 min. Plates were observed for zone of cellulose hydrolysis. Inoculum of fungal isolate showing cellulose hydrolysis was prepared.

Saw dust, sugarcane waste, coconut coir waste and newspaper wastes were used as a substrate for enzyme production. Saw dust was pre-treated with 2N NaOH and newspaper, sugarcane waste and coconut coir with distilled water. 100 ml of mineral salt medium was prepared and 20g each of the treated substrates were added. The pH of the media was then adjusted to 6.2 and sterilized in an autoclave at 121°C for 15 minutes. Media were inoculated with 1ml of inoculum of fungal isolate showing cellulose hydrolysis and incubated at 28°C in an orbital shaker at 120rpm for 120hours. After the completion of incubation period, the mixture was filtered by whatman's filter paper No.1 and the filtrate was centrifuged at 8000rpm for 5 minutes at 4°C. After centrifugation, supernatant was collected and used as crude enzyme. Amount of protein in crude enzyme was determined by Lowry's method of protein estimation. Enzyme activity in crude enzyme was determined by DNS method (Sadasivam and Manickam, 2011). To optimize cellulase production, estimation of physical parameters namely pH and temperature were carried out.

RESEARCH FINDINGS AND ANALYSIS

Different fungal isolates and pure culture of *A. niger* grown in PDA Petriplate are shown in Fig. 1 and 2, respectively. The conidial head and zone of hydrolysis of *A. niger* are shown in Fig. 3 and 4, respectively. Growth kinetics of *Aspergillus niger* is shown in Fig. 5. The activities of cellulase enzyme in four SSF extracts are shown in Fig. 6. The activity of cellulase enzyme at various pH and temperatures are shown in Fig. 7 and 8.

Different fungal species were obtained after seven days of incubation and among them, based on morphological studies and lactophenol cotton blue staining characteristics, the isolate of *Aspergillus niger* was identified and was selected and sub cultured by point inoculation. The culture of *A. niger* produced colonies of yellow to white hyphae turning the colour to black with the formation of conidia. Microscopically, *A. niger* was identified by its hyaline and septate hyphae. Asexual conidiophores were identified by observing the long and globose tip. The major distinction separating *A. niger* from the other species of *Aspergillus* was the production of carbon black or very dark brown spores. The selected fungal isolate was screened for its cellulase producing potential by using 0.1% Congo red solution. The growth of the isolate was studied by reading the absorbance at 600nm. Growth kinetics of *Aspergillus niger* showed

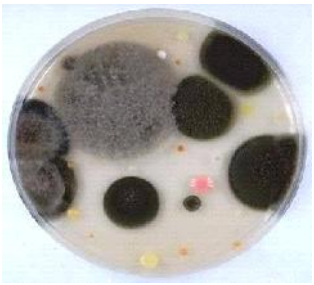

Fig. 1

Fig. 2

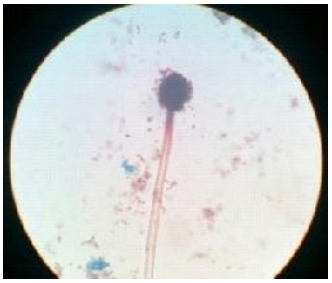

Fig. 3

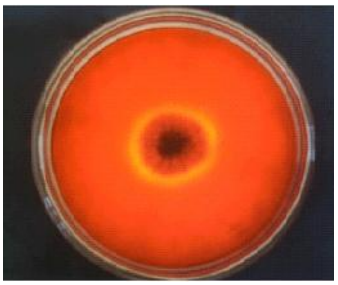

Fig. 4

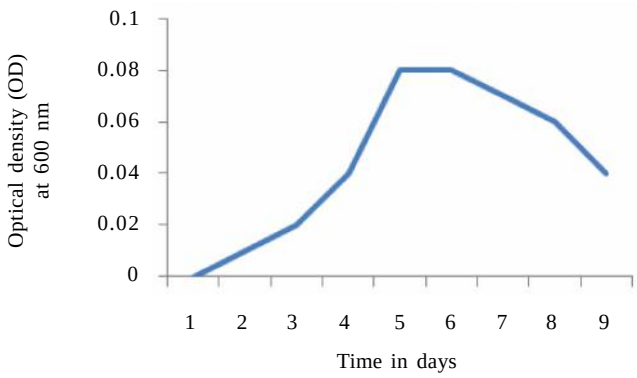

Fig 5: Growth curve of *Aspergillus niger*

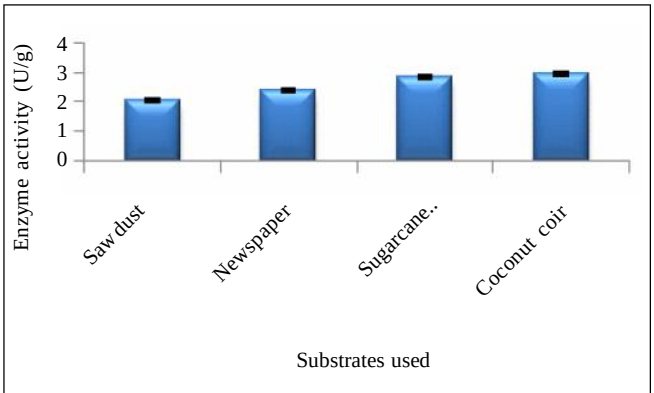

Fig 6: Enzyme activity in SSF extracts

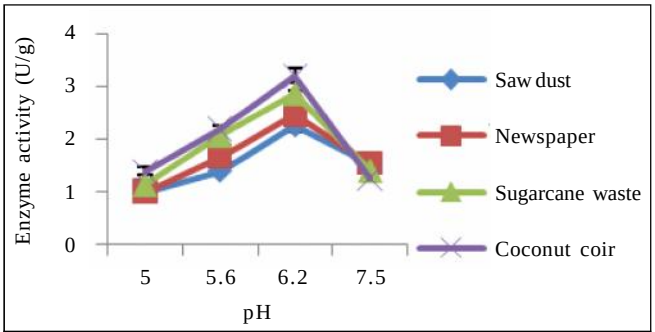

Fig 7: Cellulase enzyme activity at various pH

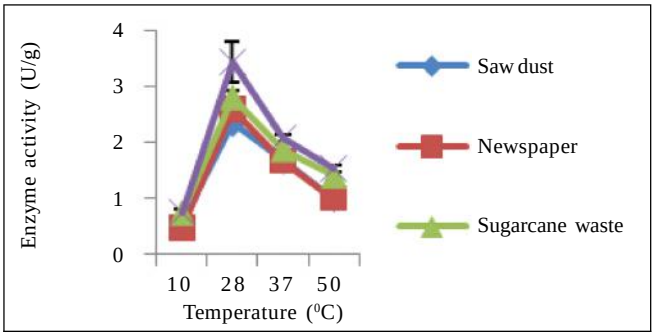

Fig 8: Cellulase enzyme activity at various temperature

that the stationary phase reached between day 4 and 6.

Aspergillus niger was used for cellulase production by solid state fermentation (SSF) technique which can improve the yield and reduces the cost of enzyme production. Filamentous fungi are the most commonly used micro-organisms in SSF because they are able to grow on solid materials with low water content. There are several reports describing use of agro industrial residues for the production of cellulose such as wheat straw, wheat bran and rice straw as substrates. The other advantages of SSF include superior productivity, simple technique, low capital investment, low energy requirement, less water output, better product recovery and lack of foam build up, and reported to be the most appropriate process for developing countries (Fadel, 2000). The isolate *A. niger* reported growth and enzyme production in all the four substrates but among the four, coconut coir showed maximum cellulase enzyme activity (3.0±0.10 U/g) followed by sugarcane waste (2.8±0.12 U/g), newspaper waste (2.4±0.15 U/g) and saw dust (2.0±0.12 U/g).

Azzaz *et al.* (2012) studied cellulose production using wheat straw as a sole of carbon source by locally isolated fungal cultures including *A.niger, Fusarium oxysporum*, *Avenaceum* and *Cephalosporium acrenonium* and reported that the highest cellulose production was obtained from *A. niger*. In the present study, the production of cellulase was noticed after 96 h of incubation and maximum production of enzyme was reported in 120 hours. The optimal pH for the production crude enzyme was determined by incubating the crude enzyme with substrate (1% CMC) prepared with different pH that are 5.0, 5.6 and 6.2 for 30 minutes. The optimal pH for the maximum biosynthesis of cellulase by *A. niger* was reported as 6.2±0.15. The optimal temperature for the production of crude enzyme was determined by incubating the crude enzyme with substrate (1% CMC) prepared with different temperatures that are 28°C, 37°C and 50°C for 30 minutes. The optimal temperature for the maximum biosynthesis of cellulase by *A. niger* was reported as 28±0.5ºC. The production of cellulase has been reported from a wide variety of bacteria and fungi (Brown, 2004 and Jahan *et al.*, 2012). However, filamentous fungi are preferred for commercial enzyme production because the level of the enzymes produced by these cultures is higher than those obtained from yeast and bacteria. Almost all the species of genus *Aspergillus* synthesize cellulase, therefore, this genus has the potential to dominate the enzyme industry.

Conclusion:

Based on the above study it can be said that all the four substrates studied can be a good source for cellulase production, and could be used for economical production of cellulase enzyme. Although good activity was seen in all the substrates, coconut coir showed maximum cellulase enzyme activity. They can be studied in order to increase the activity by trying different pretreatment procedures and optimizing the incubation time. Cellulases are one of the most widely used enzymes for the preparation of fermented foods and are used in food, beverages, textile, laundry, paper and pulp industries etc. Therefore, there has been much research aimed at obtaining new microorganisms producing cellulase enzymes with higher specific activities and greater efficiency.

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