



PRODUCTION OF CELLULASE ENZYME BY *ASPERGILLUS NIGER* UNDER SOLID STATE FERMENTATION

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ABSTRACT

Filamentous fungi are important due to their high enzyme production potential. Many enzymes produced by fungi have been related to biotechnological applications in several industrial sectors. The purpose of this study was to isolate filamentous fungi from soil using rice bran, paddy husk and chaffer grains as a feed substrate and to screen for cellulase production potential. The cellulase enzyme extract was partially purified and its protein fraction was stained and molecular weight was determined by using SDS-PAGE. In this study *Aspergillus niger* were subjected for Solid State Fermentation (SSF) for the production of cellulose. These fungi showed maximum cellulase activities at 72 hours of incubation. The physical and nutritional parameters of fermentation like pH, temperature, substrate, carbon and nitrogen sources were optimized. The optimal conditions for maximum biosynthesis of cellulase by *A. niger* were found to be at pH 6 and temperature 30°C. Among the three substrates, rice bran showed maximum cellulase production followed by paddy husk and chaffer grains. The present investigation paved the way for the production of fungal cellulase enzymes suitable for various industrial applications.

KEYWORDS

Aspergillus niger, Cellulase, Solid State Fermentation, SDS-PAGE

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1. INTRODUCTION

In recent years, environmental pollution has become a global problem. Enormous growth of industrial activities has given rise to problems such as global warming, desertification and acid deposition. These global problems are rooted in the materially rich lifestyles, which are supported by abundant and extensive use of fossil fuels in industrialized countries. Rapidly increasing industrial activities in China, India, and in other developing countries implicate that these practices will inevitably contribute to deterioration of the global environment and to destruction of the global ecosystem. The recycling of materials, minimizing the generation of waste, is a basic concept which must be implemented in order to meet the new demands of development in both industrialized and developing countries.

Numerous agricultural residues generated due to diverse agricultural practices and food processing such as rice straw, yam peels, cassava peels, banana peels among others represents one of the most important energy resources. Recycling of agricultural residue can be achieved naturally and artificially by microorganisms. Aerobic organisms such as fungi, bacteria, and some anaerobic organisms have been shown to be able to degrade some constituents of these residues. Fungi play a significant role in the degradation of cellulose under aerobic conditions (Schlegel, 1999). Hence, the present study was aimed to analyze the activities of fungal cellulase on paddy husk, rice bran and chaffer grains.

Cellulose, hemicelluloses and lignin are the major components of plant cell walls, with cellulose being the most abundant component of plant biomass among all of them, which comprises on average 35 to 50% of plant biomass, however, the cellulose fibers are embedded in a matrix of other structural biopolymers, primarily hemicelluloses and lignin, which comprise 20 to 35% and 5 to 30% of plant dry weight respectively (Lynd *et al.*, 1999). Approximately 30 individual cellulose molecules are assembled into larger units known as proto fibrils, which are crammed into larger units called microfibrils, and these are in turn assembled into the familiar cellulose fibers.

2. MATERIALS AND METHODS

2.1. GENERAL METHODS

2.1.1. Sterilization

All the glass wares were soaked in cleaning solution (100g potassium dichromate was added to 100ml of distilled water followed by addition of 500ml of concentrated sulphuric acid) for about 12 hours and washed in tap water. They were thoroughly rinsed in tap water and dried. They were sterilized at 160°C for 3 hours in hot air oven. All the media were sterilized in an autoclave at 15lb pressure for 20min.

2.1.2. Chemicals

All the chemicals used in the experiments were of analytical reagents (AG) grade and distilled water was used throughout the period of study.

2.2. ISOLATION AND IDENTIFICATION OF *Aspergillus niger* STRAIN

2.2.1. Isolation of *Aspergillus niger*

The *Aspergillus niger* were isolated from soil by serial dilution method. 1 g soil sample was dissolved in 10 ml sterilized distilled water. The soil suspension was diluted upto 10^3 to 10^5 . The sample was inoculated on potato dextrose agar (PDA) for fungi. The inoculated media were incubated at 30°C for 3-5 days. Colony developments were observed after incubation period.

Well grown *Aspergillus niger* colonies were maintained on Sabouraud dextrose agar slants and stored at 4°C.

2.2.2. Identification of *Aspergillus niger*

Identification based on cell and colony morphology characteristic as per method described by Rasper and Fennel (1965). The young colonies of *Aspergillus niger* were aseptically picked up and transferred to PDA slants. The slants were grown at 30°C for 7 days and stored at 4°C. The isolate was sub-cultured for further studies.

2.3. VIABLE SPORE COUNT

The total viable spore number on a PDA slant was determined by colony count technique. One loopful of spores were suspended in 10 ml of distilled water with 0.1% between 80, using a sterile transfer needle and diluted serially upto 10^4 .

One ml of spore suspension was poured onto sterile Petri-plates, containing sterile PDA medium and spread uniformly. The inoculated Petri-plates were incubated at 30°C for 48 hrs. A plate that developed between 7 and 200 colonies was selected for counting. The spore density was calculated as the count multiplied by the dilution factor.

2.4. CELLULASE ASSAY

Cellulase activity was determined at 40°C by using carboxymethyl cellulose (Sodium salt, Hi-Media, India) as a substrate. A reactive mixture contains 0.5 ml of 1% (w/v) substrate in 0.1 M citrate buffer (pH 4.8) and 0.5 ml of culture supernatant. The mixture was incubated at 40°C for 30 min. The reducing sugar released was measured using 3,5-dinitrosalicylic acid (DNSA) (Miller, 1959). Control was prepared with 10 min boiled enzyme. One unit of endoglucanase activity was expressed as the amount of enzyme required to release 1 μ mol

reducing sugars per ml under the above assay condition by using glucose as a standard curve.

2.5. SUBSTRATES

Paddy husk, Rice bran and Chaffer grains were used as a substrate. They were obtained from mills in Annamalai Nagar, Cuddalore district, Tamil Nadu, India. The substrates were ground into coarse powder with a blender.

2.6. SOLID STATE FERMENTATION

Solid state fermentation was carried at 30°C with substrate initial moisture content of 64% for 72 hrs using 2 ml spore suspension as inoculum. Studies were also performed to evaluate the influence of supplementation of substrate with different carbon sources such as glucose, maltose, sucrose, lactose (3% w/v) and nitrogen source such as peptone, casein, urea, yeast extract (3% w/v).

3. RESULTS AND DISCUSSION

3.1. ISOLATION AND IDENTIFICATION OF *Aspergillus niger*

The fungi *Aspergillus niger* was isolated and identified from the soil sample and the characteristics of the isolates are given below.

3.1.1. Microscopic examination

Conidiophore stipes were smooth - walled, hyaline or pigmented. Vesicles were sub-spherical, conidial heads radiate. Conidiogenous cells were biserial. Medulla twice as long as the phialides. Conidia was brown, ornamented with warts and ridges. Hyphae were septate.

3.1.2. Colony morphology on SDA plate

Colonies were black, consisting of a dense tuft of conidiophores.

From these results, the fungal isolate was identified as *Aspergillus niger*.

3.2. VIABLE SPORE COUNT

The total viable spore count on a potato dextrose agar was determined by colony count technique. The well developed spore between 7 and 200 colonies on the medium was selected for counting. The spore density was calculated as the count multiplied by the dilution factor (Table – 1).

3.3. CELLULASE ASSAY

Cellulase activity was determined at 40°C by using carboxymethyl cellulose as a substrate and the results are showed in Table - 2. The enzyme activity of the fungi *Aspergillus niger* was high at 100 mg/ml glucose concentration (0.69 at 540 nm) and very low at 10 mg/ml glucose concentration (0.09 at 540 nm). The enzyme activity was increased when the concentration of glucose was increased.

3.3. OPTIMIZATION OF CULTURE CONDITIONS

3.3.1. Effect of Temperature on cellulase production by *Aspergillus niger*

The effect of various temperatures viz., 20°C, 30°C and 40°C on cellulase production was determined and the results are furnished in Table – 3. Maximum cellulase production was observed in rice bran at 30°C (3.86 ± 0.431 mg of glucose per hour) followed by 40°C (2.65 ± 0.254 mg of glucose per hour) and 20°C (1.25 ± 0.280 mg of glucose per hour). Followed by rice bran, cellulase production was highly recorded in the paddy husk and chaffer grains.

3.3.2. Effect of pH on cellulase production by *Aspergillus niger*

The effect of various pH viz., 6.0, 6.5 and 6.7 on cellulase production was estimated and the results are given in Table – 4. Maximum cellulase production was observed in rice bran at pH 6.0 (2.43 ± 0.154 mg of glucose per hour) followed by 6.5 (2.32 ± 0.255 mg of glucose per hour) and 7.0 (2.11 ± 0.224 mg of glucose per hour). Next to rice bran, cellulase production was higher in the paddy husk and chaffer grains respectively.

3.3.3. Effect of Salinity on cellulase production by *Aspergillus niger*

The effect of salinity on cellulase production was investigated and the results are showed in Table – 5. Among the three substrates, rice bran showed maximum cellulase production followed by paddy husk and chaffer grains. Maximum cellulase production was observed in rice bran at 60 ppm (1.323 ± 0.0721 mg of glucose per hour) followed by 50 ppm (0.667 ± 0.1098 mg of glucose per hour), 80 ppm (0.245 ± 0.00372 mg of glucose per hour), 40 ppm (0.216 ± 0.0257 mg of glucose per hour), 20 ppm (0.156 ± 0.0068 mg of glucose per hour) and 0 ppm (0.109 ± 0.0059 mg of glucose per hour).

3.3.4. Effect of time on cellulase production by *Aspergillus niger*

The effect of various incubation periods viz., 24 hrs, 48 hrs, 72 hrs, 96 hrs and 144 hrs on cellulase production were studied and the results was tabulated in Table – 6. Among the three substrates, rice bran showed maximum cellulase production followed by paddy husk and chaffer grains. Maximum cellulase production was recorded in rice bran after 48 hrs (2.63 ± 0.57 mg of glucose per hour) followed by 72 hrs (2.43 ± 0.43 mg of glucose per hour), 96 hrs (2.24 ± 0.510 mg of glucose per hour) and 144 hrs (2.17 ± 0.497 mg of glucose per hour).

3.3.5. Effect of carbon sources on cellulase production by *Aspergillus niger*

The effect of various carbon sources viz., Glucose, Fructose, Lactose, Xylose and Sucrose on cellulase production tested and the results are shown in Table – 7. Highest cellulase production was observed at rice bran in the source of fructose (2.918 ± 0.428 mg of glucose per hour) followed by lactose (2.627 ± 0.515 mg of glucose per hour), xylose (2.494 ± 0.233 mg of glucose per hour) and glucose (2.411 ± 0.159 mg of glucose per hour). Lowest cellulase production was recorded in the presence of sucrose (2.267 ± 0.325 mg of glucose per hour). Followed by rice bran, cellulase production was highly recorded in the paddy husk and chaffer grains with the vallans carbon sources.

3.3.6. Effect of nitrogen sources on cellulase production by *Aspergillus niger*

The effect of various nitrogen sources viz., Yeast extract, Beef extract, Peptone, Casein and Malt extract on cellulase production was tested and the results are tabulated in Table – 8. More cellulase production was observed at rice bran in malt extract (1.26 ± 0.468 mg of glucose per hour) followed by peptone (1.10 ± 0.416 mg of glucose per hour), casein (1.07 ± 0.421 mg of glucose per hour) and beef extract (0.69 ± 0.317 mg of glucose per hour). Lowest cellulase production was recorded in the presence of yeast extract (0.55 ± 0.201 mg of glucose per hour). Followed by rice bran, cellulase production was highly recorded in the paddy husk and chaffer grains.

3.4. ESTIMATION OF PROTEIN

Protein concentration was determined by the Lowry's method and the results were given in Table - 9. The protein crude extract levels found to be 53 µg/ml, 48 µg/ml and 42 µg/ml for rice bran, paddy husk and chaffer grains, respectively.

3.5. SODIUM DODECYL SULPHATE – POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS - PAGE)

The cellulase enzyme extract was partially purified and its protein fractions were stained and molecular weight was determination by using SDS-PAGE. The crude enzyme preparation was subjected to SDS-PAGE (containing 0.2% CMC) to determine the homogeneity and molecular weight of the enzyme. The result is displayed Figure - 1. It revealed two protein bands with the molecular weight of about 83 kD and 50kD. The enzyme protein was extracted from culture supernatant by the ethanol precipitation method.

4. DISCUSSION

Cellulase is an important enzyme for the hydrolysis of agro wastes and other cellulosic wastes. The present study was carried out to evaluate the cellulase activity of the cellulolytic fungi *Aspergillus niger* isolated from soil using rice bran, paddy husk and chaffer grains as a feed substrate. To understand the activity of cellulose degrading fungi *Aspergillus niger*, it is needed to optimize under various physical and chemical parameters. Cellulase production by different organisms in submerged state fermentation has received more attention and is found to be the cost-prohibitive.

Sherif *et al.* (2008) isolated twelve *Aspergillus* species from some local soil samples. On the basis of cellulolytic activity, *Aspergillus fumigatus* was selected and used for the production of exoglucanase, endoglucanase, CMCase, -glucosidase and xylanase by adopting SSF condition using mixed substrate of rice straw amended with wheat bran.

The media optimization is an important aspect to be considered in the development of fermentation technology. The present findings are perhaps the first one about the influence of physiochemical properties on cellulase production by a fungi *Aspergillus niger*. Among physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion. The pH change observed during the growth of microbes also affects product stability in the medium (Gupta *et al.*, 2003). Optimum pH and temperature for maximum production of cellulase by cellulose fungi on paddy husk, rice bran were 6.0 and 20°C respectively. Maximum

cellulase activity in bacteria is reported around neutral pH of the medium and temperature at 30 °C but fungi vary with respect to pH and temperature to support maximum production of cellulases (Magnelli and Forchiassin, 1999; Pirt, 1975; Umekalsom *et al.*, 1997). In this study, 20 °C temperature was found optimum to support maximum production of cellulase as observed by the above workers. At higher temperature, the organisms have to spend a lot of energy for maintenance and at lower temperature, transport of nutrients is hindered. (Pirt, 1975).

The incubation period was also influences the enzyme production (Smitt *et al.*, 1996). In the present study the cellulase activity was increased steadily and reached maximum at 48 hours of incubation.

Cellulase is an inducible enzyme synthesized by a large number of microorganisms (Kubicek, 1992; Kubicek *et al.*, 1993) and it is affected by the nature of the substrate used in fermentation. Therefore the choice of an appropriate inducing substrate is of importance. To evaluate the carbohydrates to cause induction or repression of cellulase on some monosaccharides and disaccharides. Fructose among the carbon sources was found to be the best inducer and this study substantiates the earlier works: lactose as best inducers of *Aspergillus* sp. (Bagga *et al.*, 1989) and fructose is the best inducer of cellulase in *Clastiridium thermocellum* (Nochure *et al.*, 1993). Trehalose has been demonstrated as the best inducer of cellulase in *Clastiridium* sp. (Thirumale *et al.*, 2001).

The enzyme production is also affected by different organic nitrogen sources. The production of cellulase is sensitive to the nitrogen sources and nitrogen level in the medium. The result of the present study showed that the sources have different effects on the enzyme activity. Among the organic nitrogen sources tested, the enzyme activity was high with malt extract.

The cellulase enzyme extract was partially purified and its protein fraction was stained with its molecular weight determination by using SDS-PAGE. It revealed two protein bands with the molecular weights of about 83 kD and 50kD. The enzyme protein was extracted from culture supernatant by the ethanol precipitation method. The crude enzyme preparation was subjected to SDS-PAGE (containing 0.2% CMC) to determine the homogeneity and molecular weight of the enzyme. During the electrophoresis of the enzyme, two bands showing cellulolytic activity and the molecular weights of these proteins were calculated to be about 83 and 50 kd. These proteins may be isoenzymes or the different subunits of the same enzyme proteins. A cellulolytic enzyme protein is rich in acidic and aromatic amino acids. According to other research, two endoglucanase containing fractions were separated from *Aspergillus niger* (Lee *et al.*, 2001). These enzymes possess no ability to bind to or hydrolyse insoluble microcrystalline cellulose, but were active towards soluble carboxymethyl cellulose. These enzyme protein have low molecular weights and hence it has potential for industrial applications.

Eventhough, the fungal strain *Aspergillus niger* which was isolated from soil, it produced more concentration of cellulase when production medium was prepared with 5 kg substrates (rice bran, paddy husk and chaffer grains) and utilization of agroindustrial wastes was proved to be one of the method for recycling wastes and improve the economic condition.

7. CONCLUSION

Microorganisms can able to produce various extracellular and intracellular enzymes using various inexpensive sources. The effect of animal derived enzyme activity is higher than the plant derived enzymes. Cellulase is an extracellular enzyme, which is produced from various organisms including bacteria, fungi and also some Actinomycetes. Research on cellulase has progressed very rapidly over the last five decades and potential industrial applications of the enzyme especially in solid waste management have been identified. Major impediments to exploit the commercial potential of cellulases are the yield, stability and cost of cellulase production. Although, terrestrial strains of microbes have been extensively studied by many researchers. *Aspergillus niger* used for the production of cellulase and rice bran, paddy husk and chaffer grains were used as a substrate. The present investigation paved the way for potential exploitation of fungal cellulase enzymes to be applied in various industrial processes.

8. REFERENCES

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