

PRODUCTION OF A THERMOSTABLE B-GLUCOSIDASE BY A MESOPHILIC FUNGUS ASPERGILLUS NIGER NIAB 280

Muhammad Javed Iqbal, M.I. Rajoka and Kauser A. Malik

National Institute For Biotechnology and Genetic Engineering, P.O.Box. 577, Faisalabad,
Pakistan.

ABSTRACT

Aspergillus niger NIAB 280 produced extracellular B-glucosidase when grown on different lignocellulosic substrates. Wheat bran was found to be the best inducer of this enzyme. Maximum enzyme production was found at initial pH 5. When the organism was grown on easily available carbohydrates, the enzyme was induced only to a basic level essential for microbial growth. The optimum pH for enzyme activity was found to be 3 whereas optimum temperature was 55°C. The enzyme retained 100% activity when exposed to a temperature of 50°C for 5 days. When this enzyme was added to FPase from *T. reesei* and celluclast (a commercial cellulase from Novozyme), the %age saccharification as well as glucose production from lignocellulosic biomass was doubled.

INTRODUCTION

The enzymic saccharification of cellulosic materials require the synergistic activity of three enzymes namely endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and B-glucosidase (EC 3.2.1.21) (Wood, 1985, Wood and McCrae, 1982). B-glucosidase catalyses the hydrolysis of cellobiose to glucose which in turn can maximize the saccharification rate by alleviating the inhibition of cellobiohydrolase by cellobiose (Mandel, 1985. Ryu et al 1982). Due to the crucial role it plays in enzymatic saccharification of lignocellulose to monomeric sugars, cellulases produced by *T. reesei* need addition of exogenous B-glucosidase to improve the hydrolysis rate. Supplemental B-glucosidase or cellobiase from *Aspergillus phoenicus* (or other sources) has been found to cause favourable effect on cellulose hydrolysis (Ryu et al 1982, Gilbert and Tsao 1983). When cellulases are more concentrated there will be a greater chance of faster hydrolysis. Several species of *Aspergillus* produce B-glucosidase in large quantities (Gokhale et al 1984, Gokhale et al 1988, Scrivastava et al 1981). Thermal inactivation of B-glucosidase constitutes one of the major barriers to the realization of enzymic saccharification of cellulosic materials as a commercial process (Woodward and Weisman 1982). Traditionally thermostable enzymes are produced by thermophilic microorganisms. Some thermophilic fungi reported to be good cellulase producers are *Thielavia terrestris*, *Sporotrichum thermophile*, *Talaromyces emersonii* and *Aspergillus fumigatus* (Grajek, 1987). The optimum temperature for cellulolytic activities from mesophilic fungi is reported to be 40-50°C (Margritis and Merchant, 1984). In this work, a mesophilic strain of *A. niger* is reported from which B-glucosidase shows temp. optimum of 55°C, pH optimum of 3 and thermostability comparable to the B-glucosidase from thermophilic organisms. Moreover the addition of this B-glucosidase to the FPase enhances the saccharification of lignocellulosic materials.

MATERIALS AND METHODS

Microorganisms:

Aspergillus niger NIAB 280 was isolated from rice growing areas of Punjab, Pakistan (Malik and Rajoka 1973) and the role in decomposition of organic matters in saline soil has been reported (Malik and Sandhu 1973). The organism was maintained at Vogel's medium with Sigmacell 100 as carbon source. The basal medium had the following composition (g/l). Trisodium citrate 2.5, KH_2PO_4 5.0, NH_4NO_3 2.0, $(\text{NH}_4)_2\text{SO}_4$ 4.0, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2, peptone 2.0, tween 80, 1.0, the initial pH was adjusted to 5.5. $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1 prepared and autoclaved separately and added to the medium prior to inoculation.

Chemicals:

Para-nitrophenol B-D-glucopyranoside (pNPG), cellobiose, carboxymethyl cellulase Na-salt (CMC) and sigmacell 100 were purchased from sigma, USA. All other chemicals were of analytical grade. @H2 = Substrate Preparation:

The lignocellulosic (LC) substrates were ground in an electric grinder to pass a 55 mesh sieve. The powdered material was moistened with 2% alkali at a ratio of 1:6 i.e. w/v and autoclaved for 15 minutes. The alkali was removed and pH was neutralized (Rajoka and Malik 1986) with repeated washing.

Enzyme Preparation:

Solid state fermentation experiments were carried out in 250 ml Erlenmeyer flasks containing 10 grams of substrate which included x-cellulose, *Leptochloa fusca* L. Kunth (Kallar grass, salt tolerant plant raised on saline waste lands in Pakistan), *Panicum maximum*, wheat straw or wheat bran. To each flask 30 ml Vogel's medium was added. The flasks were inoculated with spore suspension prepared by scratching the spores from the culture slant in physiological saline (1 ml per flask) and incubated at 30°C. Samples were removed after twelve days at which maximum enzyme activities appeared. Enzymes were extracted with 50 ml chilled acetate buffer of pH 1-7 containing 2% tween 80. The contents were shaken for 1 hour on a gyratory shaker, centrifuged to remove mycelia and undigested substrate. The supernatant after centrifugation was analysed for enzymatic activities.

Enzyme Assays:

Endo-glucanase (CMC-ase) exo-glucanase (FP-ase) and B-glucosidase activities were determined as described by Rajoka and Malik (1986), using CMC, filter papers and pNPG. 55°C temperature and pH 3 were found optimum for B-glucosidase. Cellobiase activity was determined by incubating 0.2 ml cellobiose (10 mM), 0.2 ml acetate buffer and 0.2 ml appropriately diluted enzyme preparation. The glucose released was measured by glucose peroxidase test (Wood and McCare 1982).

Saccharification of LC Substrates:

Pretreated LC substrates were incubated with cellulase at ratio of 1:15 i.e. 1 gm of substrate and 15 IU of enzymes as FP-ase and B-glucosidase at 50°C. Total reducing sugars and glucose were tested by DNS and peroxidase test after various time intervals.

RESULTS AND DISCUSSION

In order to see the inductive effect of substrates, solid state fermentation studies were conducted. Time course studies indicated that maximum enzyme activities appeared after 12 days. Table 1 indicate the production of FP-ase and B-glucosidase measured either on pNPG or cellobiose. Wheat bran alone and in combination with Kallar grass induced B-glucosidase and cellobiase more in quantity than wheat straw, Kallar grass, *P. maximum*, a-cellulase or bagasse. When the organism was grown on easily available carbohydrates as glucose, lactose, maltose, starch, esculin, salicin and cellobiose (Fig. 1), the enzyme was induced to only a basic level essential for microbial growth. Maximum enzyme production was found to be at initial pH 5 (Fig.2).

Table 1: Production of cellulases from *Aspergillus niger* NIAB 280 after grown on different substrates in solid state fermentation.

Substrate	FP-ase	B-glucosidase	Cellobiase
	-----IU/ml-----		
a-cellulose	2.5	2.25	7.0
Bagass	1.2	1.42	10.0
Kallar grass	1.5	3.08	18.0
<i>Panicum maximum</i>	3.0	2.30	20.0
Wheat straw	2.1	3.60	Not determined
Wheat bran	1.6	7.50	36.0
Kallar grass + Wheat bran	2.5	7.35	32.0

* Cellulases were produced by growing the cells in solid state fermentation using vogel's medium at 30°C.

B-glucosidase production ability of *A. niger* NIAB 280 was compared with other strains of *A. niger* with respect to production of B-glucosidase when measured on cellobiose or pNPG as enzyme substrate (Table 2). The test strain produced more cellobiase as B-glucosidase in quantity than that produced by other strains reported in literature. Both B-glucosidase and cellobiase showed pH. Optima of 3 (Fig. 3) and temperature optima of 55°C (Fig. 4).

The thermal stability of B-glucosidase of *A. niger* NIAB 280 has been measured (Table 3). After an exposure of 120 hours at 50°C, the enzyme retained 100% of its activity and had also been compared with thermophilic fungi (Table 4). The enzyme was found more thermostable than one from thermophilic strains. The saccharification ability of cellulases from *A. niger* was studied and compared with other cellulases. When alkali treated Kallar grass was saccharified by various enzyme combinations (Fig. 5 and 6), cellulases from *A. niger* alone did more

saccharification and produced more glucose as compared to cellulases from other sources. When the B-glucosidase from *A. niger* was added to celluclast and *T. reesei* cellulases (which do not contain B-glucosidase), the percentage saccharification was doubled (Fig. 5,6).

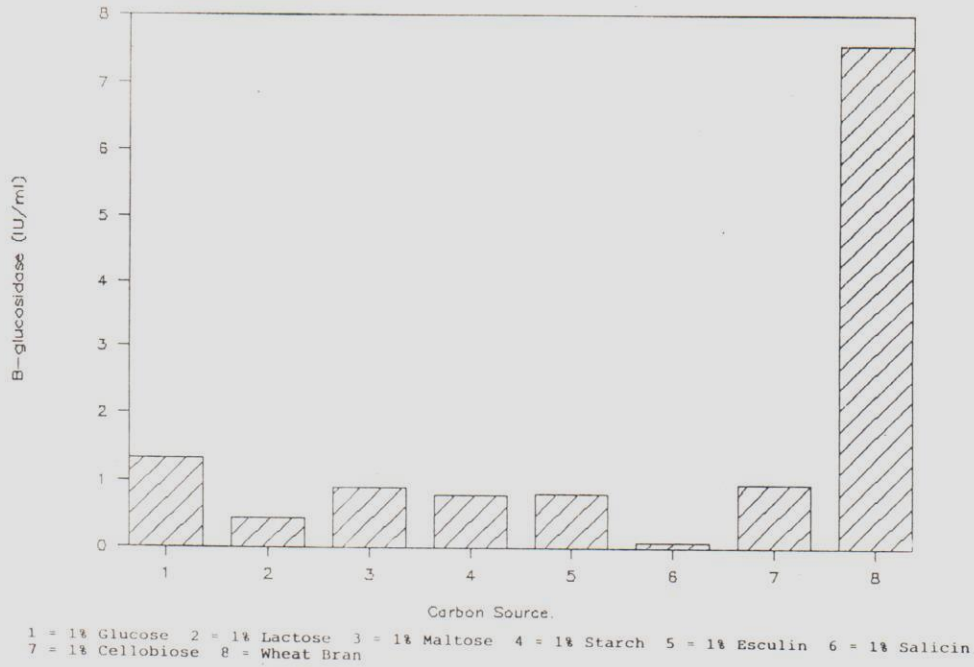


Fig. 1. Effect of different sugars on the production of β -glucosidase by *A. niger*.

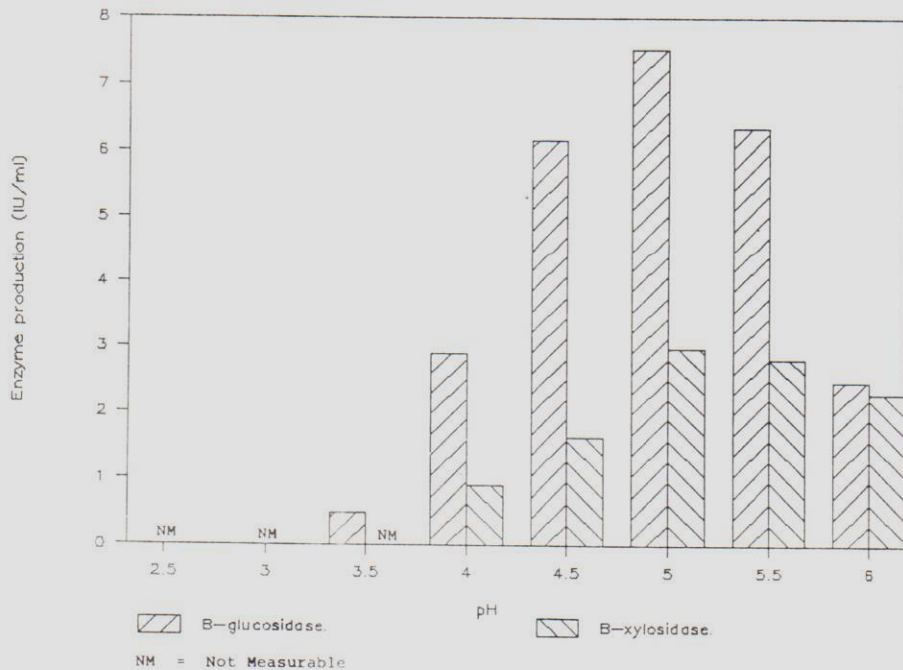


Fig. 2. Effect of pH medium on β -glucosidase and β -xylosidase production by *A. niger*.

Table 2: Comparison of B-glucosidase production by *Aspergillus niger*

Organism	Assay	B-glucosidase (U/ml)	Reference
<i>A. niger</i> QM877	Cellobiose 50°C, pH 4.8	7.4	Sternberg et al (1977)
<i>Trichoderma</i> sp & <i>Aspergillus</i> sp.	PNPG	1.6	Duff et al (1987)
<i>A. niger</i> A98 Cimerman (1986).	PNPG, 50°C pH 4.0	0.03	Gunde-Cimerman &
<i>A. niger</i> MUB 33	"	0.40	Ibid
<i>A. niger</i> NCIM 1207	PNPG, 65°C, pH 4.5	5.0	Gokhale et al (1988)
- do -	Cellobiose, 65°C pH 4.5	22.0	Gokhale et al (1984)
<i>A. niger</i> UV 10	- do -	9.8	Ibid
<i>A. niger</i> NIAB 280	PNPG, 55°C, pH 3.0	7.5	This work
- do -	Cellobiose, 55°C, pH 3.0	36.0	This work

Table 3: Thermostability of B-glucosidase from *A. niger* NAIB 280.

Storage Temp. °C	Storage Time (Hours)	Enzyme stability relative to the initial activity (%)
45	120	100
50	120	100
60	0.25	94
60	20	17
60	120	3.8
70	0.25	74
75	0.25	45
80	0.25	28

The fermentation mash was incubated at indicated temperature and time and the residual enzyme activity was determined as described in Materials and Methods.

CONCLUSION

Thermal inactivation of B-glucosidase forms one of the major barriers to the exploitation of enzymic saccharification of LC agricultural residues for production of high value product. The thermal stability of this enzyme from a mesophilic strain of *A. niger* NIAB 280 is a novel character much appreciable than the trait of this product from thermophilic organisms.

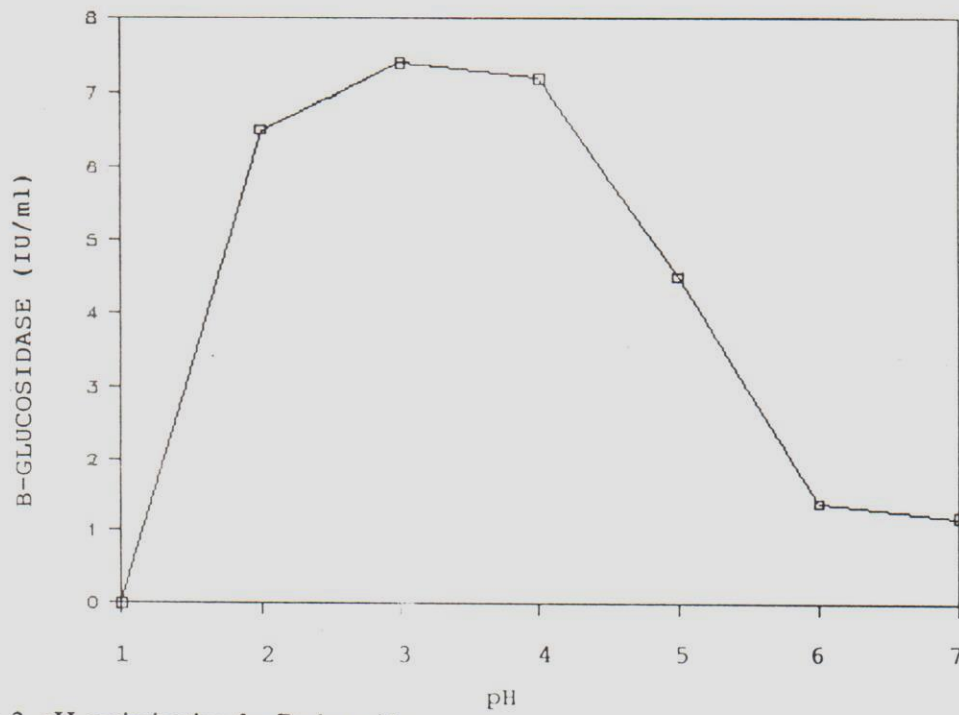


Fig. 3. pH optimization for β -glucosidase assay.

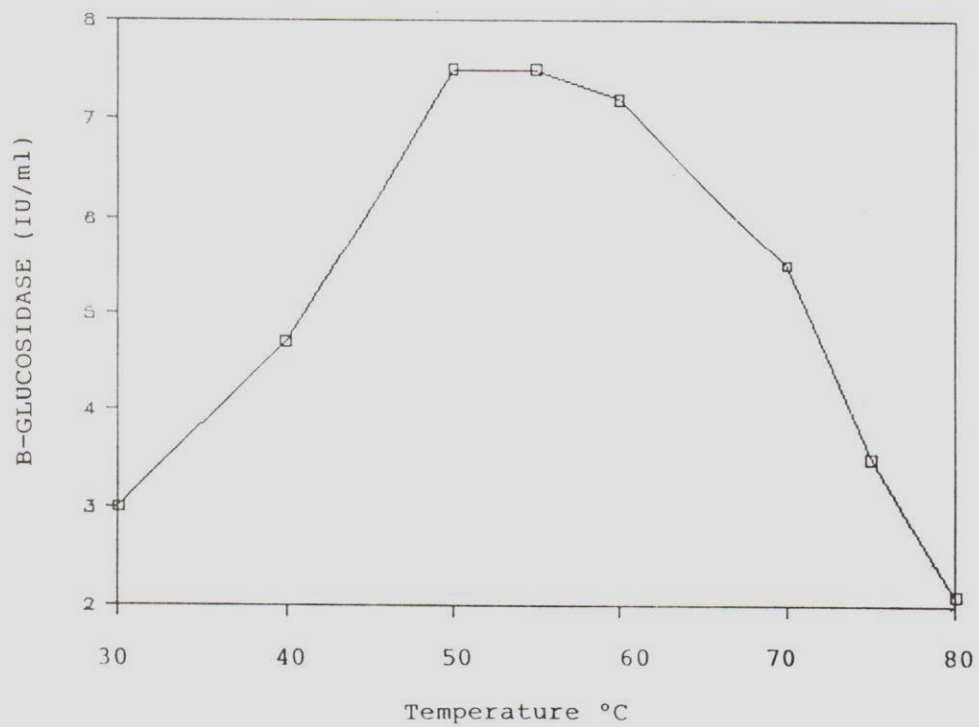


Fig. 4. Temperature optimization for β -glucosidase assay.

%age saccharification

Fig. 5. Effi
alka
Sac
Na-

Glucose mg/ml

Fig. 6. C
cc

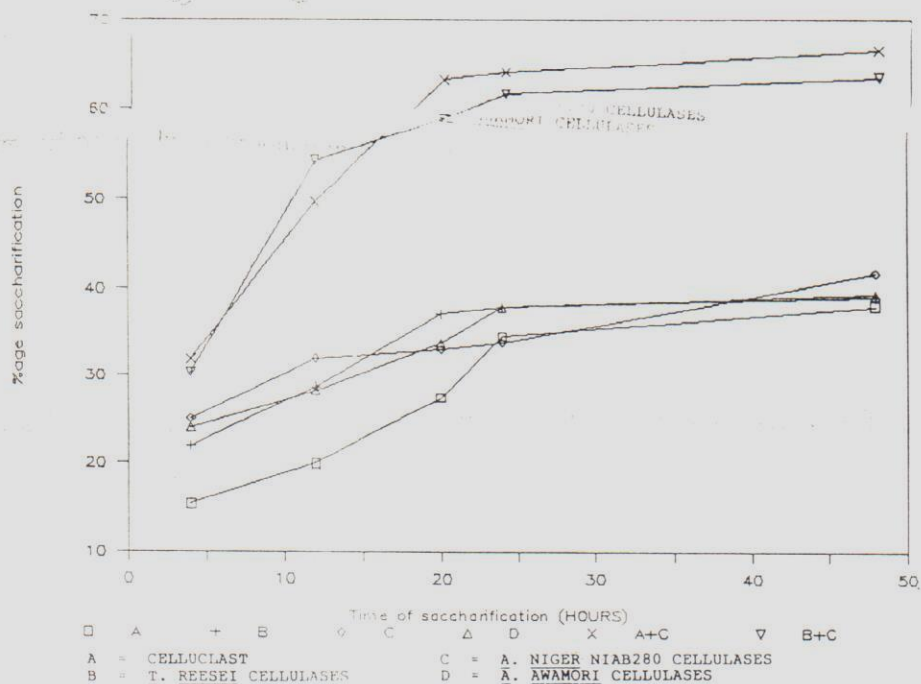


Fig. 5. Effect of addition of β -glucosidase from *A. niger* on the saccharification of alkali treated Kallar grass. 15 FPase Unites/g substrate were used. Saccharification Temp. was 50°C. Substrate (2%) was suspended in 0.05M Na-acetate buffer pH 5.0.

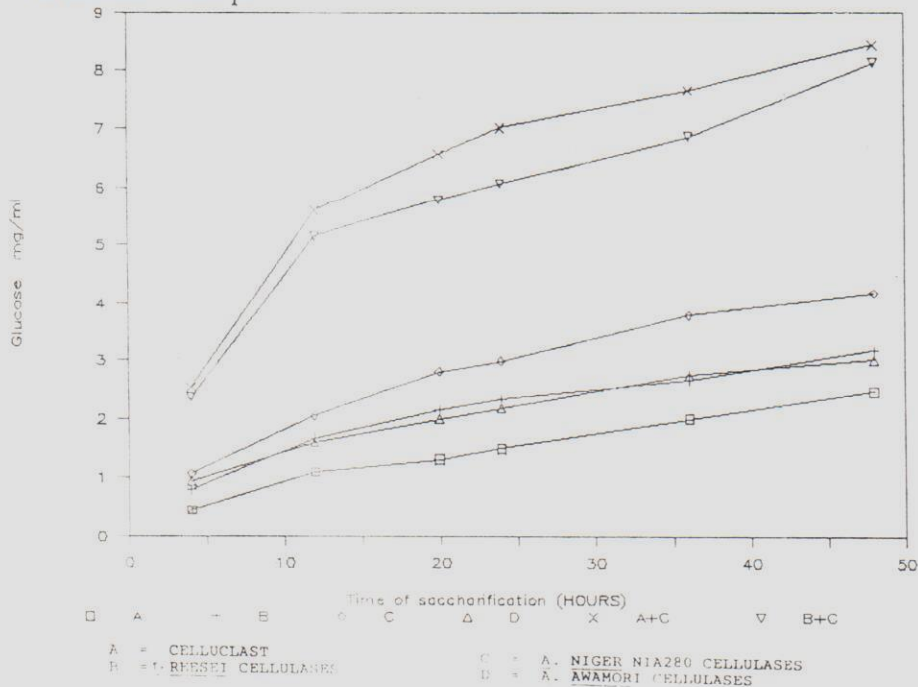


Fig. 6. Comparison of glucose production by various enzyme combinations. Other conditions were same as those in Fig.5.

Table 4: Thermal stability characteristics of B-glucosidase of various organisms (expressed as %age of original).

Organism	Relative B-gluco- sidase %	Exposure Time (Hours)	Exposure Temp °C	Reference
<u>Schizophyllum</u> <u>commune</u>	10	1	50	Desrochers et al (1981)
<u>Sporitrichum</u> <u>thermophile</u>	93	48	50	Margritis & Creese (1981)
<u>Chaetomium</u> <u>thermophile</u>	70	1	50	Luis and Becker (1973)
<u>Thielavia</u> <u>terrestris</u>	98	20	50	Margritis & Merchant (1984)
<u>Aspergillus</u> <u>niger</u> NIAB 280	100	120	50	This work

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REFERENCES

- Duff SJB, Copper DG and Fuller OM 1987. Effect of media composition and growth conditions on production of cellulases and B-glucosidase by a mixed fungal fermentation. *Enzyme Microbial Technology*. 9: 47-52.
- Gilbert, T and Tsao G 1983 *Annu. Rep. Fermentation Processes*. 6: 323-358.
- Gokhale DV, Puntambekar US Yas AK Patil SG and Deobagkar DN 1984. Hyper production of B-glucosidase by an *Aspergillus* sp. *Biotechnology Letters*. 6: 419-423.
- Gokhale DV, Puntambekar US Deobagkar DN and Peberdy, J.F. (1988) Production of cellulolytic enzyme by mutants of *Aspergillus niger* NCIM 1207. *Enzyme and Microbial Technology*. 10: 442-445.
- Grajek W, 1987. Hyperproduction of thermostable B- glucosidase by *Sporotrichum* (*Chrysosporium*) *thermophile*. *Enzyme Microbial Technology*. 9: 744-748.
- Gunde-Cimerman N and Cimerman A 1986. *Aspergillus niger* mutants for bioconversion of apple distillery wastes. *Enzyme Microbial Technology*. 8: 166-170.
- Luis AJ and Becker RA 1973 *Biochem Biophys* 125: 765.

- Malik KA and Rajoki MI 1973. Cellulolytic soil mycoflora of rice growing areas of Punjab. *Biologia* 19: 109-117.
- Malik KA and Sandhu GR 1975. Decomposition of organic matter in saline soil by fungi. *Mycopathologia et Mycologia Applicata*. 50 : 339-357.
- Mandels M, 1985. Application of cellulases. *Biochemical Society Transactions*. 13: 414-416.
- Margritis A and Merchant R 1984. Production and thermostability characteristics of cellulase and xylanase enzymes of *Thielavia terrestris*. *Biotechnology Bioengineering* 14: 299-314.
- Margritis A and Creese E 1981. *Biotechnology Letters*. 3: 47.
- Ng TK and Zeikus JG 1981. Comparison of extracellular cellulase activities of *Clostridium thermocellum* LQR1 and *Trichoderma reesei* QM9414 *Applied and Environment Microbiology*. 42: 231-240.
- Rajoka MI and Malik KA 1986. Comparison of different strains of *Cellulomonas* for production of cellulolytic and xylanolytic enzymes from biomass produced on saline lands. *Biotechnology Letters*. 8: 753-756.
- Ryu DDY, Lee S Tassinari T and Macy C 1982. Effect of compression milling on cellulose structure and on enzymatic hydrolysis kinetics. *Biotechnology Bioengineering*. 24: 1047-1067.
- Scrivastava SK, Ramachandran KB and Gopalkrishnan KS 1981. *Biotechnology Letters*. 3: 477-480.
- Sternberg DP, Vijakumar P and Reese ET 1977. B- glucosidase: microbial production and effect on enzymatic hydrolysis of cellulose. *Canadian Journal of Microbiology*. 23: 139-147.
- Wood TM 1985. Properties of cellulolytic enzyme systems. *Biochemical Society Transactions*. 407-410.
- Wood TM, and McCrae SI 1982. *Carbohydrate Research*. 57: 117-133.
- Woodward J, and Wiseman A 1982. Fungal and other B- glucosidases - Their properties and application. *Enzyme Microbial Technology*. 4: 73-79.