

**Optimisation of alkaline cellulase production from marine derived fungi, *Chaetomium* sp.,  
using agricultural and industrial wastes as substrates**

C. Ravindran\*, T. Naveenan and G. Varatharajan

Marine Biotechnology laboratory, Biological Oceanography Division,

National Institute of Oceanography (CSIR), Goa- 403 004, India

**Abstract**

Alkaline cellulases were screened from the fifty four fungal isolates obtained from mangrove leaves and mangrove wood litters as endophytes and wood litter fungi respectively. Only three fungal isolates tested positive for alkaline cellulases under plate assay, among which *Chaetomium* sp. (NIOCC 36) was found to grow in a wide range of pH (between 4 to 12). This alkaline tolerant fungi was further tested for the production of alkaline cellulases ( $\beta$ - endoglucanase,  $\beta$ - exoglucanase,  $\beta$ - glucosidase), using agricultural and industrial wastes as substrates in the submerged (SMF) and solid state fermentation (SSF) processes. Cotton seed under SSF condition showed maximum enzyme production at high alkaline pH. The results revealed that cellulase enzymes produced under alkaline cotton seed SSF condition had high stability and activity than the non alkaline conditions used at pH 5, 7, 12 and at 50 °C. The agricultural and industrial wastes used as carbon sources were economical during commercial production of cellulose. The high specific activity, and stability of cellulases under neutral to alkaline pH and high temperature will be of use in various industrial and biotechnological applications.

**Key words:** agro wastes; alkaline cellulase; marine fungi; SMF; SSF.

\* Corresponding author:

Marine Biotechnology, Biological Oceanography Division, National Institute of Oceanography (CSIR),  
Goa- 403 004, India. Email- [cravindran@nio.org](mailto:cravindran@nio.org). Tel: +91-08322450442 ; Fax: +91-08322450602

## **Introduction**

Mangrove communities are recognized as highly productive ecosystems that provide large quantities of organic matter to adjacent coastal waters in the form of detritus and live animals (fish, shellfish). The detritus serves as a nutrient source and is the base of an extensive primary source in the food web of the tropical marine ecosystem (Raghukumar et al. 1994). Marine fungi are believed to be important in the degradation of mangrove leaves and wood litters resulting in the formation of detritus. Endophytic fungi are microbes, which spend the whole or part of its life cycle colonizing inter- and/or intracellular spaces inside the healthy tissues of the host plants, typically causing no apparent symptoms of disease (Petrini 1991). Endophytes also act as wood litter fungi during favored suitable conditions (Wilson and Carroll, 1994).

Mangrove fungi have proven to be an important source of novel bioactive compounds (Lin et al. 2001) and enzymes (Pointing and Hyde, 2000). Some pH stable and active cellulases from fungi and bacteria are known, but most of them are not very active at pH 9 and 10 or not stable at pH 9 (Dusterhoft et al. 1997). Thus, considering the importance of alkaline cellulases in kraft pulping industries (Patel et al. 1993; Baraznenoka et al. 1999) a study was carried out to screen marine derived fungi from the mangrove ecosystem of Goa coast for the production of alkaline cellulase enzymes using agricultural and industrial wastes such as wheat bran, rice bran, cotton seeds, sugarcane bagasse and paper as substrates.

## **Materials and Methods**

### **Sampling of plant material and isolation of fungal endophytes**

The entire healthy leaves of the mangroves viz., *Sonneratia alba* J. Smith, *Avicennia marina* (Forsk.) Vierh. *Avicennia officianlis* L., *Avicennia alba* Blume. and *Rhizophora mucronata* Lamk. were collected and washed in running water and dried. The segments were cut equally with the sterilized cork borer from the middle portion of healthy leaves, including the midrib portion. The segments were surface sterilized (Kumaresan et al. 2002) by dipping the leaf segments in 70% ethanol for 5 seconds, followed by 4% NaOCl for 90 seconds and sterile water for 10 seconds and plated on malt extract agar (MEA) (Himedia, Mumbai, India) medium supplemented with antibiotic of 10,000 units of sodium benzyl penicillin and 0.05 g of streptomycin sulfate per 100 ml of medium to inhibit bacterial growth.

Petri dishes were incubated at  $25 \pm 2^\circ\text{C}$  for two to three weeks. The fungi grown out from the tissues were isolated and transferred to the MEA slants under sterile conditions. The salt concentrations in the leaves of the mangrove plants are known to be similar to that of sea water (Popp et al. 1984), thus, endophytic fungi were isolated and cultured in media containing sea water.

### **Isolation of wood litter fungi**

The wood pieces were collected in sterile plastic bags from mangrove swamps of Sal, Chorao and Zuari in Goa, India and washed with sterile sea water to remove the mud, and dried. The wood pieces were put in sterile polythene bags and incubated ( $25 \pm 2^\circ\text{C}$ ). They were screened under a stereomicroscope for the fungal structures at different intervals of incubation (2, 8, 16 and 32 weeks) and the fungi were isolated using a sterile needle and transferred to MEA medium containing antibiotics of 10,000 units of sodium benzyl penicillin and 0.05 g of streptomycin sulfate per 100 ml of medium (prepared with filtered sterilized sea water) to inhibit bacterial growth.

### **Identification of fungi**

The isolated fungal strains was cultured in plates and slants containing Malt Extract Agar (MEA) medium (Himedia, Mumbai, India) and incubated at room temperature and further stored in  $4^\circ\text{C}$ . The sporulated fungi were identified based on the colony morphology and microscopical features using standard taxonomic keys and monographs (Carmichael et al. 1980; Ellis, 1971).

### **Screening of alkaline cellulase using plate assay**

The cellulase assay (Teather and Wood, 1982) and enrichment procedure was done in minimal medium comprising 1%  $\text{KNO}_3$ , 0.1% yeast extract (Difco), 0.1%  $\text{KH}_2\text{PO}_4$ , 0.02%  $\text{MgSO}_4$ , 1%  $\text{Na}_2\text{CO}_3$ , 4%  $\text{NaCl}$  and 0.25% CMC, with 1.5% agar (pH 9.7). After incubation for 1 to 3 days at  $37^\circ\text{C}$  in the above mentioned medium, the Petri plates were replica plated and the parent plate was flooded with 0.1% Congo Red for 15 minutes. The plates were destained with 1M  $\text{NaCl}$  for 30 minutes. The strains that showed a clearing zone around the colony were isolated as potential alkaline cellulase producing fungi. All medium were prepared using sea water. The experiments were done in triplicates.

## **Growth characterization of the alkaline tolerant fungi**

The optimization of media and pH for alkaline cellulase -tested positive fungi was done using plate assay and broth. Different commonly used media (Himedia, Mumbai, India) like malt extract agar (MEA), potato dextrose agar (PDA), czapek dox agar (CDA), corn meal agar (CMA) were tried. The fungal inoculum from the mother plate was placed at the center of the plate. The plates were made in triplicates and incubated at room temperature. The growth of the colony was measured and growth curve was obtained. The pH of the optimized medium was adjusted for 4, 6, 8, 10, and 12. The fungus was inoculated at the center of plates of different pH. The plates were made in triplicates and incubated at room temperature. The growth of the mycelium was noted for each day and the day versus growth graph was plotted.

## **Submerged fermentation (SMF)**

Twenty milliliters of Vogel's complete medium (Vogel, 1956), pH 6.0 containing 1% carbon source (carboxy methyl cellulose (CMC) sodium salt, rice bran, cotton seed, whatman filter paper) and 2% CMC as substrates was dispensed in 100-ml Erlenmeyer flasks. Each flask was then inoculated aseptically with two mycelial discs of screened alkaline cellulase producing fungi and incubated at room temperature and 37 °C in two sets, one as static and the other as shake cultures (200 rev / min) for 8 days. The pH, growth of cells, residual substrate (CMC), extracellular cellulases including  $\text{exo-}\beta\text{-1,4-}$ glucanase,  $\text{endo-}\beta\text{-1,4-}$ glucanase and  $\beta\text{-1,4-}$ glucosidase were analysed at the end of fermentation. The growth of the cells was measured based on the wet weight and dry weight of the fungal mat obtained at end of fermentation.

Fractionations of enzyme samples were made after centrifuging the contents of each flask at 12000 rpm for 30 min at 4°C. The supernatant thus obtained was used for extracellular enzyme assay.

## **Solid state fermentation (SSF)**

Substrates (cotton seed, sugarcane bagasse, Rice bran, waste paper) with or without mineral salt solution ( $\text{gL}^{-1}$ , w/v):  $\text{NaNO}_3$ , 3.0;  $\text{KH}_2\text{PO}_4$ , 1.0; Tween 80, 1.0 mL;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5; KCl, 0.5;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01 was added to the 500 ml flask, moistened with ~ 20 ml of water. The flasks were cotton plugged and sterilized at 115°C for 60 min. After sterilization, each flask was seeded with inoculum. The final moisture content was about 80% w/w. The solid state fermentation was performed under

different conditions such as pH (pH 6 to 12); with and without nutrients and moisture conditions (80% w/w, 50% and moisture condition based on nutrient solution i.e. 33%). The culture flasks were incubated at room temperature for 8 days. The flasks were shaken twice a day. All fermentations and analysis were performed in triplicates.

### **Enzyme harvestation**

The enzyme was extracted by adding 100 mL of 0.05 M citrate buffer (pH 4.8) to the fermented substrate in each flask. The flasks were rotated on a rotary shaker at 200 rpm for 1 hr at 30°C. The fermented broth was filtered by using Whatman No.1 filter paper. Filtrate was analysed for cellulase activity.

### **Analytical methods**

Different enzyme assays for cellulase were performed using various substrates. Exo- $\beta$ -1,4-glucanase (EC 3.2.1.91) was estimated in terms of filter paper (FP) activity and endo-  $\beta$  -1,4- glucanase (EC 3.2.1.4) as carboxy methyl cellulase (CM cellulase) activity by using filter paper (1-6 cm strips of Whatman No. 1) and carboxy methyl cellulose (CMC) sodium salt as the respective substrates while  $\beta$  -1,4-glucosidase (EC 3.2.1.21) was measured using cellobiose (Soni et al. 1999). One unit of enzyme activity has been defined as the amount of enzyme required to produce 1  $\mu$ mol of the product (glucose in case of exo- and endoglucanase, cellobiose in case of  $\beta$ -glucosidase) in 1 min under assay conditions (pH 5.0; 50°C for exo- and endoglucanase, 50°C for  $\beta$ -glucosidase). The released reducing sugar (glucose) was estimated by dinitrosalicylic acid (DNS) method (Miller, 1959).

### **Effect of pH and temperature on the activity and stability of the enzyme**

Estimations of the three different cellulase activity at different pH (5 to 12) and temperature (50 to 100°C) values were carried out under standard assay conditions to determine optimum pH and temperature for enzyme activity. The pH stability of the enzyme was measured by incubating 5 IU of enzyme for one hour, at 50°C, in a buffer of desired pH. The temperature stability was determined by incubating 5 IU of enzyme, at varying temperatures for different time intervals, and then estimating the residual activity under standard assay conditions.

## Statistical analysis

All data are given as the mean  $\pm$  SD of triplicates ( $n = 3$ ). Analysis of variance (ANOVA) was performed using Microsoft Excel 2007.

## Gel electrophoresis analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 10% polyacrylamide gel by the method of Laemmli (1970). After electrophoresis, the proteins were stained with Coomassie brilliant blue R-250. The molecular weight standards used were from a high-molecular-weight calibration kit (Fermentas). CMCCase zymograms were prepared using SDS-10% polyacrylamide gels containing 0.2% CMC was incorporated into the separating gel prior to the addition of ammonium persulphate and polymerisation. After electrophoresis, the gel was stained with Coomassie Blue R dye in methanol-acetic acid-water solution (4:1:5, by volume) for 1 h and destained in the same solution without dye. For finding out the activity of CMCCase, staining was done after removing SDS by washing the gel at room temperature in Solution A (sodium phosphate buffer, pH 7.2, containing isopropanol 40%) for 1 h and Solution B (sodium phosphate buffer, pH 7.2) for 1 h, respectively. Renaturation of the enzyme proteins was carried out by leaving the gel in Solution C (sodium phosphate buffer, pH 7.2, containing 5 mM  $\beta$ -mercaptoethanol and 1 mM EDTA) at 4°C overnight. The gel was then transferred onto a glass plate, sealed in a film, and incubated at 37°C for 4-5 h. The gel was stained in a solution of 1% Congo Red for 30 min, and destained in 1 M NaCl for 15 min. Clear bands indicated CMCCase activity (Ratanakhanokchai et al. 1999).

## Results

### Occurrence of endophytes and wood litter fungi in mangrove ecosystem

Anamorphic fungi, ascomycetes and sterile forms were present as endophytic fungi. Five isolates remained unidentified since they did not produce spores, which provide the bases for identification; therefore, they have been listed under sterile forms (Table 1). Hyphomycetes dominated the total number of isolates from wood litter fungi followed by four ascomycetes (*Lulworthia* sp., *Sporormia* sp., *Chaetomium* sp. and *Corollospora* sp.) and a basidiomycete, *Halocyphina villosa* (Table 2). Among the hyphomycetes *Chrysosporium* was dominant. *Sporormia* sp., an ascomycete, was the first report from the Goa coast. The endophytes *Aspergillus* sp. (NIOCC 24) isolated from *Avicennia marina* and

*Phialophora* sp. (NIOCC 27) from *Rhizophora mucronata* were also isolated as wood litter fungi, that is NIOCC 52 from wood litters of *Avicennia marina* plant and NIOCC 49 from wood litters of *Rhizophora mucronata* respectively collected from same locations (Table 1 and 2). More number of hyphomycete fungi was found when compared to that of the other groups of fungi. Thus, in our present study we isolated 54 strains of endophytic and wood litter fungi for the screening process of alkaline cellulases (Table 1 and 2).

### **Alkaline cellulase production**

The above obtained 54 fungal strains were analyzed for their alkaline cellulase activity using a plate clearing assay as described in Material and Methods. Among the 54 strains of fungi, sterile form 4, an endophytic fungi (Table 1) and *Aspergillus* sp. 2 and *Chaetomium* sp., isolated from wood litter (Table 2) showed strong cellulase activity at pH 9.7. The other fungal strains showed average, weak and no activity of the enzymes (Table 1 and 2).

### **Growth characterization of the *Chaetomium* sp.**

The optimization of media for *Chaetomium* sp. was done using plate assay and broth. The fungus showed maximum growth in MEA medium (Fig. 1) compared to other medium. The *Chaetomium* sp. was taken for further analysis for cellulase because of its growth ability in wide range of pH between pH 4 to 12. The fungus growth was maximum at pH 8.

### **Production of cellulases using solid state fermentation conditions (SSF)**

Enzyme production was tested using various agro wastes (cotton seed, rice bran and sugarcane bagasse) as substrates under different conditions such as with and without nutrition, different pH (6-12) and moisture conditions (w/v and 33%). Cotton seed substrate used under SSF showed maximum levels of enzyme secretion, comparatively, than the other substrates such as, rice bran, sugarcane bagasse and waste paper used (Fig 2a-d). Cotton seed substrate with pH 10 condition was found optimum for all the three enzymes (Fig. 2b). Thus, the enzymes harvested from the cotton seed SSF condition was used for further analysis for testing its activity and stability under high alkaline condition.

### **Production of cellulases using submerged fermentation conditions (SMF)**

The secretion of enzymes under SMF was tested under different conditions including, temperature, shake, static and different pH (Fig. 3a – 3d). The Figure 3a shows  $\beta$ - glucosidase the enzyme with maximum secretion of 6.5 IU/ml with the 1% CMC in shake culture,  $\beta$ - endoglucanase of 6.3 IU/ml and  $\beta$ - exoglucanase of 1.5 IU/ml with 2% CMC under shaking condition. Though  $\beta$ - glucosidase was found high in 1% CMC (shake culture), the other two enzymes showed maximum levels in 2% CMC under shaking condition. Thus, 2% CMC with shaking condition under SSF was found optimum for the enzyme production among the other different conditions tested (Fig. 3a – 3d). Further, the SMF experimental results reveal that the enzyme secretion levels were found maximum in shaking SMF than static SMF condition (Fig. 3a – 3d).

### **Effect of pH and temperature on the activity and stability of the enzyme**

The conditions (cotton seeds from SSF (Fig. 2b) and CMC shake from SMF (fig. 3a)) which showed the maximum amount of enzyme levels were tested for their activity and stability at different pH and temperature (Fig. 4a-d). The results showed that cellulase enzymes produced under alkaline cotton seed SSF (Fig. 2b) condition showed high stability and activity than the non alkaline 2% CMC shake SMF (Fig. 3a) condition at pH 5, 7, 12 and at 50 °C (Fig. 4a-d).

### **Gel electrophoresis analysis**

The electrophoretic studies with extracellular fractions revealed two forms each of endoglucanase, EG I, EG II/  $\beta$ - glucosidase,  $\beta$ -Glu I,  $\beta$ -Glu II (Fig.5), under SMF and SSF conditions (Fig.5).

### **Discussion**

Fungal endophytes are known to produce cell wall degrading enzymes including cellulases, which were demonstrated by substrate utilization studies (Carroll and Petrini 1983, Kumaresan et al. 2002). The present study also shows that mangrove endophytes produce cellulases by utilizing various substrates containing cellulose. Also if the endophytes are capable of producing extracellular enzymes (White et al. 1991), it is highly likely that the endophytes can also act as wood degrading fungi. In the present investigation *Aspergillus* sp. (NIOCC 24) isolated from *Avicennia marina* and *Phialophora* sp.



(NIOCC 27) from *Rhizophora mucronata* were also present as wood litter fungi as NIOCC 52 and NIOCC 49 from wood litters of *Avicennia marina* and *Rhizophora mucronata* respectively collected from same locations (Table 1 and 2). The endophytes have been reported to produce laccase that are known to be involved in litter degradation (Kumaresan et al. 2002) and studies on wood litter fungi (Luo et al. 2005) have shown xylanolytic and cellulolytic activity on lignocellulosic substrata, which are important in the recycling of lignocellulose in the marine environment.

The cellulases of *Chaetomium* sp. (NIOCC 36) showed variation in activity depending upon the cultural conditions including shake and static culture, pH, moisture, temperature and utilization of different substrates under SMF and SSF (Figs. 2a-d and 3a-d).  $\beta$ -glucosidase secretion levels were found high when compared with that of the other two enzymes under SMF condition (Fig. 3a-3d). Similar results were observed by Soni et al. (1999) with *Chaetomium erraticum*. On the other hand, in some fungi  $\beta$ -glucosidase has been reported to be predominantly cell-free (Rapp et al. 1981). All three enzymes ( $\beta$ - endoglucanase,  $\beta$ - exoglucanase,  $\beta$ - glucosidase) showed very low level activity at higher pH of 10 and 12. On the other hand, maximum secretion of these three enzymes was under SSF with cotton seed as substrate at pH 10 (Fig. 2b). The similar results of variations among the secretion levels of cellulase enzymes under different conditions of SSF and SMF was observed with different fungi (Soni et al. 1999).

The cellulase components were characterized as endoglucanase and exoglucanase on behavior of their action upon carboxymethylcellulose and cotton (Eriksen and Goksyr, 1977). Commonly, a number of such enzymes are found secreted by fungi, varying between 2 in *Trichoderma viride* and 5 in *Sporotrichum pulverulentum* (Eriksen and Goksyr, 1977). All of these were separate proteins with different specific activities, molecular weights and isoelectric points. In *Myrothecium verrucaria*, the number of cellulases has been reported to vary between 1 to as many as 24 (Eriksen and Goksyr, 1977). The electrophoretic studies with extracellular fractions revealed two forms each of endoglucanase, EG I, EG II/  $\beta$ - glucosidase,  $\beta$ -Glu I,  $\beta$ -Glu II under SMF and SSF, which exhibited a variation in the intensities of the bands corresponding to the enzyme activity in the filtrates (Fig. 5). A similar pattern of cellulase isozymes was also observed in previous studies carried out with *Chaetomium fusiporale* (Sandhu and Puri, 1989) and *Chaetomium erraticum* (Soni et al. 1999).

In the present study all the three cellulase components were found to be active at optimum temperature of 50°C (Fig.4c, 4d) and while optimum pH ranged between 5.0 and 12 (Fig.4a, 4b). However, in different *Chaetomium* species temperature between 50–60°C and acidic pH (4.0–5.0) have been reported to be optimal for enzyme activity (Lakshmikant and Mathur, 1990). In *Neurospora crassa*, the optimum pH has been reported to be 6.0 for  $\beta$ -glucosidase and endoglucanase activity and 7.0 for exoglucanase (Yazadi et al. 1990). In several other fungi, especially *Trichoderma* and *Aspergillus* sp., pH optima are variable but in acidic (3.4–6.3) range (Soni et al. 1999).

In conclusion, the results of this study demonstrate that the *Chaetomium* sp., found alkaline tolerant up to pH 12, and its ability to use agricultural and industrial wastes as carbon sources would be economical during commercial production of cellulase. The high activity and stability of cellulase enzymes between neutral to alkaline pH and high temperature will be of use in various industrial and biotechnological applications.

### **Acknowledgements**

Authors are grateful to Director, National institute of Oceanography, Goa, India for providing the lab facilities and Project leader, OLP 0001, for his support. CR greatly thanks Prof. D. J. Bhat, H.O.D, Mycology, Department of Botany, Goa University, Goa, India, for identification of fungi and Dr. (Mrs) Wafar, retired scientist, NIO, for the identification of Mangrove plants and Dasu B. Gauns for assistance in collection of samples and media preparation.

## References

- Baraznenoka, V.A., E.G. Beckera, N.V. Ankudimovaa and N.N. Okunevb. 1999. Characterization of neutral xylanases from *Chaetomium cellulolyticum* and their biobleaching effect on eucalyptus pulp. *Enzyme and Microbial Technology* 25: 651–659
- Carmichael, J.W., W.B. Kendrick, I.L. Connors and L. Sigler. 1990. Genera of Hyphomycetes. The University of Alberta Press: Alberta, Canada
- Dusterhoft, E-M., V.A.J.M. Linssen, A.G.J. Voragen and G. Beldman. 1997. Purification, characterization, and properties of two xylanases from *Humicola insolens*. *Enzyme Microb Technol.* 20 (6): 437–45.
- Ellis, M.B. 1971. Dematiaceous Hyphomycetes. UK, Wallingford, CAB International.
- Eriksen, J. and J. Goksqyr. 1977. Cellulases from *Chaetomium thermophile* var. *dissitum*. *Eur. J. Biochem.* 77: 445-450
- Kumaresan, V., T.S. Suryanarayanan and J.A. Johnson. 2002. Ecology of Mangrove endophytes. In; Fungi of marine environments (ed. K.D. Hyde), *Fungal Diversity Research series* 7: 145 – 166.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.
- Lakshmikanth, K and S.N. Mathur. 1990. Cellulolytic activities of *Chaetomium globosum* on different substrates. *World J. Microbiol. Biotechnol.* 6: 23–26.
- Luo, W., L.P. Lilian Vrijmoed and E.B. Gareth Jones. 2005. Screening of marine fungi for lignocellulose-degrading enzyme activities. *Bot. Mar.* 38: 379–386.
- Lin, Y., X. Wu., S. Feng., G. Jiang., J. Luo., S. Zhou., L.L.P. Vrijmoed., E.B.G. Jones., K. Krohn., K. Steingröver and F. Zsila. 2001. Five unique compounds: xyloketales from mangrove fungus *Xylaria* sp. from the South China Sea coast. *Journal of Organic Chemistry* 66: 6252 - 6256.
- Patel, R. N., A.C. Grabski and T.W. Jeffries. 1993. Chromophore release from kraft pulp by purified *Streptomyces roseiscleroticus* xylanases. *Appl Microbiol Biotechnol.* 39: 405–12.
- Petrini, O. 1991. *Fungal endophytes of tree leave*. In: J.A. Andrews, S.S. Hirano (Eds) Microbial ecology of leaves. Springer-Verlag, New York, 179 – 197.

- Pointing, S.B and K.D. Hyde .2000. Lignocellose-degrading marine fungi. *Biofouling* 15: 221 - 229.
- Popp, M., F. Larher., and P. Weigel. 1984. Chemical composition of Australian mangroves. III. Free amino acids, total methylated onium compounds and total nitrogen. – *Z. Pflanzenphysiol. 114S*: 15–25.
- Raghukumar, S., S. Sharma., C. Raghukumar., V. Sathe-Pathak and D. Chandramohan. 1994. Thraustochytrid and fungal component of marine detritus. IV. Laboratory studies on decomposition of leaves of the mangrove *Rhizophora apiculata* Blume. *J. Exp. Mar. Biol. Ecol.* 183: 113 – 131.
- Ratanakhanokchai, K., K.L. Kyu and M. Tanticharoen. 1999. Purification and properties of a xylan-binding endoxylanase from alkaliphilic *Bacillus* sp. strain K-1. *Appl. Environ. Microbiol.* 65: 694–697.
- Sandhu, D.K and R. Puri. 1989. Expression of  $\beta$  -glucosidase and endo- $\beta$ -1,4-glucanase during development of *Chaetomium fusisporale* and their characterization. *J. Basic Microbiol.* 29: 519–526.
- Soni, R., D.K. Sandhub and S.K. Soni. 1999. Localisation and optimisation of cellulase production in *Chaetomium erraticum*. *Journal of Biotechnology* 73: 43–51.
- Teather, R.M and P.J. Wood. 1982. Use of Congo red-polysaccharide interactions in enumeration and characterisation of cellulolytic bacteria from the bovine rumen. *Appl Environ Microbiol* 43: 777 – 780.
- Vogel, H.J. 1956. A convenient growth medium for *Neurospora* (Medium N). *Microb. Gen. Bull.* 13: 42–43.
- White, I.F and G. Morgan-Jones. 1991. Substrate utilization in selected *Acremonium*, *Atkinsonella* and *Balansia* species. *Mycologia* 83: 601-610.
- Wilson, D and G.C. Carroll. 1994. An endophyte of Oregon white oak: infection studies of *Discula quercina*. *Mycologia* 86: 635 – 647.
- Yazadi, M.T., A. Radford., J.N. Keen and J.R. Woodward. 1990. Cellulase production by *Neurospora crassa*: Purification and characterization of cellulolytic enzymes. *Enzyme Microb. Technol.* 12: 120–123

## Figure captions

Fig. 1: Optimization of growth medium for *Chaetomium* sp using different media, MEA-

Malt extract agar, PDA-Potato dextrose agar, CDA-Czapekdox agar, CMA-Corn Meal agar. The results are mean  $\pm$  S.D. of three parallel measurements.

Fig. 2a-d: Production of exoglucanase, endoglucanase and  $\beta$ -glucosidase by *Chaetomium*

sp. under SSF (solid state fermentation) using different substrates at different conditions. 2a. Rice bran with pH 12 showed the maximum level of  $\beta$ - endoglucanase secretion. 2b. The conditions pH 10 and pH 12 of Cotton seed showed maximum secretion levels of all three  $\beta$ -exoglucanase,  $\beta$ -endoglucanase and  $\beta$ -glucosidase 2c.  $\beta$ - exoglucanase was found maximum with Sugarcane bagasse pH10. 2d.  $\beta$ -glucosidase secretion levels were found maximum with pH 12 paper. Data represent mean  $\pm$  S.D. (n=3).

Fig. 3a-d: Production of exoglucanase, endoglucanase and  $\beta$ -glucosidase by *Chaetomium*

sp. under SMF (submerged fermentation) using different substrates at different conditions. 3a.  $\beta$ - glucosidase found maximum with 1% CMC shake, and  $\beta$ - endoglucanase and  $\beta$ -exoglucanase was maximum with 2% CMC shake. 3b.  $\beta$ -glucosidase found maximum with whatman shaking. 3c.  $\beta$ -glucosidase was found maximum with rice bran shake. 3d.  $\beta$ -glucosidase was found maximum with cotton seed shake. Data represent mean  $\pm$  S.D. (n=3). CMC-Carboxymethyl cellulase

Fig. 4a-d: Analysis of cellulase activity and stability of *Chaetomium* sp., at different

temperature and pH for the experimental conditions which showed the maximum enzyme secretion levels. 4a. Alkaline cellulases ( $\beta$ - endoglucanase,  $\beta$ - exoglucanase,  $\beta$ - glucosidase) from cotton substrate SSF tested under different pH. 4b. Non alkaline cellulases ( $\beta$ -endoglucanase,  $\beta$ - exoglucanase,  $\beta$ - glucosidase) from 2% CMC shake SMF tested under different pH. 4c Alkaline cellulases ( $\beta$ - endoglucanase,  $\beta$ - exoglucanase,  $\beta$ - glucosidase) from cotton substrate SSF tested under different temperature. 4d. Non alkaline cellulases ( $\beta$ -endoglucanase,  $\beta$ - exoglucanase,  $\beta$ - glucosidase) from 2% CMC shake SMF tested under different temperature. Data represent mean  $\pm$  S.D. (n=3).

Fig. 5: Zymograms showing isozymes of cellulase enzymes. 1. Crude protein of 2%

CMC shaking of SMF, 2. Crude extract of alkaline cotton seeds of SSF

Table 1: Plate assay of alkaline cellulase activity of mangrove endophytic fungi

Isolate	Fungus	Cellulase (pH 9.7)
NIOCC 1	<i>Cladosporium cladosporioides</i> (Fries) de Vries	—
NIOCC 2	<i>Aureobasidium pullulans</i> (De Bary) Arnaud	—
NIOCC 3	Hyphomycetes 1	—
NIOCC 4	<i>Cladosporium</i> sp. 1	—
NIOCC 5	Hyphomycetes 2	—
NIOCC 6	<i>phoma</i> sp.	—
NIOCC 7	Hyphomycetes 3	++
NIOCC 8	<i>Chrysosporium</i> sp.	—
NIOCC 9	<i>Periconia</i> sp.	—
NIOCC 10	Sterile form 1	—
NIOCC 11	Hyphomycetes 4	—
NIOCC 12	<i>Sporormia</i> sp.	—
NIOCC 13	Sterile form 2	—
NIOCC 14	Sterile form 3	—
NIOCC 15	<i>Cladosporium</i> sp. 2	—
NIOCC 16	Hyphomycetes 5	—
NIOCC 17	<i>Acremonium</i> sp.	—
NIOCC 18	<i>Nodulisporium</i> sp.	—
NIOCC 19	<i>Aspergillus</i> sp.	—
NIOCC 20	<i>Penicillium</i> sp.	—
NIOCC 21	<i>Curvularia</i> sp.	—
NIOCC 22	<i>Aspergillus</i> sp. 2	++
NIOCC 23	Sterile form 4	+++
NIOCC 24	<i>Aspergillus</i> sp. 3	—
NIOCC 25	<i>Alternaria</i> sp.	—
NIOCC 26	Sterile form 5	—
NIOCC 27	<i>Phialophora</i> sp.	—

Table 2: Plate assay of alkaline cellulase activity of mangrove wood litter fungi.

Isolate	Fungus	Cellulase (pH 9.7)
NIOCC 28	<i>Chrysosporium</i> sp.1	—
NIOCC 29	Hyphomycetes 1	—
NIOCC 30	<i>Cirrenalia</i> sp.	—
NIOCC 31	<i>Chrysosporium merdarium</i> (Link : Fries) Carmichael	—
NIOCC 32	<i>Chrysosporium</i> sp.2	—
NIOCC 33	Hyphomycetes 2	—
NIOCC 34	Sterile form 1	—
NIOCC 35	Sterile form 2	—
NIOCC 36	<i>Chaetomium</i> sp.	+++
NIOCC 37	<i>Sporormia</i> sp.	—
NIOCC 38	<i>Lulworthia</i> sp. 1	—
NIOCC 39	<i>Corollospora</i> sp.	—
NIOCC 40	<i>Phialophorophoma litoralis</i> Linder	—
NIOCC 41	Hyphomycetes 3	—
NIOCC 42	<i>Trichocladium</i> sp. 1	—
NIOCC 43	<i>Trichocladium</i> sp. 2	—
NIOCC 44	Sterile form 3	++
NIOCC 45	<i>Penicillium</i> sp. 1	—
NIOCC 46	<i>Pencillium</i> sp 2	—
NIOCC 47	<i>Alternaria</i> sp.	—
NIOCC 48	<i>Halocyphina villosa</i> Kohlm. et E. Kohlm	—
NIOCC 49	<i>Phialophora</i> sp. 1	—
NIOCC 50	Hyphomycetes 4	—
NIOCC 51	<i>Phialophora</i> sp. 2	—
NIOCC 52	<i>Aspergillus</i> sp. 1	+
NIOCC 53	<i>Aspergillus</i> sp. 2	+++
NIOCC 54	<i>Trichoderma</i> sp.	—

**Figure 1**

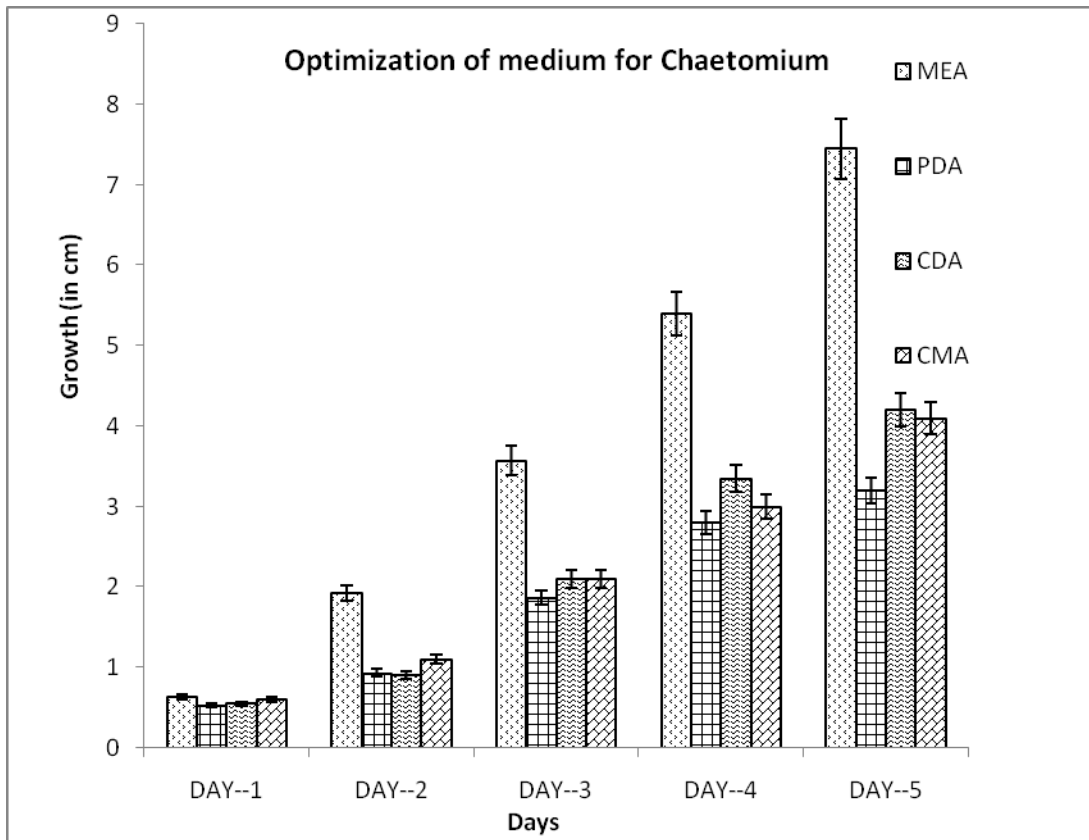




Figure 2a

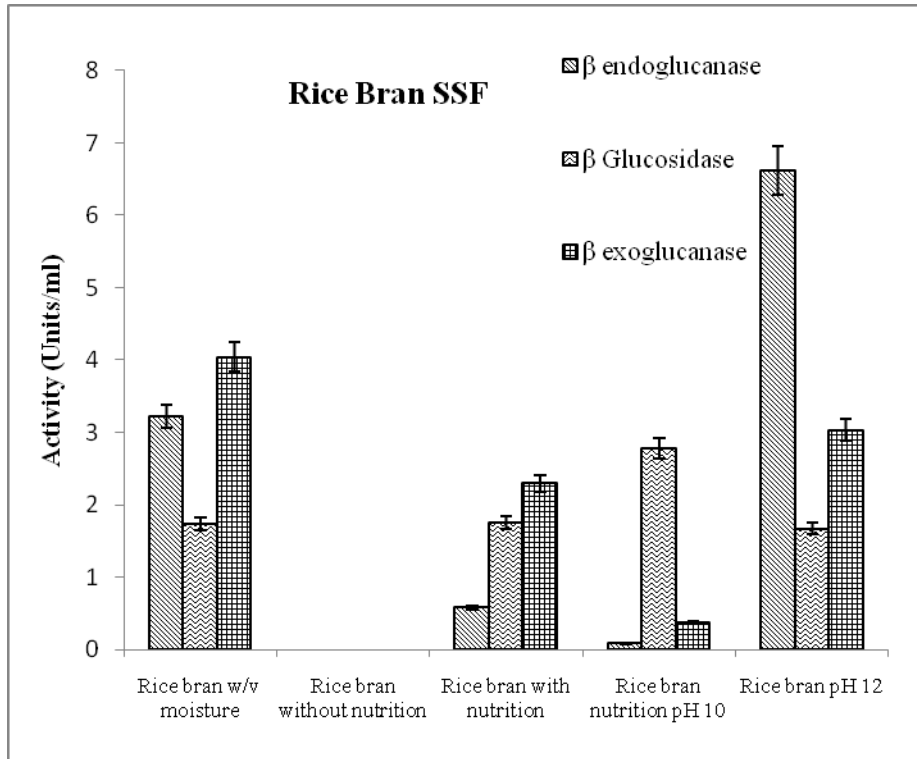


Figure 2b

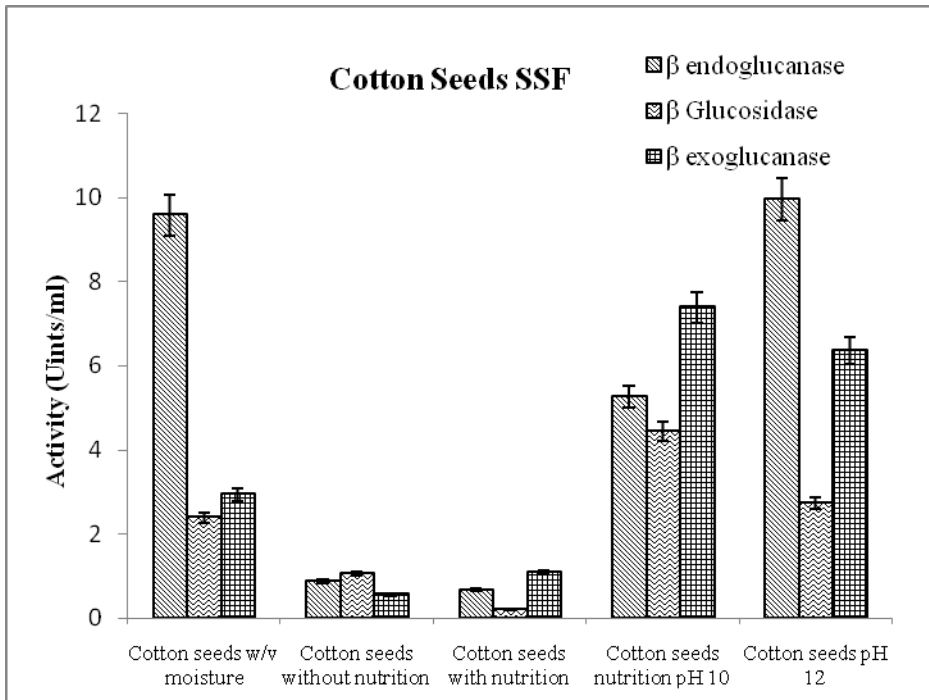


Figure 2c

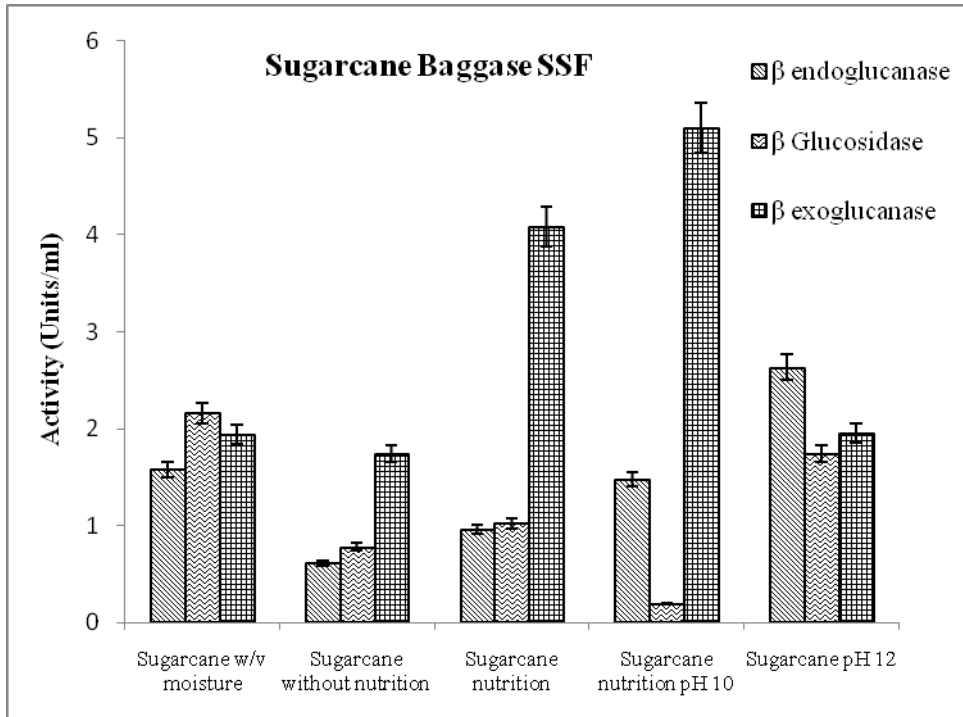


Figure 2d

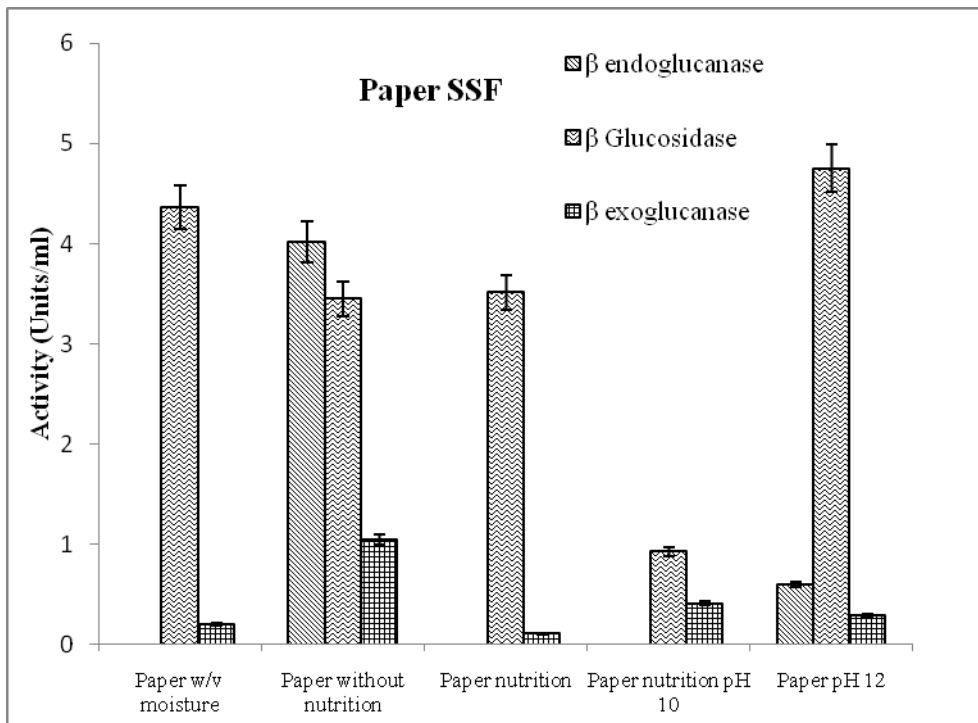


Figure 3a

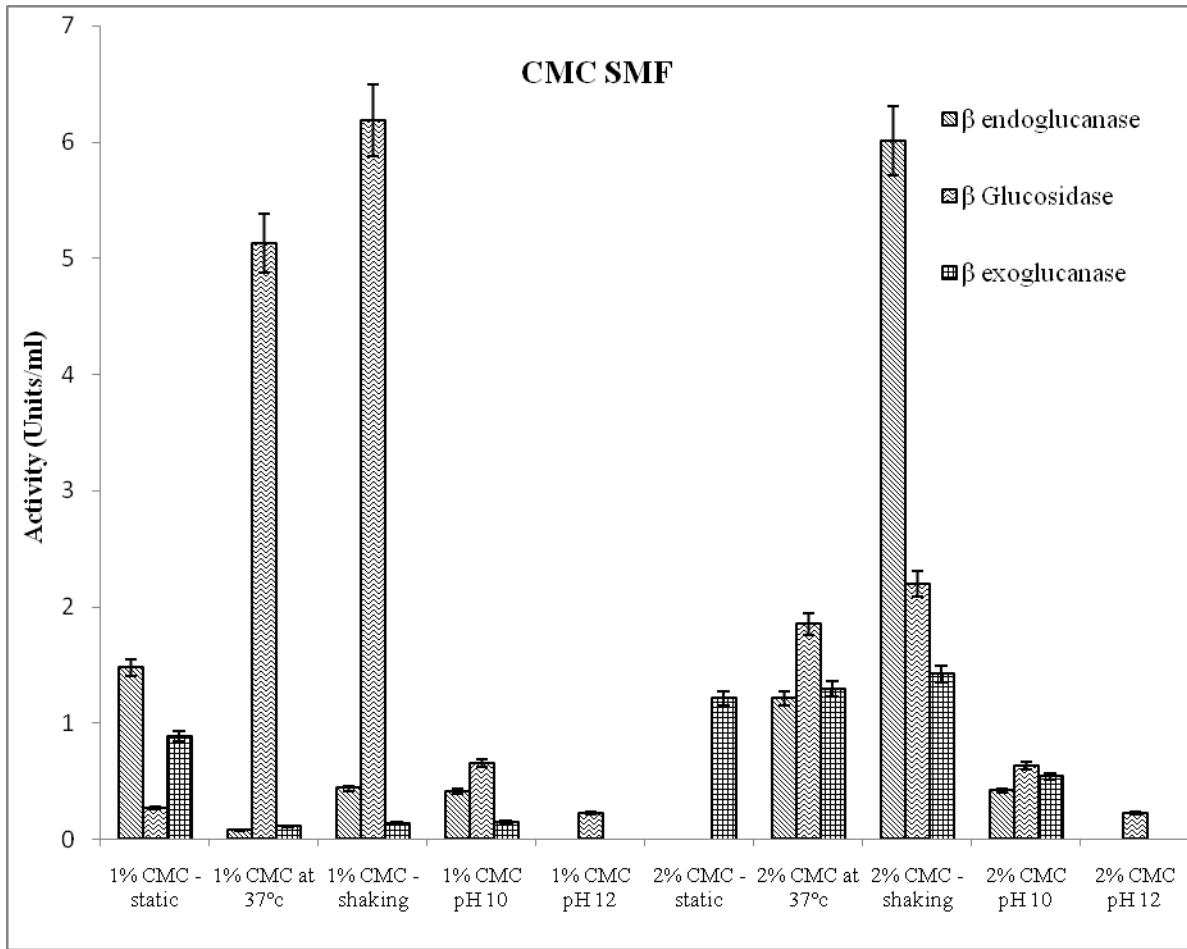


Figure 3b

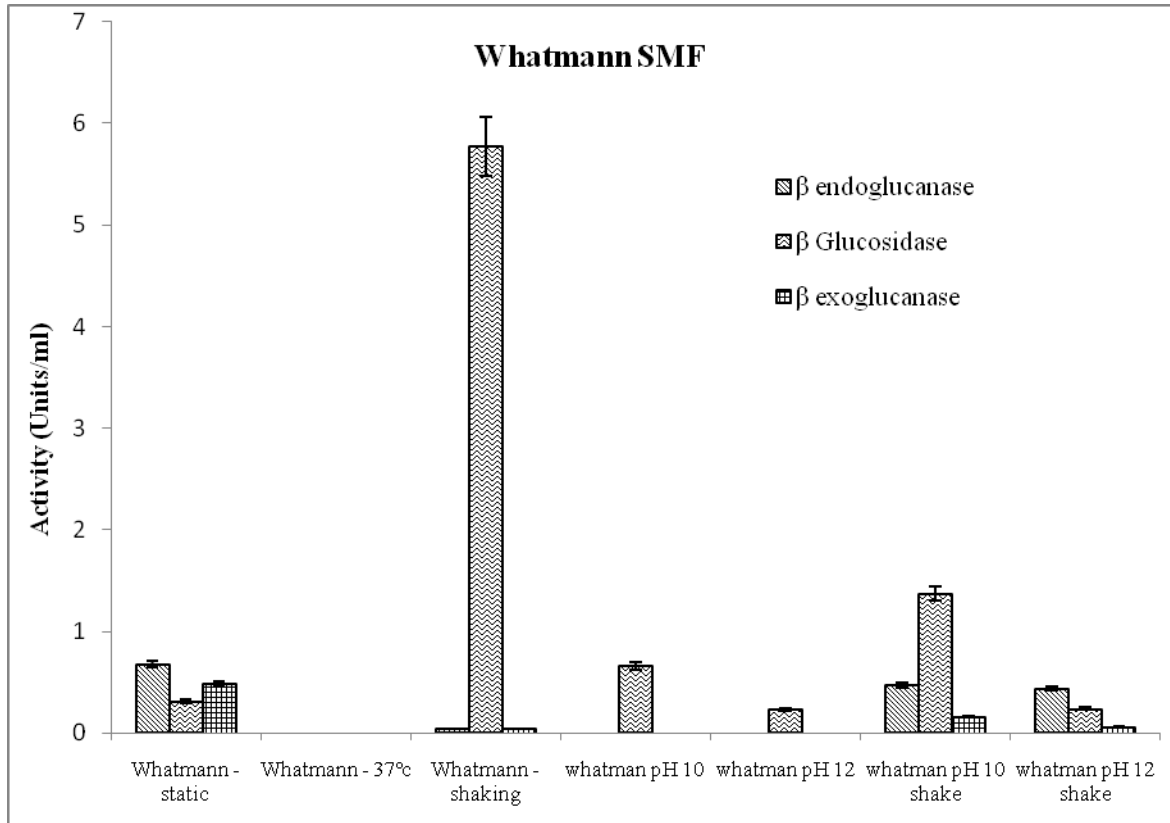


Figure 3c

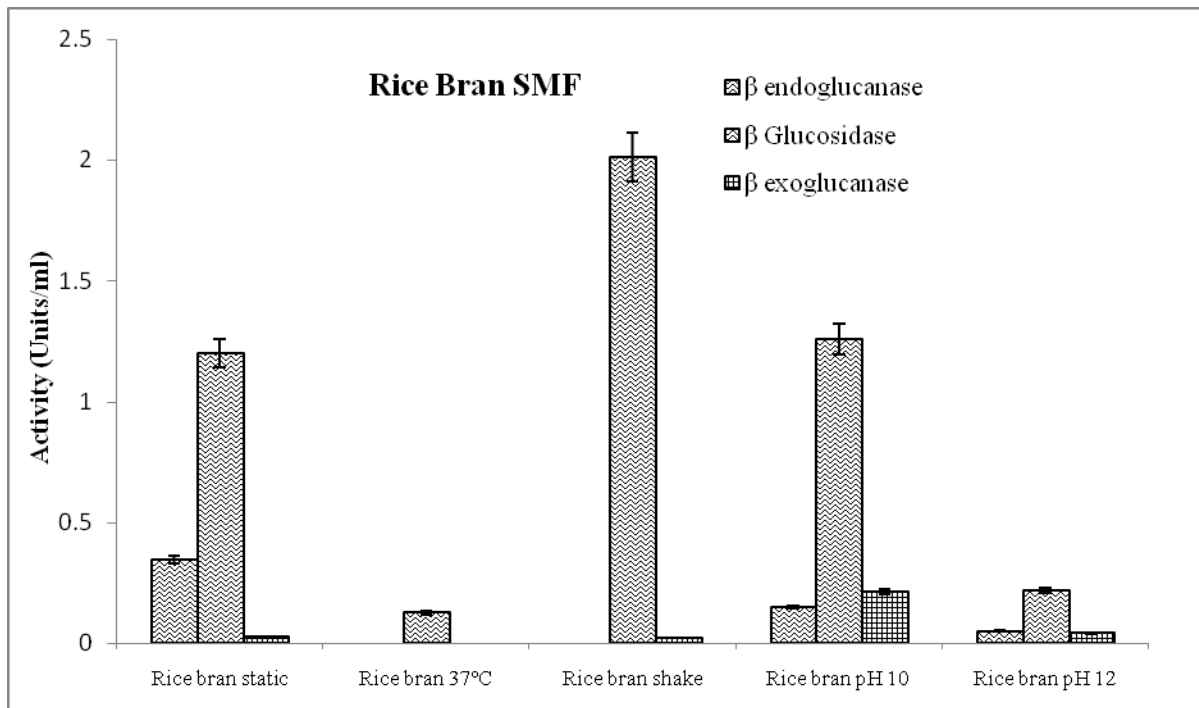


Figure 3d

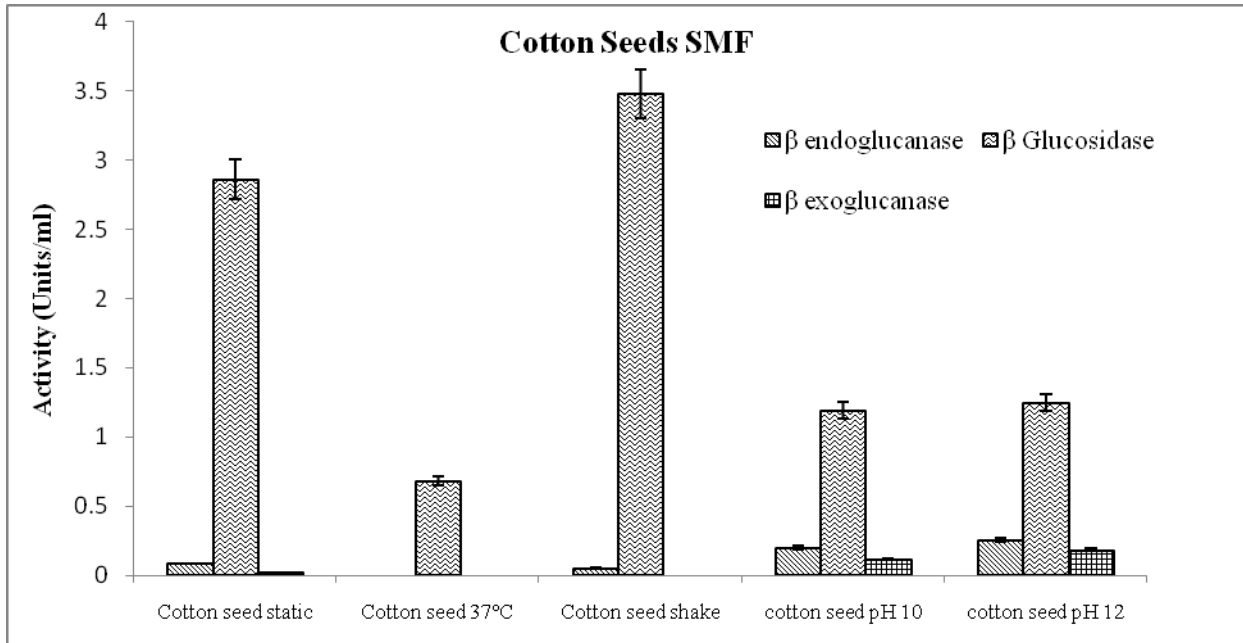


Fig. 4a

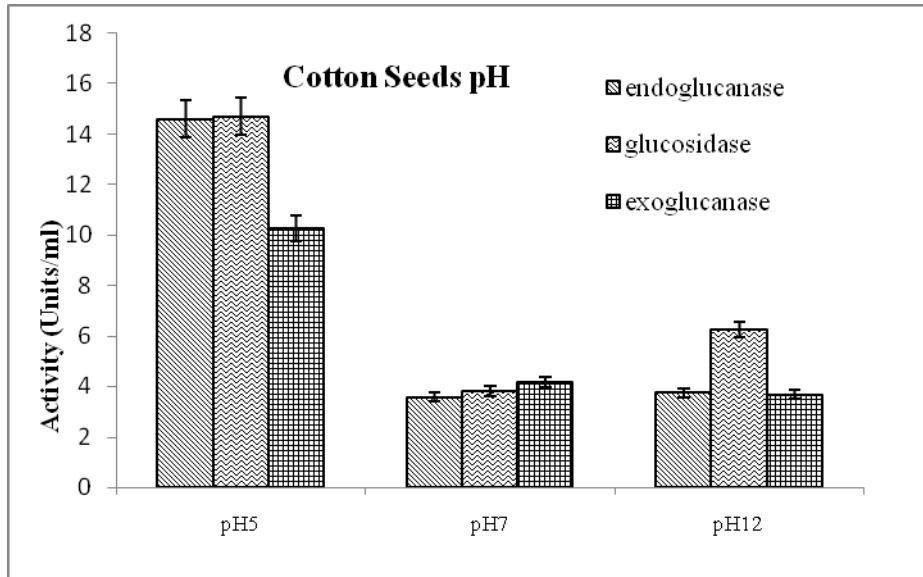


Fig. 4b

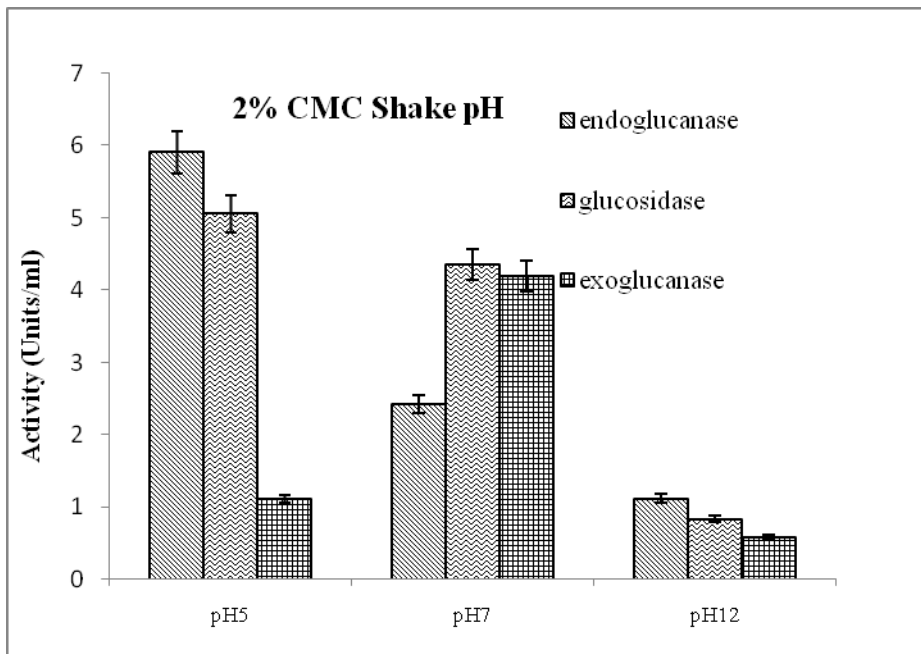


Fig. 4c

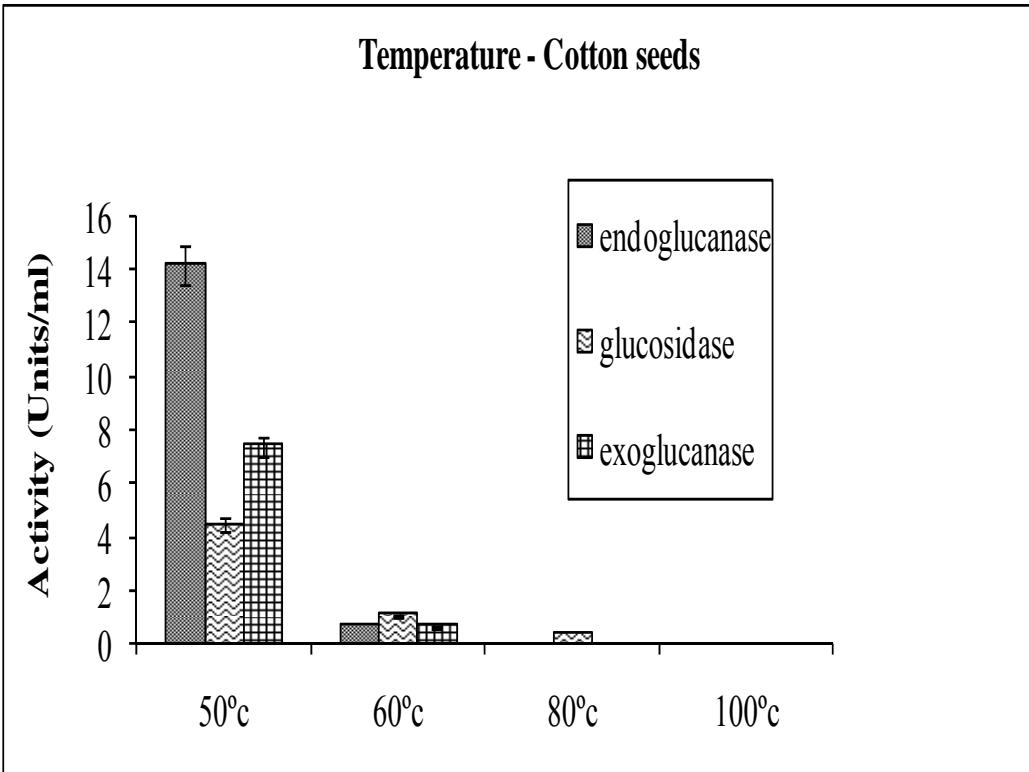


Fig. 4d

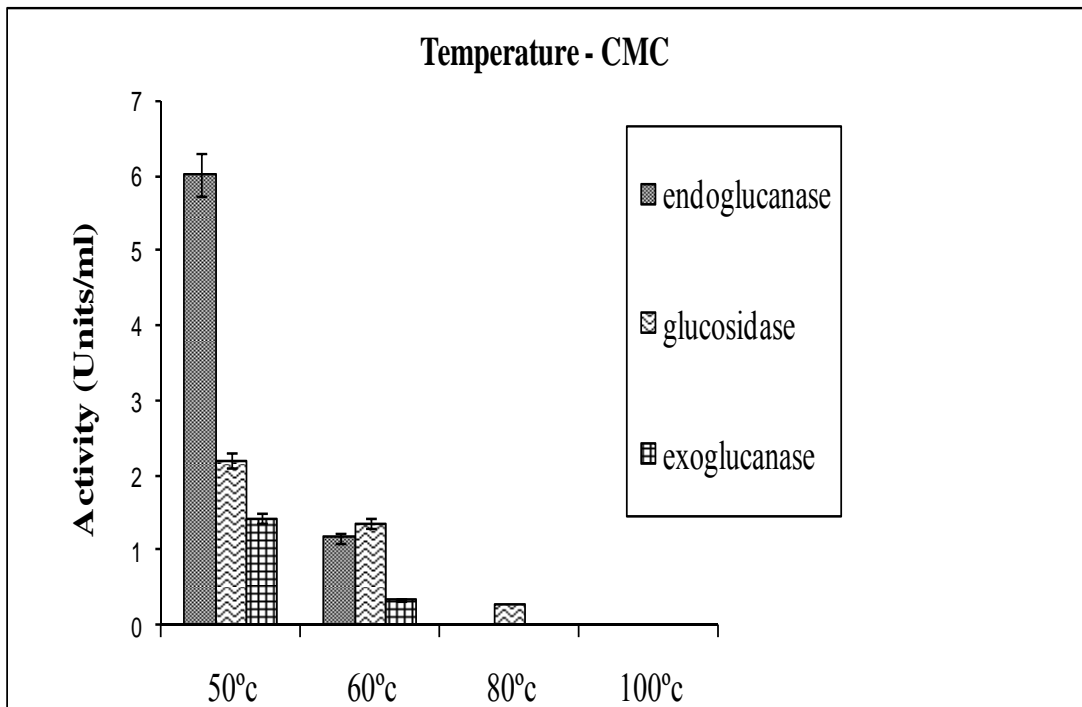


Figure 5

