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Production and Optimisation of Cellulases on Pre-treated Groundnut Shell by *Aspergillus niger*

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Abstract : *The objective of this study was to optimise the different cultural conditions i.e. temperature, pH and incubation period and to determine the influence of different biochemical parameters i.e. effect of acid and alkali pre-treatment, amino acids and nitrogen sources by Aspergillus niger on groundnut shell as a lignocellulosic substrate for production of cellulase enzyme using submerged fermentation. The cellulase enzyme production was analyzed by measuring the amount of glucose liberated using the dinitrosalicylic acid assay method. The optimum pH was about 4.5, the optimum temperature was 30°C and the optimum incubation period was 5 days for the production of cellulase on groundnut shell substrate by Aspergillus niger. Alkali pre-treatment was found to increase the cellulase enzyme production as compared to untreated and acid pre-treated substrate. Among nitrogen sources peptone showed the most pronounced effect than other. Metheonine and aspargine were found to be stimulatory for cellulase activity. This study reports groundnut shell as potent inducer of cellulase enzyme by Aspergillus niger.*

Key Words: *Aspergillus niger, Cellulase, Endoglucanase, Exoglucanase , Groundnut shell, submerged fermentation.*

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Introduction :

Enzymes, the most remarkable and highly specialized proteins, have extraordinary catalytic power. They catalyse hundreds of stepwise reactions that degrade nutrient molecules, conserve and transform chemical energy, and make biological macromolecules from simple precursors. Cellulases are among the industrially important hydrolytic enzymes and are of great significance in present day biotechnology. Cellulases are widely used in the food, feed, textile and pulp industries (Nakari and Pentilla, 1996).

Cellulase is produced by several microorganisms, mainly by bacteria and fungi (Immanuel *et al.* 2006).

Fungi are the main cellulase producing microorganisms, although a few bacteria and actinomycetes have also been reported to yield cellulase activity.

Cellulase is an enzyme complex which breaks down cellulose to beta-glucose. Cellulose is a long chain polymer, made up of repeating units of glucose, a simple sugar, joined together with β-1,4 glycosidic linkages. Cellulase refers to a family of enzymes which act in concert to hydrolyze cellulose.

Several different kinds of cellulases are known, which differ structurally and mechanically. Three general types of enzymes make up the cellulase enzyme complex. **Endoglucanase** breaks internal bonds to disrupt the crystalline structure of cellulose and expose individual cellulose polysaccharide chains. These are capable of hydrolyzing the β (1-4)bonds randomly along the cellulose chain. **Exoglucanase** cleaves 2-4 units from the ends of the exposed chains produced by endocellulase, resulting in the tetrasaccharides or disaccharide such as cellobiose. **Cellobiase or beta-glucosidase** hydrolyses the β (1-4) bonds in cellobiose, giving two molecules of glucose.

Aspergillus niger is used as an organism source by many researchers for the media optimization and fermentation of cellulases family. Researchers have taken different kinds of substrates for designing of media protocols for higher yield. For the production of cellulases wheat bran, (Kang *et al.,* 2004) wheat straw, rice straw, corn cob, cotton flower shell, groundnut shell wheat and sorghum straw , water hyacinth blend saw dust (Acharya, *et al.* 2008) and so many type of substrates are used by researchers.

Lignocelluloses are the most abundant natural organic raw materials present on the earth. Groundnut shell (*Arachis hypogea* L) is an important oil crop seed of India. The pod or dry pericarp contains about 25-40% shell. Chemical composition of groundnut shell is as follows: cellulose, 65.7%; carbohydrates, 21.2%; protein, 7.3%; minerals, 4.5%; lipids, 1.2 %. Groundnut shell is used as manure, substrate for culture termites, mushroom cultivation, production of extracellular enzymes and mass inoculum. This study reports groundnut shell as a potent inducer of cellulase enzyme by the cellulolytic fungi *Aspergillus niger.*

Methods :

Collection and preparation of substrate : Groundnut shell was collected from local suppliers from Daldali, Bakarganj, Patna. Shell (1Kg) was dipped in water (5 L) to remove any amount of soluble sugar present in the substrate, dried at 80°C for 36 hr in hot air oven and then chopped into small pieces (Krishna C., 1999). It was grounded in an electric grinder, sieved and kept at room temperature.

Isolation of *Aspergillus niger* : *Aspergillus niger* was isolated from soil using serial dilution technique and pour plate technique on PDA plates. The plates were incubated at 26°C for three days. The colonies showing morphological characteristics of *Aspergillus niger* were identified. The culture of *Aspergillus niger* was preserved on PDA slants and stored at 4^0 C for further use.

Inoculum Preparation: Fungal culture was grown on PDA medium and the spores were harvested aseptically from 5 days old PDA slants. Sterile distilled water (2ml) was added to the fungal slants and shaken vigorously. It was used as inoculum.

Production of Cellulases (submerged fermentation): 25 ml production medium (Mandel

Cellulase is produced by several microorganisms, mainly by bacteria and fungi (Immanuel *et al.* 2006).

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S.No.	pH	Endoglucanase activity (IU/ml/min)	Exoglucanase activity (FPU/ml/min)
1.	4	0.395	0.069
2.	4.5	0.424	0.037
3.	5	0.408	0.031
4.	5.5	0.387	0.027

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were noticed at temperatures 26^{0}C and 37^{0}C. Hence, 30^{0}C is considered to be the optimum temperature for cellulase production for this study.

Table 2. Effect of temperature on production of cellulases by *Aspergillus niger*

S.No.	Temperature ($^\circ$C)	Endoglucanase activity (IU/ml/min)	Exoglucanase activity (FPU/ml/min)
1.	26	0.381	0.026
2.	30	0.424	0.037
3.	37	0.391	0.031

Effect of incubation period (days) on cellulase production : For optimizing the incubation period *Aspergillus niger was* incubated at temperature 30^{0}C for 3 days, 4 days and 5 days. The enzyme was extracted and the Endoglucanase and the exoglucanase activities of the cellulase produced at different incubation period were recorded (Table 3, Fig.3). Optimum Endoglucanase activity (0.408 IU/ml/min) and the exoglucanase activity (0.037FPU/ml/min) were observed after 5 days of incubation. A decrease in enzyme activities is marked after 3 days. It can be because the fungal spores are not fully developed. A sharp decrease in enzyme activity is observed after 8 days and 10 days of incubation. It can be due to the exhaustion of nutrients and accumulation of toxics in the media.

Table 3. Effect of incubation period (days) on production of cellulases by *Aspergillus niger*

S. No.	Incubation Period (days)	Endoglucanase activity (IU/ml/min)	Exoglucanase activity (FPU/ml/min)
1.	3	0.356	0.027
2.	5	0.408	0.037
3.	8	0.318	0.028
4.	10	0.281	0.019

Effect of Biochemical parameters : The Endoglucanase enzyme activity and exoglucanase enzyme activity of pre- treated groundnut substrate 0.25 N HCl and 0.25 N NaOH and untreated groundnut substrate were recorded (Table 4, Fig.4).

The Endoglucanase activity of untreated groundnut substrate was 0.408 IU/ml/min and the exoglucanase activity was 0.026 FPU/ml/min. The pre-treatment of substrate both with the acid and alkali shows an increase in the Endoglucanase and exoglucanase enzyme activities as compared to the untreated substrate. The Endoglucanase activity was 0.786 IU/ml, 0.630 IU/ml and exoglucanase activity was 0.034 FPU/ml/min and 0.053 FPU/ml/min for alkali pre-treated and acid pre-treated substrate respectively. Both the Endoglucanase and exoglucanase enzyme activity of alkali pre-treated substrate was greater than the acid pre-treated substrate.

Table 4. Effect of acid and alkali pre-treatment on production of cellulases by *Aspergillus niger.*

S.No.	Type	Endoglucanase activity (IU/ml/min)	Exoglucanase activity (FPU/ml/min)
1.	Untreated	0.408	0.026
2.	Alkali pre-treated	0.786	0.054
3.	Acid pre-treated	0.630	0.035

Among amino acids alanine, metheonine, threonine, aspargine and alanine were added in the medium (0.2w/v). The control was also maintained without adding amino acids. The Endoglucanase enzyme activity and exoglucanase enzyme activity of the amino acid treated medium were recorded (Table 5, Fig. 5).

Aspargine (1.602 IU/ml/min), alanine (1.062 IU/ml) and methionine (1.493 IU/ml/min) were found to be better in influencing the Endoglucanase activity, whereas, arginine (0.555 IU/ml/min) and threonine (0.441 IU/ml/min) were found to be suppressive in action. *Aspergillus niger* shows maximum exoglucanase activity in the presence of methionine (0.185 FPU/ml) followed by asparagine (0.171 FPU/ml/min). Amino acids being the building blocks of protein have a profound influence in the cellulase synthesis of fungi. Cellulolytic activity of *Aspergillus niger* is significantly influenced in

presence of asparagine and methionine. The increased production might be due to the enhanced synthesis of cellulolytic enzymes in presence of amino acids. Same parameters were used by Vyas *et. al*. (2005) in cellulase production from *Aspergillus terrus* on groundnut shell substrate.

Table 5. Effect of amino acids on production of cellulases on pre-treated groundnut shell by *Aspergillus niger*

S.No.	Amino acid	Endoglucanase activity (IU/ml/min)	Exoglucanase activity (FPU/ml/min)
1.	Control	0.646	0.168
2.	Alanine	1.062	0.096
3.	Methionine	1.493	0.185
4.	Threonine	0.441	0.037
5.	Asparagine	1.602	0.171
6.	Arginine	0.555	0.039

Various organic (peptone and urea) and inorganic (Ammonium sulphate, Ammonium nitrate and Potassium nitrate) were individually evaluated for maximum cellulase production. Each nitrogen source was added in equivalent amount (0.2%) to the production medium. The control was also added without adding nitrogen.The Endoglucanase enzyme activity and exoglucanase enzyme activity of the medium treated with different nitrogen source were recorded (Table 6, Fig.6).

Peptone shows the maximum Endoglucanase activity (1.342 IU/ml/min) and exoglucanase activity (0.490 FPR/ml/min) followed by ammonium sulphate, urea, ammonium nitrate and potassium nitrate. Same parameters were used by Vyas *et. al*. (2005) in cellulase production from *Aspergillus terrus* on groundnut shell substrate. He found maximum cellulase activity for ammonium sulphate (0.2%).

Acharaya *et al* also found maximum cellulase activity of *Aspergillus niger* at peptone (0.1%). Nitrogen is the major constituent of protoplasm and building blocks of enzymes. Stimulation of Endoglucanase activity by ammonium salt may be due to their direct entry in protein synthesis (Mandels, 1975).

Table 6. Effect of nitrogen sources on production of cellulases on pre-treated groundnut shell by *Aspergillus niger*

S. No.	Nitrogen sources	Endoglucanase activity (IU/ml/min)	Exoglucanase activity (FPU/ml/min)
1.	Control	0.646	0.168
2.	Ammonium sulphate	0.919	0.277
3.	Ammonium nitrate	0.725	0.323
4.	Potassium nitrate	0.703	0.353
5.	Peptone	1.342	0.490
6.	Urea	0.744	0.279

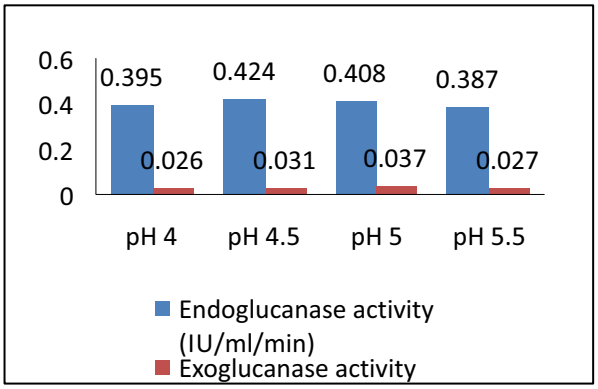

Fig 1. Graph showing effect of pH on cellulase production by *Aspergillus niger*

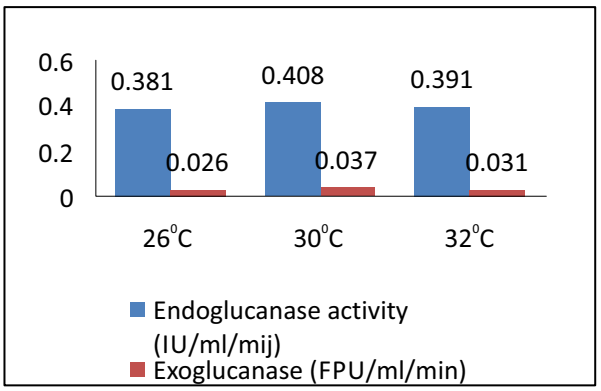

Fig 2. Graph showing effect of temperature on cellulase production by *Aspergillus niger*

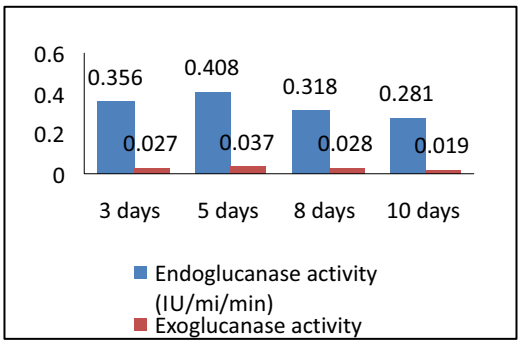

Fig 3. Graph showing effect of incubation period (days) on cellulase production by *Aspergillus niger*

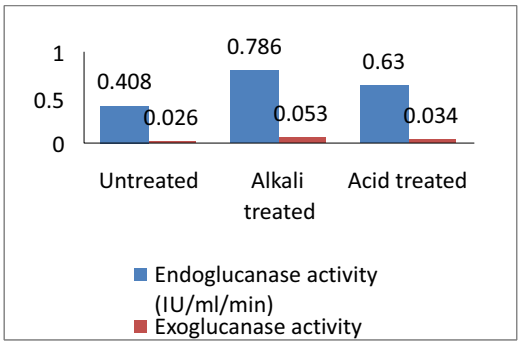

Fig 4. Graph showing effect of alkali and acid treatment of groundnut substrate on cellulase production by *Aspergillus niger*

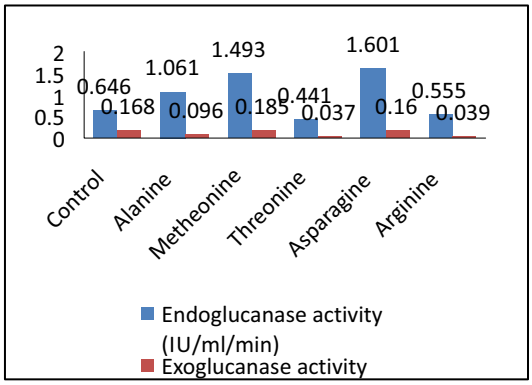

Fig 5. Graph showing effect of amino acid cellulase production by *Aspergillus niger*

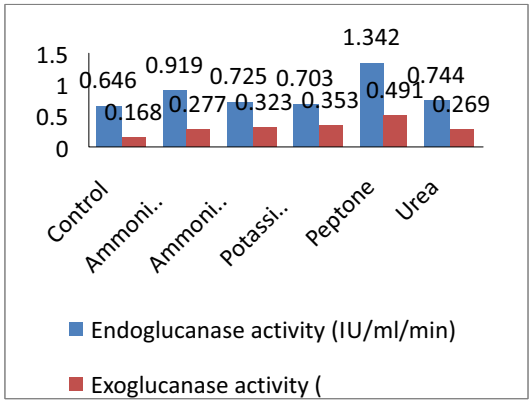

Fig 6. Graph showing effect of nitrogen on sources on cellulase production by *Aspergillus niger*

Conclusion :

From the present findings it can be concluded that certain additional biochemical parameters significantly affect the cellulase production and raises the possibility of groundnut shell as a substrate for large scale production of cellulase enzyme.

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