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Studies on influence of natural biowastes on cellulase production by *Aspergillus niger*

Author Details

M. Usha Kiranmayi (*Corresponding author*)	Department of Microbiology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur - 522 510, India e-mail: ugali1974@gmail.com
Sudhakar Poda	Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur - 522 510, India
P.B.B.N. Charyulu	Department of Microbiology, Sri Krishnadevaraya University, Anantapur - 515 001, India
M. Vijayalakshmi	Department of Microbiology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur - 522 510, India
P.V. Krishna	Department of Zoology and Aquaculture, Acharya Nagarjuna University, Nagarjuna nagar, Guntur - 522 510, India

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Abstract

The objective of this study was to determine the influence of natural biowaste substrates such as banana peel powder and coir powder at varying environmental parameters of pH (4-9) and temperature (20-50°C) on the cellulase enzyme production by *Aspergillus niger*. The cellulase enzyme production was analyzed by measuring the amount of glucose liberated in IU ml^{-1} by using the dinitrosalicylic acid assay method. The substrates were pretreated with 1% NaOH (alkaline treatment) and autoclaved. The maximum activity of the enzyme was assayed at varying pH with temperatures being constant and varying temperatures with pH being constant. The highest activity of the enzyme at varying pH was recorded at pH 6 for banana peel powder (0.068 ± 0.002 IU ml^{-1}) and coir powder (0.049 ± 0.002 IU ml^{-1}) and the maximum activity of the enzyme at varying temperature was recorded at 35°C for both banana peel powder (0.072 ±0.001 IU ml^{-1}) and coir powder (0.046 ±0.003 IU ml^{-1}). At varying temperatures and pH the high level of enzyme production was obtained at 35°C and pH 6 by using both the substrates, respectively. However among the two substrates used for the production of cellulases by *Aspergillus niger* banana peel powder showed maximum enzymatic activity than coir powder as substrate.

Key words

Natural biowaste, Cellulase activity, *Aspergillus niger*

Introduction

The primary product of photosynthesis in the terrestrial environment is cellulose which is the abundant renewable bioresource produced (~ 100 billion dry tons yr^{-1}) in the biosphere (Jarvis, 2003 ; Zhang and Lynd, 2004). Majority of the carbon flow that is operated from the fixed carbon sinks to atmospheric CO_2 is the carbon from the cellulose that is subjected to degradation by the cellulases produced by numerous microorganisms (Berner, 2003). The carbon liberated by the biodegradation of cellulose is very important in several agricultural and waste treatment processes (Angenent *et al.,* 2004; Das and Singh, 2004 ; Haight, 2005; Hamer, 2003; Humphrey *et al*., 1977; Vanwyk, 2001) and could be widely used to produce sustainable biobased products and bioenergy to replace depleting fossilfuels (Demain *et al.,* 2005; Moreira, 2005; Angenent *et al.,* 2004; Kamm and Kamm, 2004; Hoffert *et al.,* 2002; Lynd, 1996; Lynd *et al.,* 1991,1999,2002; Wyman, 1999). Recently the use of microbial enzymes form the plant biomass hydrolysis has been encouraged by the increase demand for the biofuels (Huitron *et al*., 2008). Additionally, studies have shown that the use of biobased products and bioenergy can achieve zero net carbon dioxide emission (Demain, 2004; Demain *et al.,* 2005; Hoffert *et al.,* 2002; Lynd *et al.,* 1991, 1999). National interest can be potentially

benefited by implementing the developmental technologies that are efficient and cost effective in converting the agricultural and biowaste to fermentable sugars through: (1) improved strategic security, (2) decreased trade deficits, (3) healthier rural economies, (4) improved environmental quality, (5) technology exports and (6) a sustainable energy resource supply (Caldeira *et al.,* 2003).

Effective conversion of recalcitrant lignocelluloses to fermentable sugars requires three sequential steps: (1) size reduction, (2) pretreatment/fractionation and (3) enzymatic hydrolysis (Zhang and Lynd, 2004; Wyman, 1999). One of the most important and difficult technological challenges is to overcome the recalcitrance of natural lignocellulosic materials, which must be enzymatically hydrolyzed to produce fermentable sugars (Demain *et al.,* 2005; Moreira, 2005; Wyman, 1999). Cellulases are relatively costly enzymes and a significant reduction in cost will be important for their commercial use in biorefineries. Cellulase-based strategies that will make the biorefinery processing more economical include: increasing commercial enzyme volumetric productivity, producing enzymes using cheaper substrates, producing enzyme preparations with greater stability for specific processes, and producing cellulases with higher specific activity on solid substrates.

Since the biowaste is a good source of carbon in the form of cellulose, many microorganisms including fungi such as *Trichoderma, Pencillium, Aspergillus* spp. etc use cellulose obtained due to the degradation of the biowaste by producing the cellulolytic enzymes (Lakshmikant and Mathur, 1990). Similarly cellulolytic property of bacterial species like *Pseudomonas*, *Cellulomonas*, *Bacillus, Micrococcus*, *Cellovibrio* and *Sporosphytophaga* spp. were also reported (Immanuel *et al.,* 2006). The specific cellulolytic activity shown by the microbial species is found to be depending on the source of occurrence (Saxena *et al.,* 1993). Many studies on degradation of the cellulosic waste materials such as baggase, corncob (Ojumu *et al.,* 2003) saw dust (Solomon *et al.*, 1990) have been reported. Since the coir fiber and banana peel powder are the major biowastes in the coir industry and agriculture respectively, literature related to the influence of coir fiber and banana peel powder as a carbohydrate source on cellulase production is scanty. Therefore, the present investigation was carried out to study the influence of above mentioned natural biowastes on *Aspergillus niger* at varying temperatures with constant pH and varying pH with constant temperature.

Materials and Methods

Isolation: Ten different soil samples were collected from different areas of the Kaza and Mangalagiri villages of Guntur district, Andhra Pradesh. Soil was enriched by pretreating soil sample with 1% cellulose incubated for 30 days at room temperature. One gram of dried sample was taken in 100 ml of distilled water agitated vigorously in rotatory shaker for 30 min and further dilutions were carried out up to 10^{-5}. Serially diluted soil suspension (0.1 ml) was spread over Mandal's agar medium containing (g l^{-1}) Urea =0.3 g, $(NH_4)_2SO_4$ =1.4 g, K_2HPO_4 = 2 g, $CaCl_2$. $2H_2O$ = 0.4 g, Mg SO_4.$7H_2O$ = 0.3 g, peptone = 1 g, Tween 80 = 2 ml, $FeSO_4$. $7H_2O$ = 5 mg, $MnSO_4$. $7H_2O$ = 1.6 mg, $ZnSO_4$. $7H_2O$ = 1.4 mg, $CoCl_2$.$6H_2O$ = 2 mg, Cellulose = 10 g, Sorbose = 4 g, Agar = 20 g at pH 5. The inoculated plates were incubated at 30°C for 4 days. After incubation selective colonies of fungi were identified based on the diameter of zone produced on Mandel's agar plates after flooding with 1% congo red solution.

Fungal colonies obtained were purified and maintained over potato dextrose agar medium and was identified by 18s rRNA analysis. To assay the cellulase activity of the pure culture, the strain was inoculated in the production medium containing Mandal's (Mandel and Weber, 1969) mineral salts solution consisting (g l^{-1}) Urea = 0.3 g, $(NH_4)_2SO_4$ = 1.4 g, K_2HPO_4 = 2 g, $CaCl_2$. $2H_2O$ = 0.4 g, Mg SO_4.$7H_2O$ = 0.3 g, peptone = 1 g, yeast extract = 0.25 g, maize steep liquor = 10 g, cellulose = 2 g, $FeSO_4$. $7H_2O$ = 5 mg, $MnSO_4$. $7H_2O$ = 1.6 mg, $ZnSO_4$. $7H_2O$ = 1.4 mg, $CoCl_2$.$6H_2O$ = 2 mg.

Cellulolytic activity of the strain was assessed by adopting the procedure of Miller (1959). For cellulase assay, 0.5 ml of the enzyme filtrate was added to 1.5 ml of 0.5% carboxy methyl cellulose, in 50 mM sodium phosphate buffer (pH 5) . The reaction mixture was incubated at 30°C for 30 min. The reaction was stopped by addition of dinitrosalicylic acid (DNS) reagent followed by boiling. The developed color was read at 540 nm using spectrophotometer. The amount of released sugar was quantified using glucose as standard, hence one unit of enzyme activity was defined as the amount of enzyme releasing 1 µmol of glucose equivalent from substrate per minute.

Influence of pH and temperature on enzyme activity was recorded by growing the strain at different pH levels from 4 to 9 and temperature ranging between 25 to 55°C. The optimal pH and temperature achieved was recorded with commercial cellulose as substrate.

To investigate the effect of biowaste substrates on cellulase activity the production medium was supplemented with pretreated banana peel powder and coir powder each at a concentration of 2% as the sole carbon source with the other ingredients of medium remained same. The culture conditions were optimized to study the effect of varying pH (4, 5, 6, 7, 8 and 9) with constant temperature and varying temperatures (25, 30, 35, 40, 45, 50 and 55°C) with constant pH on the cellulase activity of the fungal isolate.

Data obtained on cellulase enzyme production under different cultural conditions were statistically analyzed with one-way analysis of variance (ANOVA).

Results and Discussion

On the basis of morphology and microscopic observations and also the 18s rRNA sequencing studies the fungus was identified as *Aspergillus niger.* The observations of the cultures were based on the characteristic features described by Vries and Visser (2001).

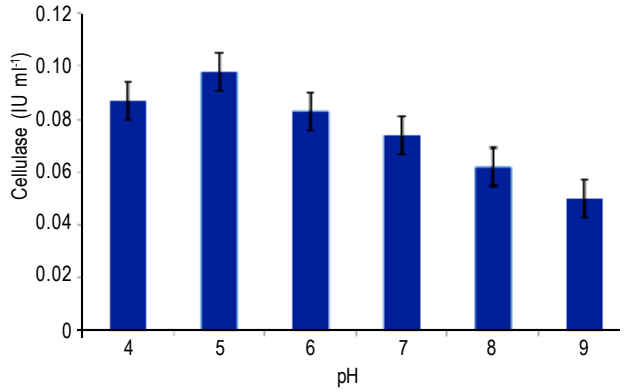

Fig. 1: Effect of varying pH (4-9) on cellulase production by *Aspergillus niger.* Statistically significant at 5%

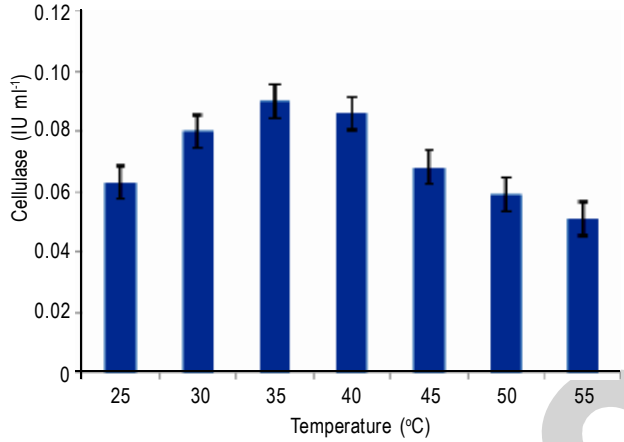

Fig: 2: Effect of varying temperature (25-55°C) on cellulase production by *Aspergillus niger.* Statistically significant at 5%

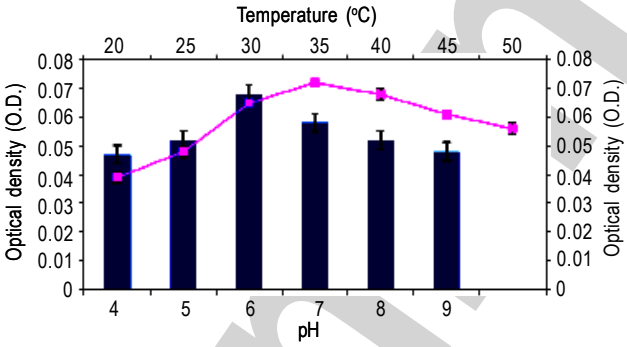

Fig. 3: Effect of banana peel powder on cellulase activity of *Aspergillus niger* at varying pH with constant temperature (35°C) and varying temperatures with constant pH. Statistically significant at 5%.

Effect of pH on cellulase activity: The enzyme activity increased gradually from pH 4, (0.087 ± 0.002 IU ml^{-1}) and reached maximum at pH 5 (0.098 + 0.003 IU ml^{-1}).The optimum pH for maximum cellulase activity was found to be 5 and there was a considerable decrease in the enzyme activity with the increase in pH 6 (0.083 ± 0.002 IU ml^{-1}), pH 7 (0.074 ± 0.001 IU ml^{-1}); pH 8 (0.062 ± 0.002 IU ml^{-1}) pH 9 (0.050 ± 0.001 IU ml^{-1}) and the maximum activity was

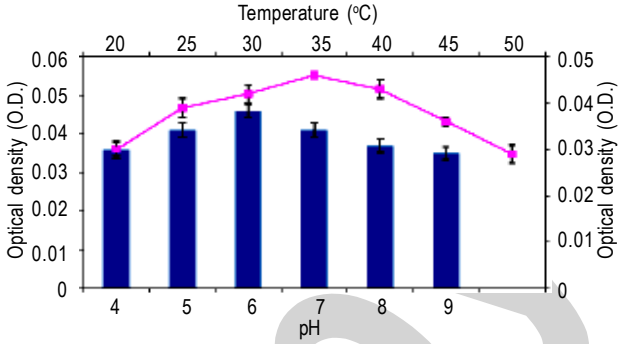

Fig. 4: Effect of the coir powder on cellulase activity of *Aspergillus niger* at varying pH with temperature constant and varying temperatures with pH constant. Statistically significant at 5%.

observed after 8th day of incubation. It was reported that the optimum pH for cellulase activity of *Aspergillus niger* was between pH 6 to 7 (Akiba *et al*., 1995). Acharya *et al.* (2008) and Sohail *et al.* (2009) also reported that the cellulase activity was maximum at pH 4. Such different results may appear because of the difference within the same genus.

Effect of temperature on cellulase activity: There was a considerable increase in the cellulase activity from temperatures 25°C (0.063 ± 0.002 IU ml^{-1}) and 30°C (0.080 ± 0.003 IU ml^{-1}). The enzyme showed a good activity at 35°C (0.090 ± 0.003 IU ml^{-1}). The optimum temperature for cellulase activity was found to be at 35°C. However the enzyme activity was reduced from 40°C (0.086 ± 0.002 IU ml^{-1}) and further decrease in the activity was observed at temperatures 45°C (0.068 ± 0.002 IU ml^{-1}), 50°C(0.059± 0.003 IU ml^{-1}) and 55°C (0.051± 0.001 IU ml^{-1}). The maximum activity was observed after 8th day of incubation. Further it was also reported that the optimum temperature for the cellulase production by *Aspergillus niger* was 35°C (Sohail *et al*., 2009). In addition there were reports that the cellulase production by *Aspergillus niger* was observed over a wide rage of temperatures between 30 to 50°C (Ziad *et al*., 2008; Milala *et al*., 2005).

Effect of banana peel powder on cellulase enzyme production: The production of the cellulase enzyme by *Aspergillus niger* in the presence of the biowaste substrate (Banana peel powder) showed maximum (0.068 ± 0.002 IU ml^{-1}) at pH 6 with temperature 35°C constant after 8th day of incubation where the pH and temperature was considered to be optimum. But the enzyme activity at pH 4 and 5 with temperature 35°C (0.047 ± 0.002 IU ml^{-1}, 0.052 ± 0.003 IU ml^{-1}) showed decreased activity than at pH 6, further increase in the pH above 6 showed reduced enzyme activity from pH 7-9 (0.058±0.002, 0.052 ± 0.003 and 0.048 ± 0.002 IU ml^{-1}) (Fig. 3).

In addition, the activity of the enzyme by the isolate at varying temperatures using banana peel powder as the substrate showed maximum activity at 35°C (0.072 ±0.001) with pH 6 constant after 8th day of incubation which was found to be optimum, further when the temperatures were increased from 40- 50 the enzyme activity decreased (0.068 ± 0.004, 0.061 ±0.002 and 0.056 ± 0.0001 IU

ml^{-1}). Decrease in temperature below 35°C showed reduced enzymatic activity at temperatures 20, 25 and 30°C (0.039 ± 0.003, 0.048 ± 0.002 and 0.065 ± 0.002) (Fig. 3). Reports showed that the induction of cellulase enzyme production by the celluloytic organisms is influenced by carbon sources as substrate. In addition environmental factors also influence the growth of the organism and maximum production of cellulase at certain optimum temperature and pH (Aboul-Enein, 2010). The major requirement of the commercial potential of cellulases is the yield stability and cost of cellulase production. Therefore, research should aim in exploiting the potential existing new cellulases from nature (Coral *et al*., 2002). Agriculture residues such as corn stove, wheat straw, rice straw and baggase used in cellulase production (Rao *et al*., 1983; Chalal and Chalal, 1996) to reduce the enzyme cost.

Effect of coir powder on cellulase enzyme production: The effect of pH with constant temperature and varying temperatures with constant pH on cellulase activity of the isolate was determined after 8th day of incubation with coir powder as the substrate as shown in Fig. 4. The cellulase activity at varying pH 4, 5, 6, 7, 8 and 9 with constant temeprature 35°C was found to be 0.036 ± 0.001, 0.041 ± 0.003, 0.049 ± 0.002, 0.041 ± 0.002, 0.037 ± 0.002, 0.035 ± 0.003 IU ml^{-1}, respectively. Optimum pH for the cellulase activity was found at pH 6 (0.049 ± 0.002 IU ml^{-1}) (Fig. 4). It was reported that the optimal pH for cellulase from *A. niger* was between 6.0 and 7.0 (Akiba *et al*., 1995).

The activity of the cellulase enzyme was optimized by incubating the flasks with the production medium at varying temperatures (20, 25, 30, 35, 40, 45 and 50°C) with constant pH 6. The cellulase activities was found to be 0.030 ± 0.001, 0.039 ± 0.002, 0.042 ±0.001, 0.046 ±0.003, 0.043 ± 0.001, 0.036 ±0.003 and 0.029 ± 0.002 IU ml^{-1}. The optimum cellulase activity was found at temperature 35°C with constant pH 6 after 8th day of incubation. The reduction of sugar conversion as well as enzyme production was observed at the temperature above and below 28°C.

The important cellulolytic fungus like *Trichoderma* sp. (Mandels and Reese, 1985); *Penicillium* sp. (Brown *et al.,* 1987); *Sporotrichium* sp (Erikkson and Johnsrud, 1983); *Aspergillus* sp (Kazuhisa Miyamoto, 1997) etc have been reported to have cellulolytic activity. The amount of carbon produced by cellulase is variable since the production of the cellulases is influenced by substrates (carbon source) on the growth of the cellulolytic organisms. The present study aimed at using two different biowaste substrates such as coir powder and banana peel waste. In addition, the production of cellulases is influenced by factors such as temperature and pH (Immanuel *et al.,* 2006).

The results showed that the optimum pH and temperature was found to be 5 and 35°C. In addition, the cellulase production when performed at varying pH and constant temperature 35°C and varying temperature and constant pH 6, the results showed that cellulase enzyme production was maximum in the presence of banana peel powder

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