

Optimization of Endoglucanase Production by *Aspergillus niger* VTCC-F021

Thi Hoa Pham, Dinh Thi Quyen, Ngoc Minh Nghiem,

Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Distr. Caugiay, 10600 Hanoi, Vietnam

Abstract: Among 63 *Aspergillus niger* strains collected at the Vietnam Type Culture Collection, the strain *Aspergillus niger* VTCC-F021 showed the highest endoglucanase production. The endoglucanase production by *A. niger* VTCC-F021 attained the maximum in CPY medium containing (w/v) 0.4% peptone, 0.1% extract, 0.1% K₂HPO₄, 0.05% KCl, 0.05% MgSO₄·7H₂O, 0.001% FeSO₄·7H₂O and 1% carboxymethylcellulose (CMC), after 120 hours of cultivation at 37°C, agitated at 200 rpm. The optimum temperature and initial pH for the endoglucanase production by the *A. niger* VTCC-F021 strain was 37°C and pH 5. Among tested carbon sources (coffee shell, corncob, dried tangerine skin, glucose, lactose, peanut shell, rice bran, sucrose, and sugar-cane bagasse), yeast extract and sugar-cane bagasse at the concentration of 0.1% (w/v) showed the highest glucanase production with an activity of 6.88 U/ml (100%) and 6.62 U/ml (96%), respectively. Peptone and soybean powder were the best among tested nitrogen sources (NH₄)₂NO₃, (NH₄)₂SO₄, fish powder, peptone, soybean powder, urea) with glucanase activity of 5.32 U/ml (100%) and 5.11 U/ml (96%), respectively. *A. niger* VTCC-F021 produced the glucanase production optimum in the optimal CPY medium containing 0.4% (w/v) of sugar-cane bagasse as the carbon source and 1.4% (w/v) of soybean powder as the nitrogen source.

Key words: *Aspergillus niger*, CMC (carboxymethyl cellulose), endoglucanase production, optimization of culture conditions.

INTRODUCTION

Cellulase is a complex enzyme system that is used to break up cellulose into glucose or other oligosaccharide compounds (Chellapandi and Jani, 2008). The cellulase system in fungi is considered to comprise three hydrolytic enzymes: endo-(1,4)-β-D-glucanase (endoglucanase, endocellulase, CMCase [EC 3.2.1.4]), which cleaves β-linkages at random, commonly in the amorphous parts of cellulose, exo-(1,4)-β-D-glucanase (cellobiohydrolase, exocellulase, microcrystalline cellulase, avicelase [EC 3.2.1.91]), which releases cellobiose from nonreducing or reducing end, generally from the crystalline parts of cellulose and β-glucosidase (cellobiase [EC 3.2.1.21]), which releases glucose from cellobiose and short-chain cellooligosaccharides (Bhat and Bhat, 1997).

In recent years, one of the most important biotechnological applications is the conversion of agricultural wastes and all lignocellulosics into products of commercial interest such as ethanol, glucose and single cell protein (Ojumu *et al.*, 2003). The key element in bioconversion process of lignocellulosics to these useful products is the hydrolytic enzymes mainly cellulases (Ojumu *et al.*, 2003; Fan *et al.*, 1987; Immanuel *et al.*, 2007; Kansoh *et al.*, 1999; Wu and Lee, 1997). Lignocellulosics are abundant sources of carbohydrate, continually replenished by photosynthetic reduction of carbon dioxide by sunlight energy (Fan, *et al.*, 1987). Thus, they are the most promising feedstock for the production of energy, food and chemical (Ojumu *et al.*, 2003; Wu and Lee, 1997). The bioconversion of cellulosic materials is now a subject of intensive research as a contribution to the development of a large-scale conversion process beneficial to mankind (Hanaee *et al.*, 1997). Such process would help alleviate shortages of food and animal feeds, solve modern waste disposal problem and diminish man's dependence on fossil fuels by providing a convenient and renewable source of energy in the form of glucose.

Cellulase production by members of the genus *Aspergillus* using different agricultural wastes was reported such *A. niger* (Ojumu, *et al.*, 2003; Immanuel *et al.*, 2007; Acharya *et al.*, 2008; Milala *et al.*, 2005; Usama and Hala, 2008), *A. fumigatus* (Immanuel *et al.*, 2007), *A. flavus* (Solomon *et al.*, 1990), *A. nidulans* (Usama and Hala, 2008), *A. humicola* (Nipa *et al.*, 2006).

Corresponding Author: Dinh Thi Quyen, Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Caugiay District 10600 Hanoi Vietnam
E-mail: quyen@ibt.ac.vn, quyendt2000@yahoo.com
Tel.: +84-04-37568260; Fax: +84-04-38363144

Since, the production of cellulases is a major factor in the hydrolysis of cellulosic materials, it is important to make the process economically feasible. A variety of studies was done on the cellulase production from lignocellulosics, especially from sugar-cane bagasse (Kansoh *et al.*, 1999; Milala *et al.*, 2005; de Pauval *et al.*, 1999, Solomon *et al.*, 1999). Reducing the costs of enzyme production by the optimization of the fermentation medium and cultivation condition is the goal of basic research for industrial application. The aim of this present work was to optimize some cultivation conditions for the endoglucanase production by *A. niger* VTCC-F021 collected at the Vietnam Type Culture Collection.

MATERIALS AND METHODS

Filamentous Strains and Culture Medium:

Sixty-three *Aspergillus niger* strains from the Vietnam Type Culture Collection (Institute of Microbiology and Biotechnology, Vietnam University Hanoi) were screened for the endo-glucanase production by growing at 37°C in three milliliters on CPY medium containing (w/v) 0.4% peptone, 0.1% yeast extract, 0.1% K₂HPO₄, 0.05% KCl, 0.05% MgSO₄·7H₂O, 0.001% FeSO₄·7H₂O, and 0.5% CMC (carboxymethyl cellulose); with initial pH 6.5.

Chemicals:

Carboxymethyl cellulose was from Biochemika, peptone from Merck, yeast extract from Fluka. All other reagents were of analytical grade unless otherwise stated.

Endoglucanase Assay:

Endoglucanase activity was examined relatively by the halo radius of enzyme diffusion on agar plates containing substrate: After 96 hours of growth in the CPY medium containing 0.5% (w/v) CMC, 50 ml of the culture supernatant was dropped on agar plates containing 0.5 % (w/v) CMC and incubated for 24 hours at 4°C to diffuse enzyme. After that the agar plates were incubated for further 24 hours 37°C and stained with 1% (w/v) lugol dye.

Endoglucanase activity towards carboxymethyl cellulose was measured by the appearance of reducing end groups in solution of CMC using Nelson method (Nelson, 1944). The endoglucanase activity was determined by incubating the enzyme (0.5 ml) and 0.5% (w/v) CMC (0.5 ml) in 0.5 M sodium acetate buffer, pH 5.0 at 45°C for 5 min. The reaction was stopped by the addition of 1.0 ml of combined copper reagent and heated for 20 min in a boiling water bath. The tubes were cooled down to room temperature and 1.0 ml of arsenomolybdate reagent was added to all the tubes. The reaction mixtures were spin at 8000x g for 5 minutes and the optical density (OD) was read at 660 nm against the blank.

Glucose was used as the standard reduced sugar for concentration estimation. One unit of glucanase activity was defined as the amount of enzyme which released 1 µmol glucose per minute under assay conditions.

Endoglucanase Production:

A. niger VTCC-F021 was grown in the CPY medium, pH 6.5 at 30°C in orbital shaker with 200 rpm. One ml culture sample was obtained every 12 hours for glucanase activity estimation.

Optimization of Substrate Concentration:

To determine the influence of CMC as an inducer on the endoglucanase production, *A. niger* VTCC-F021 was cultivated in a 100 ml shaking flask containing 20 ml CPY medium, pH 6.5 supplemented with CMC at various concentration from 0.1 to 2.0%, at 28 ± 2°C, agitated 200 rpm. After 96 hours of cultivation, endoglucanase activity was measured.

Optimization of Culture Temperature:

Optimization of temperature was carried out by incubating the CMC containing fermentation medium CPY at 28°C, 30°C and 37°C, in orbital shaker at 200 rpm. After regular intervals, enzyme assay was performed.

Optimization of pH:

To determine optimal initial pH, *A. niger* VTCC-F021 was cultivated in a 100-ml shaking flask containing 20 ml optimized CPY medium with different pH ranges from 3.5 to 8.0. The initial pH of the medium was adjusted by using 1 N HCl or 1 N NaOH. The flasks were kept in orbital shaker at 37°C at 200 rpm and for 120 hours of cultivation.

Optimization of Carbon Source and its Concentration:

To optimize the carbon source, *A. niger* VTCC-F021 was cultivated in a 100 ml shaking flask containing 20 ml CPY medium except for yeast extract, which was replaced by one of various carbon sources (coffee shell, corncob, dried tangerine skin, glucose, lactose, peanut shell, rice bran, sucrose, sugar-cane bagasse), at 37°C and agitated at 200 rpm. After 120 hours of cultivation, the endoglucanase activity was determined. After optimization of carbon sources for the highest endoglucanase production by *A. niger* VTCC-F021, the strain was cultivated in medium with the optimal carbon source at various concentrations from 0.1 to 1.4% (w/v).

Optimization of Nitrogen Source and its Concentration:

To optimize the nitrogen source, *A. niger* VTCC-F021 was cultivated in a 100-ml shaking flask containing 20 ml carbon source optimized CPY medium except for peptone, which was replaced by one of various nitrogen sources ((NH₄)₂SO₄, NH₄NO₃, fish paste, soybean powder and urea). All cultures were agitated at 200 rpm, at 37°C, and after 120 hours of cultivation the endoglucanase activity was estimated. After optimization of nitrogen sources for the highest endoglucanase production by *A. niger* VTCC-F021, the strain was cultivated in the optimized CPY medium with the optimal nitrogen source at different concentration from 0.2 to 2% (w/v).

RESULTS AND DISCUSSION

Screening *A. niger* Strains for Endoglucanase Production:

Among 63 *Aspergillus niger* strains from the Vietnam Type Culture Collection, the *A. niger* VTCC-F021 strain showed the highest endoglucanase production both on agar plate (Fig. 1A) as well as through the endoglucanase assay with an activity of 1.98 U/ml, whereas all the rest produced endoglucanase with an activity from 0.01 to 1.94 U/ml in the CPY medium. This strain was selected for optimization of culture conditions for the endoglucanase production.

Endoglucanase Production Course by *A. niger* VTCC-F021:

The endoglucanase production by *A. niger* VTCC-F021 increased linearly from 15% (0.3 U/ml) at 24 hours to the maximum of 100% (2.0 U/ml) at 120 hours cultivation (Fig. 1B). This result was coincident with other reports. The maximum production of the cellulase by other fungi was reported also very low just around 2 U/ml (*Phanerochaete chrysosporium*, 2.40 U/ml (Khan *et al.*, 2007); *Trichoderma harzianum*, 1.88 U/ml; *A. humicola*, 1.62 U/ml (Nipa *et al.*, 2006); *Trichoderma* spp., 1.53 U/ml), and achieved at 96 hours of the fermentation period. Ojumu *et al.*, (2003) found that the highest level of cellulase activity occurred at 120 hours of fermentation by *A. flavus*, but *A. fumigatus* produced the highest cellulose level at 144 hours of growth (Grigorevski-Lima *et al.*, 2009).

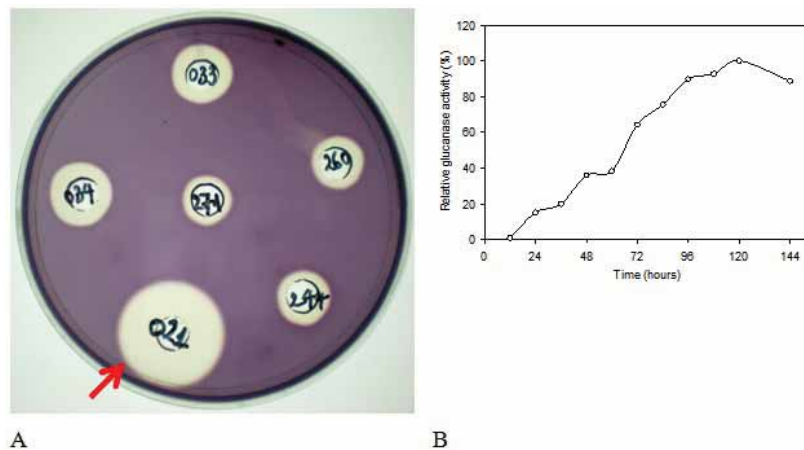


Fig. 1: Endoglucanase activity on agar plates containing 0.5% (w/v) CMC and staining with lugol. 021, 033, 034, 244, 269, 271 are abbreviated for *A. niger* VTCC-F021, *A. niger* VTCC-F033, *A. niger* VTCC-F034, *A. niger* VTCC-F244, *A. niger* VTCC-F269, and *A. niger* VTCC-F271 (A). Endoglucanase production course by the *A. niger* VTCC-F021 strain (B).

Effect of Cultivation Temperature on Endoglucanase Production:

A. niger VTCC-F021 produced the highest endoglucanase level (2.09 U/ml) at the cultivation temperature of 37°C (Fig. 2A) among three tested temperatures 28°C, 30°C, and 37°C. Maximum temperature for the CMCase production by other *Aspergillus* species was also 37°C as reported by many authors (*A. humicola* (Nipa *et al.*, 2006); *Aspergillus* species, *A. niveus*, *A. niger*, *A. terreus*, *A. nidulans* (Jahangeer *et al.*, 2005). The optimum production temperature for the three cellulases (b-glucosidase, CMCase, Avicellase) was 35°C for *A. niger* (Usama and Hala, 2008). The maximum cellulase production by *A. terreus* (Singh *et al.*, 1996) was obtained at a lower temperature of 28°C.

Effect of CMC Concentration on Endoglucanase Production:

CMC is a substrate for the endoglucanase and showed the induction effect on the endoglucanase production. The addition of CMC at the concentration from 0.1% to 1% to the CPY medium increased the endoglucanase production gradually from 3% (0.08 U/ml) to the maximum 100% (2.53 U/ml). The addition of more CMC (1.1-2%) to the culture medium decreased the endoglucanase production gradually to 39%. No addition of CMC showed nearly no endoglucanase production, just 0.5% (0.013 U/ml) in comparison to the maximum production (Fig. 2B). The addition of 1% CMC also induced the maximum cellulase production by *A. terreus* (Singh *et al.*, 1996).

Effect of Initial Medium pH on Endoglucanase Production:

A. niger VTCC-F021 produced the highest level of the endoglucanase (7.34 U/ml) in the optimized mineral medium at the initial pH 4.5 (Fig. 2C). At the initial pH 4.0 and 5.0, the glucanase production was also quite high (80%, 5.84-5.86 U/ml) in comparison to the optimum pH. Beneath pH 4.0 and above 5.0, the enzyme production decreased to 33-68% (2.45-4.97 U/ml) (Fig. 2C). This result was matched with other reports that the optimum pH was 4.0-4.5 for the cellulase production by *A. niger* (Acharya *et al.*, 2008) and 4.8 for the cellulase production by *Aspergillus* strains (Jahangeer *et al.*, 2005). The optimal pH for a cellulase production by an *A. niger* strain was 4.5 pH and 7.5 (Coral *et al.*, 2002) and between 6.0 and 7.0 (Akiba *et al.*, 1995).

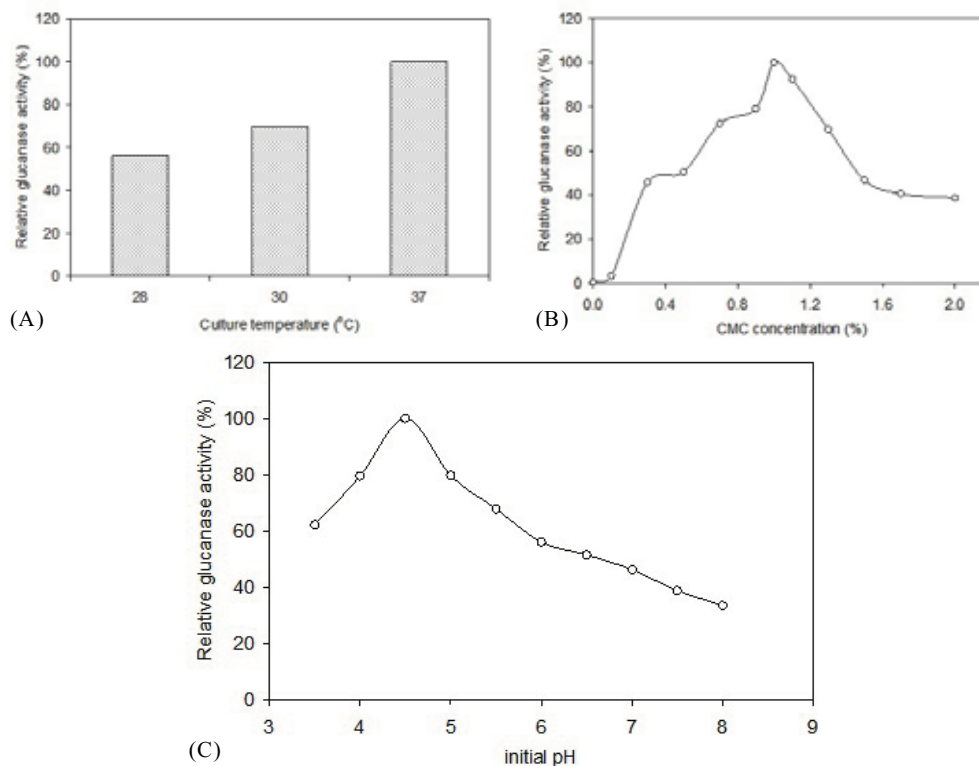


Fig. 2: Effect of the cultivation temperature (A), CMC concentration (B), initial medium pH (C) on the endoglucanase production by *A. niger* VTCC-F021.

Effect of Carbon Sources and its Concentration:

Among the investigated carbon sources (coffee shell, corncob, dried tangerine skin, glucose, lactose, peanut shell, rice bran, sugar-cane bagasse, yeast extract), yeast extract and sugar-cane bagasse was the most appropriate carbon source for the glucanase production (6.88 U/ml, 100%, 6.62 U/ml, 96%, respectively) by *A. niger* VTCC-F021 (Fig. 3A). Other carbon sources reduced the enzyme production by about half in comparison to the yeast extract as carbon source (Fig. 3A), especially the glucanase production was reduced to 11% (0.74 U/ml) when *A. niger* VTCC-F021 was grown in the CPY medium containing rice bran as the carbon source. Sugar-cane bagasse also was a suitable carbon source for the cellulase production by other strains (*A. flavus* Lin Isolate NSPR 101 (Ojumu *et al.*, 2003; Solomon *et al.*, 1999), *Trichoderma reesei* RUT C30 (Naveen *et al.*, 2008)).

After the sugar-cane bagasse was fixed as the best among tested carbon sources for the glucanase production by *A. niger* VTCC-F021, its concentration was varied from 0.1 to 1.4% in the CPY medium. The glucanase production increased from 53% (3.52 U/ml) in the CPY medium containing 0.1% sugar-cane bagasse to the maximum 100% (6.66 U/ml) in the CPY medium containing 0.4% sugar-cane bagasse (Fig. 3B). The glucanase production decreased gradually to 48% in the CPY medium containing 1.4% sugar-cane bagasse. The highest levels of endoglucanase (CMCase) by *A. fumigatus* was obtained using sugarcane sugar-cane bagasse (1%) and corn steep liquor (1.2%) in submerged fermentation (Grigorevski-Lima *et al.*, 2009).

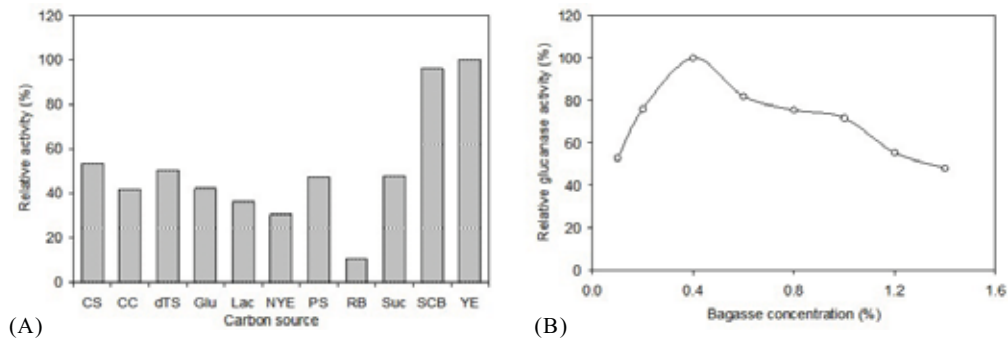


Fig. 3: Effect of carbon source (A) and sugar-cane bagasse concentration (B) on the endoglucanase production by *A. niger* VTCC-F021.

CS, coffee shell; CC, corncob; dTS, dried tangerine skin; Glu, glucose; Lac, lactose; NYE, no yeast extract; PS, peanut shell; RB, rice bran; Suc, sucrose; SCB, sugar-cane bagasse; YE, yeast extract.

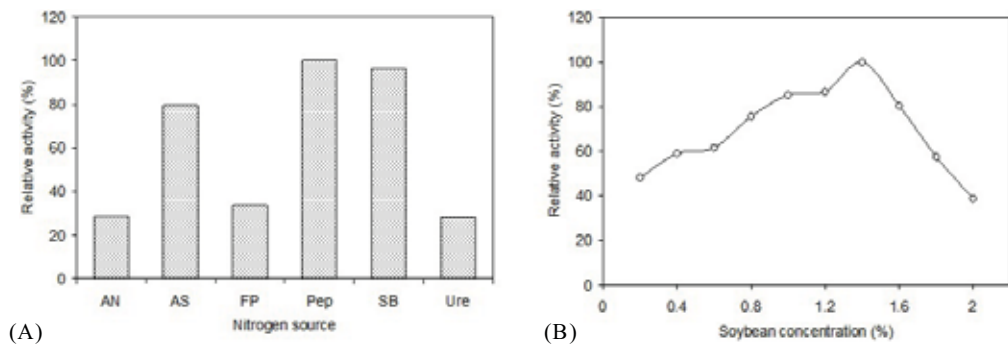


Fig. 4: Effect nitrogen source (A) and soybean powder concentration (B) on the endoglucanase production by the *A. niger* VTCC-F021 strain.

AN, ammonium nitrate; AS, ammonium sulphate; FP, fish powder; Pep, peptone; SBP, soybean powder; Ure, urea.

Effect of Nitrogen Source and its Concentration:

Among the examined nitrogen sources (Fig. 4A), peptone was the best nitrogen source for the endoglucanase production by *A. niger* VTCC-F021 (5.32 U/ml). However, soybean powder was an appropriate

nitrogen source for the endoglucanase production (5.11 U/ml). *A. niger* VTCC-F021 produced just only one third of the enzyme production in CPY medium containing inorganic nitrogen sources (NH_4NO_3 , $(\text{NH}_4)_2\text{SO}_4$, and urea) in comparison to peptone. Soybean powder was substituted for peptone in the CPY medium and its concentration varied from 0.2 to 2% (w/v). *A. niger* VTCC-F021 produced the highest endoglucanase amount (7.19 U/ml) when the soybean powder was used with the amount of 1.4% (Fig. 4B). The lowest (0.2%) and highest (2%) amount of the soybean powder in the CPY medium reduced the endoglucanase production to 48% (3.48 U/ml) and 39% (2.79 U/ml) in comparison to the optimal amount of the soybean powder of 1.4%.

Conclusions:

Aspergillus niger VTCC-F021 produced the endoglucanase production maximum at the culture temperature of 37°C, with the initial pH of 4.5 and after 120 hours of cultivation. Yeast extract and peptone were the best carbon and nitrogen source for the endoglucanase production, respectively, however the endoglucanase productions observed with sugar-cane bagasse and soybean powder were nearly the same. The enzyme production was maximum in the culture medium CPY containing 0.4% sugar-cane bagasse as carbon source and 1.4% soybean powder as nitrogen source, and 1% CMC as inducer.

ACKNOWLEDGEMENT

The study was supported by the Priority Program of Development and Application of Biotechnology in Agriculture and Rural Development Towards 2020, Vietnam Ministry of Agriculture and Rural Development (Project: *Production and application of highly qualitative multi-enzyme products by recombinant microbes to improve the effective use of animal food*, 2007-2010). Dinh Thi Quyen gratefully acknowledged a grant for equipment from the International Foundation of Science (F/3411-3, 2008).

REFERENCES

- Acharya, P.B., D.K. Acharya, H.A. Modi, 2008. Optimization for cellulase production by *Aspergillus niger* using saw dust as substrate. Afr. J. Biotechnol., 7: 4147-4152.
- Akiba, S., Y. Kimura, K. Yamamoto, H. Kumagai, 1995. Purification and characterization of a protease-resistant cellulase from *Aspergillus niger*. J. Ferment. Bioeng., 79: 125-130.
- Bhat, M.K., S. Bhat, 1997. Cellulose degrading enzymes and their potential industrial applications. Biotechnol. Adv., 15: 583-620.
- Chellapandi, P., H.M. Jani, 2008. Production of endoglucanase by the native strains of *Streptomyces* isolates in submerged fermentation. Braz. J. Microbiol., 39: 122-127.
- Coral, G., B. Arikan, M.N. Unaldi, H. Guvenmes, 2002. Some properties of crude carboxymethyl cellulase of *Aspergillus niger* Z10 wild-type strain. Turk. J. Biol., 26: 209-213.
- de Paula, E.H., L.P. Ramos, M. de Oliveira Azevedo, 1999. The potential of *Hemicola grisea* var. *Thermoidea* for bioconversion of sugarcane bagasse. Bioresour. Technol., 68: 35-41.
- Fan, L.T., M.M. Gharpuray, Y.H. Lee, 1987. Cellulose hydrolysis. 1-68, Springer-verlag.
- Grigorevski-Lima, A.L., F.N.M. Da Vinha, D.T. Souza, A.S.R. Bispo, E.P.S. Bon, R.R.R. Coelho, R.P. Nascimento, 2009. *Aspergillus fumigatus* thermophilic and acidophilic endoglucanases. Appl. Biochem. Biotechnol., 155: 18-26.
- Hanaee, J., F.R. Schilling, G.M. Partzsch, H. Brasse, G. Schwarz, F.A. Macias, J.M.G. Molinillo, A. Torres, R.M. Varela, D. Castellano, M. Kumakura, 1997. Preparation of immobilized cellulase beads and their application to hydrolysis of cellulosic material. Process Biochem., 32: 555-559.
- Jahangeer, S., N. Khan, S. Jahangeer, M. Sohail, S. Shahzad, A. Ahmad, S.A. Khan, 2005. Screening and characterization of fungal cellulase isolated from the native environmental source. Pak. J. Bot., 37: 739-748.
- Kansoh, A.L., S.A. Essam, A.N. Zeinat, 1999. Biodegradation and utilization of bagasse with *Trichoderma reesei*. Polym. Degrad. Stab., 62: 273-278.
- Khan, M.D.M.H., S. Ali, A. Fakhru'l-Razi, M.D.Z. Alam, 2007. Use of fungi for the bioconversion of rice straw into cellulase enzyme. J. Environ. Sci. Health Part. B., 42: 381-386.
- Immanuel, G., C. Bhagavath, P.I. Raj, P. Esakkiraj, A. Palavesam, 2007. Production and partial purification of cellulase by *Aspergillus niger* and *A. fumigatus* fermented in coir waste and sawdust. Internet J. Microbiol., pp: 3.
- Milala, M.A., A. Shugaba, A. Gidado, A.C. Ene, J.A. Wafar, 2005. Studies on the use of agricultural wastes for cellulase enzyme production by *Aspergillus niger*. Res. J. Agric. Biol. Sci., 1: 325-328.

Naveen, K.M.S., K.S. Rajeev, P. Ashok, 2008. Cellulase production under solid-state fermentation by *Trichoderma reesei* RUT C30: Statistical optimization of process parameters. *Appl. Biochem. Biotechnol.*, 151: 122-131.

Nelson, N., 1944. A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.*, 153: 375-380.

Nipa, M.N., S. Sultana, M.A. Hakim, 2006. Induction of cellulase biosynthesis by cellobiose octaacetate in *Aspergillus humicola*. *Microbiol. J.*, 23: 174-176.

Ojumu, T.V., B.O. Solomon, E. Betiku, S.K. Layokun, B. Amigun, 2003. Cellulase production by *Aspergillus flavus* Linn isolate NSPR 101 fermented in sawdust, bagasse and corncorb. *Afr. J. Biotechnol.*, 2: 150-152.

Singh, S., D.K. Sandhu, J.K. Brar, A. Kaur, 1996. Isozyme polymorphism of cellulases in *Aspergillus terreus*. *J. Basic. Microbiol.*, 36: 289-296.

Solomon, B.O., S.K. Layokun, P.K. Nwesiogwe, P.O. Olutiola, 1990. Hydrolysis of sawdust by cellulase enzyme derived from *Aspergillus flavus* Linn Isolate NSPR 101 beyond the initial fast rate period. *JNSChE.*, 9: 1-2.

Solomon, B.O., B. Amigun, E. Betiku, T. Ojumu, S. Layokun, 1999. Optimization of cellulase production by *Aspergillus flavus* Linn isolate NSPR 101 grown on bagasse. *JNSChE.*, 16: 61-68.

Usama, F.A., S.S. Hala, 2008. Production and partial purification of cellulase complex by *Aspergillus niger* and *A. nidulans* grown on water hyacinth blend. *Appl. Sci. Res.*, 4: 875-891.

Wu, Z., Y.Y. Lee, 1997. Inhibition of the enzymatic hydrolysis of cellulose by ethanol. *Biotechnol. Lett.*, 19: 977-979.