Production of Cellobiohydrolase by *Penicillium funiculosum* NCL1 under Submerged and Solid State Fermentation using Agricultural Waste Residue

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The purpose of this work was to study the production of cellobiohydrolase production by a *Penicillium funiculosum* NCL both in SSF and SMF using different agricultural residues (wheat bran, sugarcane bagasse, rice bran and rice straw) as substrates. Maximum cellobiohydrolase activity (13 U/gds) was produced by the culture grown on wheat bran substrate when compared to the cultures grown on sugarcane baggase, ricebran and rice straw. Time course of cellobiohydrolase production in SSF showed that maximum cellobiohydrolase activity of 13.2 U/gds was obtained in SSF at 144 h. *Penicillium funiculosum* NCL1 was grown under SSF and SmF conditions with different carbon sources. A maximum level of cellobiohydrolase was produced at 144 h in SSF whereas this strain produced maximum cellobiohydrolase at 96 h in SmF. Among the carbon sources tested wheat bran enhanced the cellobiohydrolase production by *P. funiculosum* NCL1. This strain produced higher level of cellobiohydrolase at acidic pH of the medium. The effect of various carbon sources on cellobiohydrolase was examined. Avicel was found to be a potent inducer for cellobiohydrolase production by *P. funiculosum* NCL1.

Key words: *Penicillium funiculosum*, SSF, Wheat bran, Fermentation.

Enzymes are among the most important products obtained for human needs through microbial sources. These enzymes are commonly used in many industrial applications, and the demand for more stable, highly active and specific enzymes is growing rapidly. Cellulases have wide application in the textile industry, substituting conventional stonewash methods (biostoning). They also have applications in the detergent industry for reducing fuzz and pilling of fabrics, and as general cleaning agents for cotton garments (Bhat, 2000; Olson and Stanley, 1990).Fungal cellulases have proved to be a better than other microbial cellulases. Generally, a typical cellulolytic complex includes a variety of hydrolytic and oxidative enzymes. Depending on their mode of action, cellulolytic enzymes fall into one of two main groups, endogluconase or cellobiohydrolase (Beguin and Aubert, 1994). The complete degradation of cellulose to glucose requires the

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action of at least three types of enzymes: Endo-β-1,4 -glucanase, Exo-β-1,4 -glucanase (cellobiohydrolase) and β-glucosidase (Beguin and Lemaire *et al*, 1996).Cellobiohydrolase is the essential component of the cellulase system to hydrolyze cellulose ,consisting of both crystalline and amorphous cellulose .

Agricultural residues are an important source of nutrients for several microorganisms used in fermentative processes. They serve as an adequate substrate for microbial growth with the consequent formation of products of industrial interest (Keskar, 1992; Haltrich *et al*., 1995). Compared to the submerged fermentation, SSF possesses several advantages such as higher fermentation productivity, higher end-concentration of products, higher product stability, lower catabolic repression, cultivation of microorganisms on water-insoluble substrates or mixed cultivation of various fungi, and lower demand of sterility due to the low water activity (Hölker *et al*., 2004). Cellobiohydrolase has broad applications in cellulose recycling as well as industrial and commercial purposes (Han EL., *et al.,* 2008). This enzyme is mainly produced by fungi. Microorganisms mostly reported as producers of cellobiohydrolase and other two cellulases were members of the genera *Trichoderma, Aspergillus*, *Fusarium,Penicillium* and *Humicola* such as *Trichoderma reesei ,T. harzianum, T. viride, Aspergillus niger*, etc. (Ratha J 2010).Most processes of cellobiohydrolase production involved solid state fermentation (SSF) rather than submerged fermentation (Sun, Y *et al*.,2002). Since commercial endoglucanase is still relatively high cost (Singhania, R.R. *et al*., 2010), strategies for effective bioproduction of cellobiohydrolase is highly required. *Penicillium* strains have been reported as producers of cellulolytic complexes with improved synergy due to their high production of 5ØýÞ-glucosidase and endoglucanase. Although *P. funiculosum* can efficiently produce cellulases from pretreated agro industrial residues. These enzymes can be produced by several microorganisms. Studies have shown that the filamentous fungus *Penicillium funiculosum* has great potential for cellulases production, depicting the enzymatic activities (Castro *et al*., 2010 and Maeda *et al*., 2010). In this context present study shows the Production of cellobiohydrolase by *Penicillium funiculosum*

NCL1 under submerged and solid state fermentation using agricultural waste residue

MATERIALS AND METHODS

Organism and culture condition

Penicillium funiculosum NCL1 was obtained from National Chemical Laboratory, Pune, India. Stock cultures were kept on potato dextrose agar and subcultured monthly. *P. funiculosum* NCL1 spores were inoculated in Reese Basal medium at a final concentration of 108 spores/ml. Flasks were incubated in an orbital shaker (220 rpm) at 30 ºC for 96h. The mycelia were recovered by filtration on a nylon filter (30 ¼m spore) washed with 0.9% (w/v) NaCl and dried by pressing between two filter papers.

Pre-treatment of substrates

The procured cellulosic substrates such as rice husk, rice bran, coir waste, wheat bran and saw dust were ground to fine powder and the substrates were individually treated with 1% (w/v) NaOH solution in the ratio of 1:10 (substrate: solution) for 1h and was brought to neutral pH by washing thoroughly with distilled water and dried at room temperature. The treated substrates were autoclaved at 121°C for 1 h (18).

Submerged fermentation (SmF)

Submerged fermentation was carried out in 250 ml Erlenmeyer flasks containing 100 ml of fermentation medium. Reese medium for cellulase production in Smf (g/l) consisted of: $NaNO_3$, 10; K_2HPO_4, 3.3; $MgSO_4$, 1.6; $FeSO_4$, 0.0003; KCl, 0.4; Peptone, 1.6 and Yeast extract, 1.6. Wheat bran (10g) was used as substrate . The medium was sterilized by autoclaving at 121°C for 15 min. Each flask was inoculated with 1ml of the above said inoculum. The cultures were incubated on a rotary shaker (120 rpm) at 30°C for 72 h.

Solid state fermentation (SSF)

Solid state fermentation was carried out in 250 m l Erlenmeyer flasks that contained 10 g of agricultural waste residues (Wheat bran ,baggase,rice bran ,rice straw and 15 ml of distilled water (moistening agent). The flasks were sterilized at 121°C for 15 min and cooled to room temperature. About 1ml of inoculum was added, mixed well and incubated at 30°C in a humidified incubator for 96 h. The flasks were periodically mixed by gentle shaking.

Enzyme extraction

At 72 h the contents in the individual flasks were extracted with 20 ml of 0.05 M sodium acetate buffer (pH 4.8) by mechanical squeezing. The extract was centrifuged at 10,000 rpm for 20 min. The clear supernatant was assayed for cellobiohydrolase activity. All the fermentation trials were carried out in triplicates. For fermentation under submerged conditions, 1% wheat bran was used as substrate.

Enzyme assay

The total cellulase activity (filter paper activity, FPase) was assayed by incubating 500 ¼l of crude enzyme with 1 ml of 0.05 M sodium citrate buffer containing Whatman No. 1 filter paper (50 mg, 1 x 6 cm) at 50°C for 1 h. The amount of reducing sugars released was determined by the 3, 5 dinitrosalicylic acid (DNS) method. Then, the amount of glucose in the final reaction solution was measured with a UV VIS spectrophotometer at 600 nm with glucose as the standard. In addition, the total protein in the crude enzyme extract was determined by the Bradford method with bovine serum albumin (BSA) as the standard

Enzyme activity

One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 µmole of products (reducing sugar or glucose) from the appropriate substrates per minute under the assay conditions. In this study, the enzyme activities are expressed as units per gram of EFB (U/g)

Optimization of process parameters in SmF and SSF

Evaluation of optimized culture conditions in SSF and SmF using *Penicillium funiculosum NCL1*

The protocol adopted for the standardization of fermentation parameters was to evaluate the effect of an individual parameter. The parameters optimized were: substrates (wheat bran, rice bran, rice straw, sugarcane bagasse) temperature (20 to 40 °C), pH (3 to 7),fermentation period [(24 to 192 h in SmF) and (24 to 120 h in SSF)].

Effect of pH and temperature on the activity of crude cellobiohydrolase

The effect of pH on the cellobiohydrolase activity was determined by measuring the relative activity using different buffers. Sodium acetate (pH 5.0-5.5), sodium phosphate (pH 6.0-8.5) and sodium

carbonate (pH 9.0-9.5) buffers were used for the estimation of relative activity at different pH. The maximum activity was considered as 100%, and used as reference in determining relative activities at different pH values. The effect of temperature on the reaction rate was determined by performing the standard reaction at different temperatures in the range of 30-90°C. The relative activity was expressed considering maximum activity as 100%. Determination of pH stability and thermostability The stability as a function of pH was determined by measuring the residual activity at each pH after 1 h of incubation at room temperature. The retention of activity was assayed at optimal pH and temperature. The relative activity was expressed considering the activity before incubation as 100%. Thermostability of cellobiohydrolase was determined by incubating the enzymes at different temperatures (30-90°C) for 30 min. The residual activity was estimated under optimal conditions and the relative activity was calculated by taking activity before incubation as 100%.

RESULTS

Effect of different substrates on cellobiohydrolase production

Pretreated agricultural residues such as wheat bran, rice bran, rice straw and bagasse were used as substrates for cellobiohydrolase production under Solid state ferementation (SSF) (Fig. 1). Maximum cellobiohydrolase activity (13 U/gds) was produced by the culture grown on wheat bran substrate when compared to the cultures grown on sugarcane baggasse (10 U/gds); rice straw (7 U/gds) and rice bran (3 U/gds) as substrates.
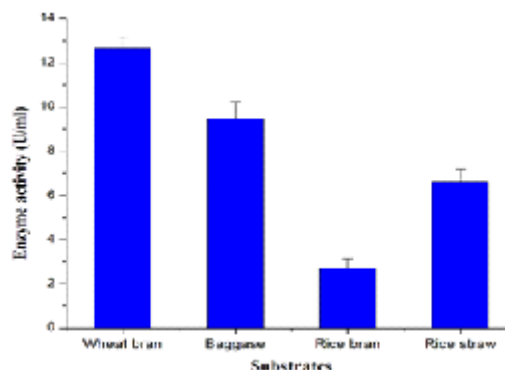

Fig. 1. Production of cellobiohydrolase by *P. funiculosum* in SSF

Kinetics of cellobiohydrolase production in SSF

The cellobiohydrolase production was increased significantly in the fermentation medium with wheat bran compared to other substrates and therefore wheat bran was selected as substrate in subsequent experiments. Time course of cellobiohydrolase production in SSF was studied by withdrawing samples at 24 h intervals and assaying for cellobiohydrolase activity **(Fig. 2)**. A maximum cellobiohydrolase activity of 13.2 U/gds was obtained in SSF at 144 h. The cellobiohydrolase activity started to decline thereafter and at 196 h, the activity was only 2.3 U/gds. Similarly, the specific activity was found maximum at 172 h (5.43 U/mg) and declined at 196 h (0.8 U/mg).
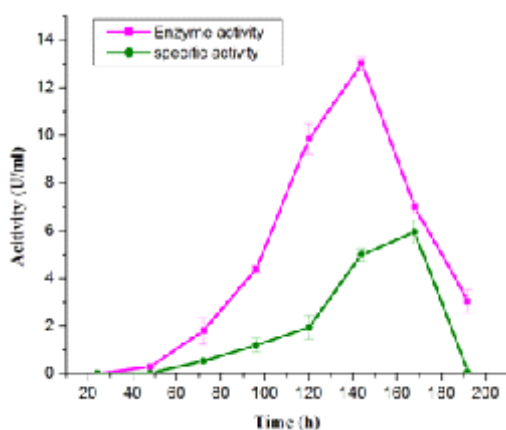

Fig. 2. Kinetics of cellobiohydrolase production by *P. funiculosum* in SSF

Characterization of cellobiohydrolase produced by *P. funiculosum* NCL1 in SSF.

The activity and stability profiles of cellobiohydrolase produced by *P. funiculosum* in SSF were examined at different pH and temperature (Fig. 4). The cellobiohydrolase exhibited maximum activity at pH 5.0 (13.2 U/gds), but only 20% of activity at pH 7.0. The cellobiohydrolase did not show activity at pH 9.0. With respect to their stability, the cellobiohydrolase was stable over wide range of pH from 3.0 to 7 and showed maximum stability at pH 6.0. The maximum cellobiohydrolase activity (13.2 U/gds) was observed at 50 °C but the cellobiohydrolase exhibited only 20% of activity at 80°C. The cellobiohydrolase was stable up to 60 °C.

Effect of initial pH of the medium on cellobiohydrolase production

To determine the optimum pH of the medium for fungal growth and cellobiohydrolase production, *P. funiculosum* was grown on fermentation media at different initial pH from 5.0 to 9.0. Fermentation trials with wheat bran as substrate were performed at 30°C for 144 h (Fig. 3). The cellobiohydrolase production was maximum (12.2 U/gds) from the culture grown in the medium with an initial pH 5.0. This strain also produced 50% of maximum cellobiohydrolase activity (6.4 U/gds) at pH 6.0. At the intial pH 4, this strain also produced nearly 10 U/gds
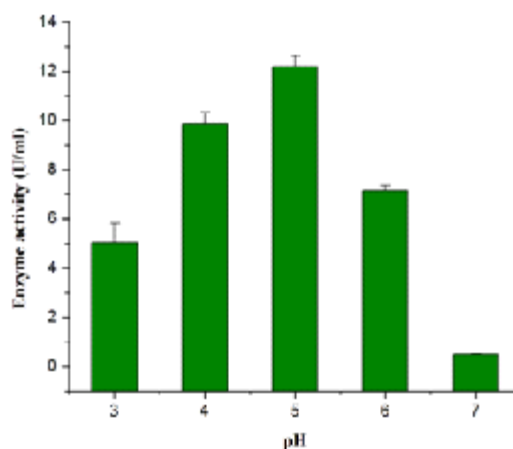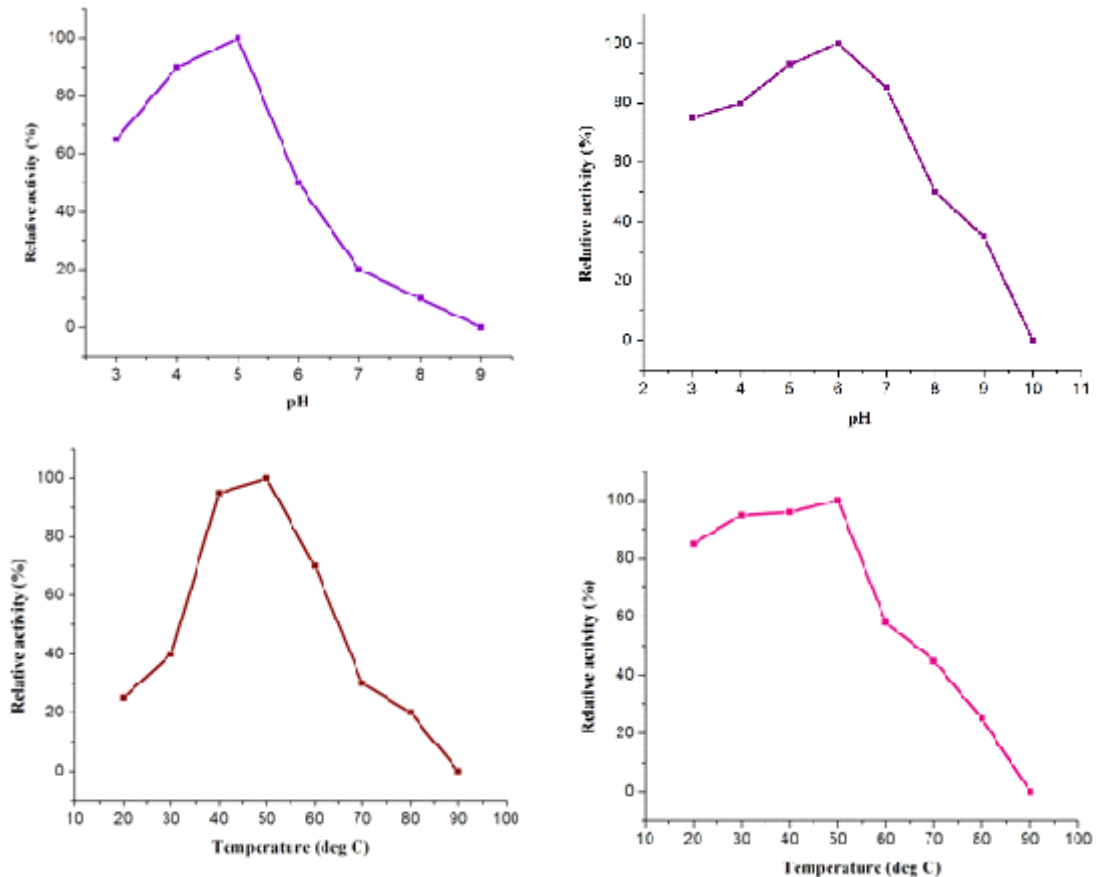

Fig. 3. Effect of initial pH of the medium on cellobiohydrolase production by *P. funiculosum* under SSF

P. funiculosum was grown in SSF with 10 g wheat bran as solid support and as substrate with 60% moisture content for 72 h at 30 °C. Appropriately diluted crude enzyme extract was assayed for cellobiohydrolase activity and stability at different pH and temperature. (A) Cellobiohydrolase activity was determined in different buffers (pH 3.0 to 9.0) at 50°C. (B) The stability of the cellobiohydrolase was determined by incubating the enzyme extract in different buffers (pH 3.0 to 9.0) for 1 h at 50°C and assaying the residual activity at pH 5.0. (C) Cellobiohydrolase activity of enzyme extract was measured at different temperatures (30°C to 80°C) at pH 5.0. (D) The stability of cellobiohydrolases was determined by pre-incubating the enzyme at different temperatures (30°C to 80°C) at pH 5.0 for

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Fig. 4. Effect of pH and temperature on the activity and stability of cellobiohydrolase produced by *P. funiculosum* in SSF

30 min and the residual cellobiohydrolase activity was determined at temperature 50°C, pH 6.0. Relative activity (%) in percentage was expressed in comparison to the maximum activity. Data shown are average of triplicate assays with SD within 10% of the mean values.

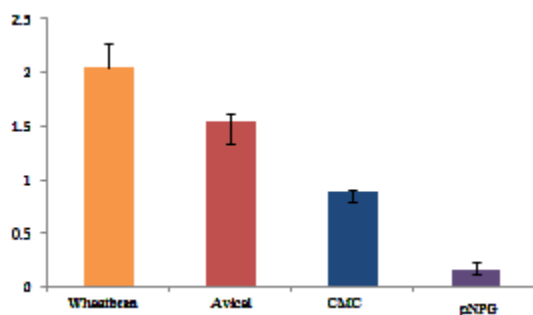**Cellobiohydrolase production by *P. funiculosum* in SmF**

Effect of different substrates on cellobiohydrolase Production

   P. funiculosum was grown under submerged fermentation (SmF) condition with different substrates (1% w/v) such as wheat bran, avicel, CMC and cellulose. In SmF also wheat bran was found to be the best substrate for the production of cellobiohydrolase by *P. funiculosum* (Fig. 5). The highest titre cellobiohydrolase (2.3 U/ml) was produced when wheat bran was used as substrate whereas lower activities were produced if other cellulase sources were used (Avicel- 1.5 U/ml; CMC- 0.9 U/ml and pNPG 0.25 U/ml).

Time course of cellobiohydrolase production by *P.funiculosum* in SmF

Time course of cellobiohydrolase production by *P.funiculosum* in SmF with wheat bran (1% w/v) as the substrate was studied (Fig.6). At 24 h intervals, samples were withdrawn and assayed for cellobiohydrolase activity in the culture supernatant. At 96 h of SmF, maximum of 2.6 U/ml cellobiohydrolase activity was obtained and started declining thereafter. The production of cellobiohydrolase was decreased to 1.2 U/ml at 120 h and 0.8U/ml at 144 h. The specific activity of cellobiohydrolase (2U/mg) was also maximum at 96 h of fermentation and declined subsequently.

P. funiculosum was grown in fermentation medium supplemented with different carbon sources (1%) at pH 5.0 and at 30°C. After 96 h of fermentation, the culture supernatant was assayed for cellobiohydrolase activity at pH 5.0 and 50°C.

Fig. 5. Effect of different substrates on cellobiohydrolase production by *P. funiculosum* in SmF

Effect of initial pH of the medium on cellobiohydrolase production by *P.funiculosum* in SmF
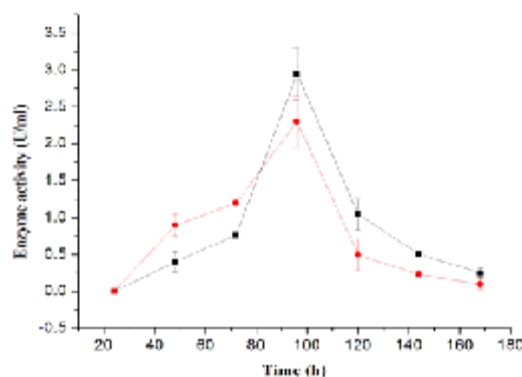
P.funiculosum was grown in a medium containing wheat bran (1%) as substrate at various initial pH from 3.0 to 7.0, at 30°C with agitation (160 rpm). The initial pH of the medium was adjusted with either acetic acid or 10% Na_2CO_3. After 96 h of fermentation, the samples were taken and assayed for cellobiohydrolase activity

Characterization of cellobiohydrolase from SmF

The activity and stability profiles of cellobiohydrolase with respect to pH and temperature were studied (Fig. 8).Maximum activity (2.3U/ml) was observed at pH 5.0, whereas at pH 7.0 showed only 10% of activity. The
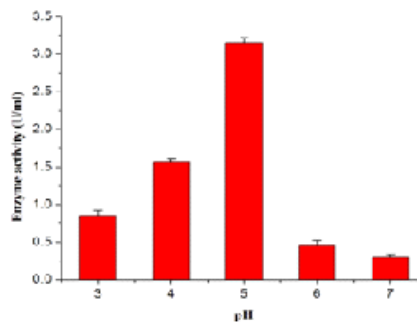
Effect of initial pH of the medium on cellobiohydrolase production by *P.funiculosum* in SmF

To determine the optimum growth pH for cellobiohydrolase production, *P. funiculosum* was grown in medium with different initial pH from 3.0 to 7.0 and wheat bran as substrate. After 96 h of fermentation, the culture supernatant was assayed for cellobiohydrolase activity. If the initial pH of the medium was acidic, after the fermentation the final pH of the medium was increased to alkaline pH (7.5 to 8.2). Maximum cellobiohydrolase (3.2 U/ml) was produced by *P. funiculosum* grown in the medium with initial pH 5.0 (Fig. 11). At pH 7.0, this strain produced only 10% of the maximum level of cellobiohydrolase.
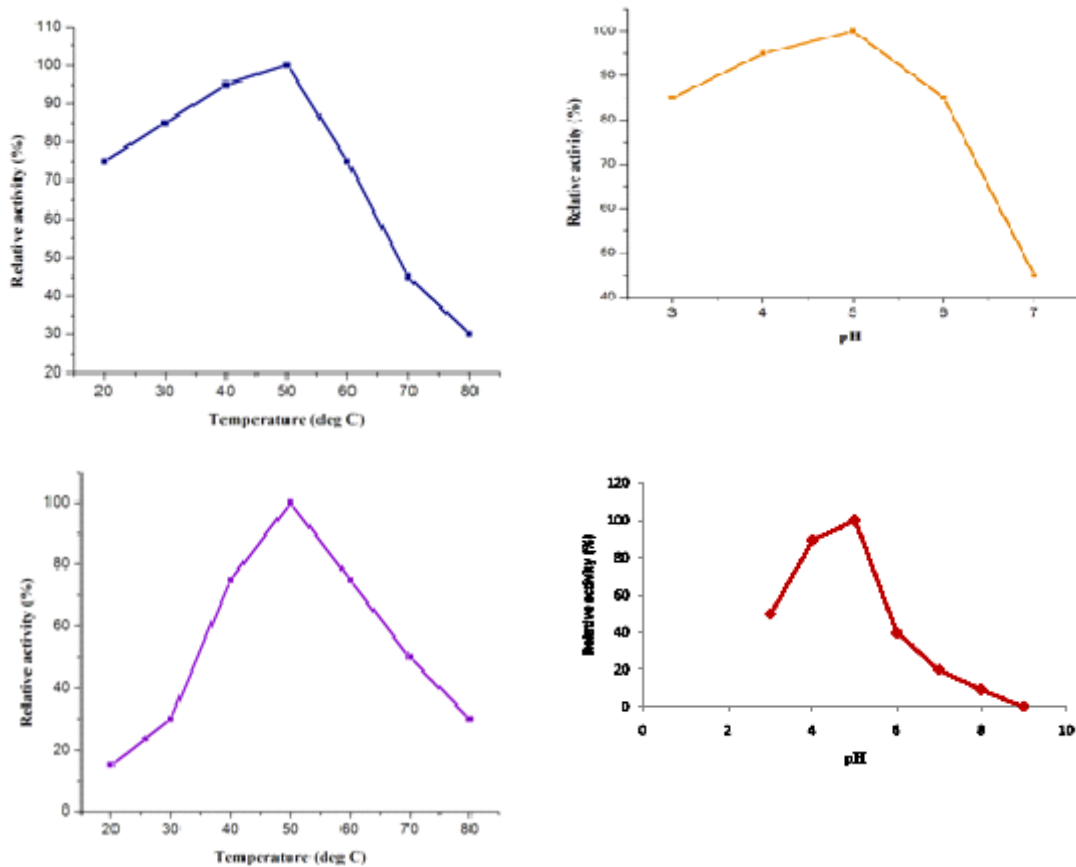

P. funiculosum was grown in 1% wheat bran medium at pH 5.0 and at 30 °C with agitation (160 rpm). At 24 h intervals, mycelia free culture filtrate was collected and used to determine cellobiohydrolase activity and specific activity.

Fig. 6. Time course of cellobiohydrolase production by *P.funiculosum* in SmF

P.funiculosum was grown in a medium containing wheat bran (1%) as substrate at various initial pH from 3.0 to 7.0, at 30°C with agitation (160 rpm). The initial pH of the medium was adjusted with either acetic acid or 10% Na_2CO_3. After 96 h of fermentation, the samples were taken and assayed for cellobiohydrolase activity.

Fig. 7. Effect of initial pH of the medium on cellobiohydrolase production by *P.funiculosum* in SmF

P. funiculosum was grown in SmF medium with 1% wheat bran as substrate for 96 h. The culture supernatant was used to assay the cellobiohydrolase activity. (A) Cellobiohydrolase activity was determined in different buffers (pH 3.0 to 9.0) at 50°C. (B) The culture supernatant was incubated in different buffers for 1 h at 30°C. The residual cellobiohydrolase activity was measured at pH 5.0. (C) The cellobiohydrolase activity in the culture supernatant was measured at different temperatures (20°C to 80°C) at pH 6.0. (D) The stability of cellobiohydrolase was determined by preincubating the culture supernatant at different temperatures (30°C to 80°C) for 30 min and the residual cellobiohydrolase activity was measured at pH 5.0 and 50°C. Relative activity (%) in percentage was expressed in comparison to the maximum activity. Data shown are average of triplicate assays with SD within 10% of the mean values.

Fig. 8. Effect of pH and temperature on the activity and
stability of cellobiohydrolase produced by *P.funiculosum* in SmF

cellobiohydrolase was stable over wide range of pH from 3.0 to 6.0; the maximum stability was at pH 5.0. Maximum activity of cellobiohydrolase (2.3 U/ml) was observed at 50 °C but exhibited only 50% activity at 70 °C. The cellobiohydrolase showed stability up to 50 °C and retained 40% of activity at 70 °C

DISCUSSION

SSF has gained renewed attention from industry because it becomes a more alternative to submerged fermentation in the production of some chemicals and enzymes with simpler cultivation equipments, lower capital investment, higher productivity per reactor volume, easier aeration, reduced bacterial contamination (Roopesh *et al.*, 2006). Agricultural residues are important source of nutrients for several microorganisms used in fermentative processes. They serve as adequate substrate for microbial growth with the consequent formation of products of industrial interest (Keskar, 1992; Haltrich *et al.*, 1995). Compared to the submerged fermentation, SSF possesses several advantages such as higher fermentation

productivity, high concentration of products, higher product stability, lower catabolic repression, cultivation of microorganisms on water-insoluble substrates or mixed cultivation of various fungi, and lower demand of sterility due to the low water activity (Hölker *et al*., 2004). Under SSF, *P. funiculosum* NCL1 also produced maximum level of cellobiohydrolase compared to SmF. The fermentation time required for maximum cellobiohydrolase production was higher in SSF compared to SmF. In SSF, higher level of cellobiohydrolase was produced in six days whereas SmF took four days to attain maximum cellobiohydrolase production. Although the time required for cellobiohydrolase production is high in SSF than SMF, the cellobiohydrolase production was found maximum in SSF. Incubation time necessary for optimal production was observed at 72 h in SSF. Short incubation period for enzyme production offers the potential for inexpensive production of enzyme (Sonjoy *et al.,*1995). In SmF, the specific activity was not drastically decreased when compared to SSF. But the maximal cellobiohydrolase activity of *Trichoderma ressei* was attained at 5 days of incubation (Khushal *et al*., 2011). The maximum level of cellobiohydrolase was reached in nine days in liquid culture for *Aspergillus niger* (Abidah *et al.,*2011).

P. funiculosum* NCL1 produced maximum cellobiohydrolase activity when wheat bran was used as substrate in both SmF and SSF. In SmF, the commercial like avicel, CMC, pNPG supported less than wheat bran in cellobiohydrolase production. The strain NCL1 used various natural hemicellulosic agricultural residues such as wheat bran, rice bran and bagasse, but yielded high activities of cellobiohydrolases with only wheat bran.. *P.funiculosum* NCL1 produced highest level of cellobiohydrolase on wheat bran, which is inexpensive and abundant and require minimum pretreatment (only coarse grinding). Therefore, wheat bran appeared to be of most economic and technical potential for large scale production of cellobiohydrolases.

The initial pH of the medium has been found to influence the synthesis of many enzymes and their secretion across the cell membrane (Moon and Parulekar, 1991). The effect of the initial pH (from 3.0 to 7.0) on cellobiohydrolase production was studied. The initial pH value of the medium strongly influenced the cellobiohydrolase production in many fungi (Malik *et al*, 2010, Abidah *et al*, 2011). Acid pH levels (5.0–6.5) generally favored fungal cellulase production. After pH value of 5.5, the production of cellulases decreased which might be due to the fact that cellulases are acidic proteins and are greatly affected by the neutral pH values (Juhasz *et al*., 2004; Chandra *et al*., 2009).

CONCLUSION

In this study *Penicillium funiculosum* NCL1 was grown under SSF and SmF conditions with different carbon sources. A maximum level of cellobiohydrolase was produced at 144 h in SSF whereas this strain produced maximum cellobiohydrolase at 96 h in SmF. However, the amount of cellobiohydrolase produced under SSF always greater than that was produced in SmF condition.

REFERENCES

1. Abidah, M. N., Jamaliah, M. J., Abdul, M., Abdul, M., Farah, D.and Abu, B. Effect of Various Cultivation Methods on Cellobiohydrolase Production from *Aspergillus niger. International Journal on Advanced Science Engineering Information Technology.* 2012; **27** : 65-70

2. Beguin P, Aubert J The biological degradation of cellulose. *FEMS Microbiology Review.* 1994; **13**: 25-58.

3. Beguin, P., Lemaire, M, The cellulosome: An exocellular,multi-protein complex specialized in cellulose degradation. *Critical Review Biochemistry Molecular Biology.* 1996; **31**: 201-236.

4. Bhat, M.K.. Cellulases and related enzymes in

biotechnology. Biotechnology Advances. 2000; **18**: 355-383.

5. Castro, A.M., Ferreira, M.C., Cruz, J.C., Pedro, K.C.N.R. Carvalho, D.F. Leite, S.G.F. and Pereira, N. High-yield endoglucanase production by *Trichoderma harzianum* IOC-3844. Cultivated in pretreated sugarcane mill byproduct. *Enzyme Research*, vol. 2010., 1-8.

6. Haltrich D ,Sebesta B ,Steiner W. Induction of xylanase and cellulose in Schizophyllum commune enzymatic degradation of insoluble carbohydrates .*ACS symp Ser* 1995; **618**: 305-18 .

7. Hölker, U., Höfer, M. and Lenz, J. Biotechnological advantages of laboratory–scale solid–state fermentation with fungi. *Applied Microbiology and Biotechnology*.2004., **64**: 175–186.

8. Kesker,S.S. High activity xylanase from thermotolerant Streptomyces T7 cultural conditions and enzyme properties . *Biotechnology Letters*. 1992;**14**: 481-6.

9. Khushal, B. and Praveen, Vadlani, V. Cellulolytic Enzymes Production via Solid-State Fermentation: Effect of Pretreatment Methods on Physicochemical Characteristics of Substrate. *Enzyme Research* .2011; **4**: 1196-1201

10. Maeda, R.N., Serpa, V.I., Rocha, V.A.L., Mesquita, R.A.A., Santa Anna, L.M.M., Castro, A.M., Driemeier, C.E., Pereira, N. and Polikarpov, I. Enzymatic hydrolysis of pretreated sugar cane bagasse using *Penicillium funiculosum* and *Trichoderma harzianum* cellulases. *Process Biochemistry*, 2011; **46**: 1196-1201.

11. Moon, S.H., Parulekar, S.J., A parametric study of protease production in batch and fed batch cultures of *Bacillus firmus*. *Biotechnology Bioengineering*. 1991; **37**: 467-483.

12. Olson, L., and P. Stanley. 1990. Compositions and methods to vary color density Patent WO 90/02790 1990.

13. Ratha, J. and Chaowaree, A. Selection of nutrient parameters for endoglucanase production from rice bran by *Penicillium* sp. using Plackett-Burman design .*KKU Research. Journal*. 2012; **17**: 965-971

14. Singhania RR, Soccol CR, Pandey A. Application of tropical agro-industrial residues as substrate for solid-state fermentation processes. In: Pandey A, Soccol CR, Larroche C, editors. Current Developments in Solid-state Fermentation. Springer; New York, USA: 2008. 412-442.

15. Sonjoy, S., Bill Bex, Houston, K.H. Cellular activity of *Trochoderma ressei* (RUT-C30) on municipal solid waste. *Applied Biochemistry Biotechnology*. 1995., **15**: 145-153.

16. Sun Y, Cheng J. Hydrolysis of lignocellulosic material from ethanol production: A review. *Bioresour Technol*. 2002; **83**:1–11.