Regulation of Endo-β-D- Xylanase and β- Xylosidase Synthesis in *Humicola lanuginosa*

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Summary: *Humicola lanuginosa* produced endo-β-xylanase and β-xylosidase when grown on different monosaccharides, disaccharides, commercial cellulose, cellulosic residues, xylan and carboxymethyl cellulose (CMC) in shaken cultures. Among mono-saccharides, only xylose induced the cells to produce both enzymes. The organism synthesized endo-xylanase and β-xylosidase maximally on xylan followed by wheat bran. Among nitrogen sources, corn steep liquor (CSL) supported the highest yields of endo-β-xylanase and β-xylosidase (1200 and 250 IU g^{-1} substrate respectively), which were 1.6- and 3.5- fold enhancement over the activities achieved on basal Vogel's medium. In the presence of CSL, even mono-saccharides acted as inducers of both enzymes. This implied that synthesis of enzymes was regulated by both substrate and nitrogen sources. Additional of vitamin mixture did not enhance enzymes production. Optimal production of enzymes was observed over a temperature range of 45-50 °C. These studies revealed that the thermophilic mould required less activation energy, enthalpy and entropy of activation for endogenous metabolism during growth and product formation compared with those of mesophilic and thermo-tolerant organisms reported in literature.

Introduction

Renewable natural resources such as xylans are abundant in many agricultural wastes [1]. Fungi and bacteria producing high β-xylanase and β-xlyosidase activities have attracted considerable attraction because of potential applications in biobleaching, reduction of ecological harmful effluents from bleaching plants, food and animal feed [2]. Pre-bleaching with cellulase-less xylanases namely endo-β-xylanases (EC 3.2.1.8) and β-D-xylosidases (EC 3.2.1.37) facilitates the chemical extractability of lignin from crude pulp by hydrolysis of lignin-carbohydrate complexes, thus, leading to significant savings of chemicals conventionally consumed for bleaching [1, 3]. The production of xylanases is influenced by induction and catabolite repression, which alters transcription with CreA protein, a transcriptional repressor of genes involved in metabolic processes other than glucose [4-6]. To reach commercial viability, enzyme production must be increased by induced mutations, gene cloning or introducing more potent strains and selecting most effective substrate. In Pakistan, many cellulosic residues namely sugarcane bagasse, wheat straw, wheat bran and corn cobs are produced to as much as 50 million tons every year [7] and could be utilized for bulk production of cellulase-less xylanases using thermophilic moulds.

Thermophiles represent an obvious source of thermo-stable enzymes, it being reasonable to assume that such character will confer on their proteins a high thermal stability [8]. Enzymes isolated from these microorganisms are not only thermo-stable, but are also often resistant to and active in the presence of organic solvents and detergents [8-11]. The technological use of thermophiles still faces several challenges, since knowledge on the diversity, genetics and physiology of such organisms is generally poor [11]. Thermopiles or the genes derived from them are, however, still the preferred source for thermo-stable enzymes. Thermophilic strains of *H. lanuginosa* secrete large amounts of cellulase-less thermo-stable xylanases and have been applied for bio-bleaching of pulp [1, 9, 12]. These characteristics prompted us to study regulation of their synthesis by carbon and

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nitrogen sources and fermentation temperature to understand the mechanism of their endogenous synthesis and to maximize the production of endo-xylanase and β-xylosidase.

Results and Discussion

Time Course Production of Xylanases and Carbon Source Utilization

Enzyme production and substrate utilization from one representative substrate namely, xylose in shaken cultures is presented in Fig. 1. This figure and others (not presented) revealed that optimal production of xylanase and β-xylosidase was reached within 120 h while other thermo-philic fungi produced maximum activity after 140 h of fermentation [10, 12]. These curves also indicated that production of both xylanases was apparently growth-associated. Extensive screening of potential inducers of xylanases using un-induced (glucose grown) cells indicated that a basal level of xylanases under all growth conditions essential for cellular metabolism was induced by mono-saccharides. Xylan and lingocellulosic substrates caused enhancement of xylanases and β-xylosidase synthesis. It has been observed that during growth on such polymeric carbon sources, high amount of mRNA for a particular enzyme is produced [13]. Normally, easily metabolizable carbon sources support high specific growth rate (μ) and cause catabolic repression (Table-1) due to formation of CreA protein [4] as reported by other workers [5]. During growth of the organism on different cellulosic and lignocellulosic substrates, reducing sugars accumulated slowly in the growth medium (Table-1) as unmetabolized principles and induced xylanases [13]. As their release from these substrates is slow and their final quantity in the fermentation mash was different and permitted larger variation on synthesis of xylanases (Table-1). It appeared that β-xylosidase and xylanase synthesis varied under an induction mechanism that enhanced the product formation rate of β-xylosidase and xylanase up to several-fold in induced over non-induced cultures and growth dependent repression mechanism which suppressed synthesis of these enzyme on mono-saccharides.

Effect of Nitrogen Sources

Among the various nitrogen sources (ammonium nitrate, ammonium sulfate, sodium glutamate, sodium nitrate, urea and corn steep liquor)

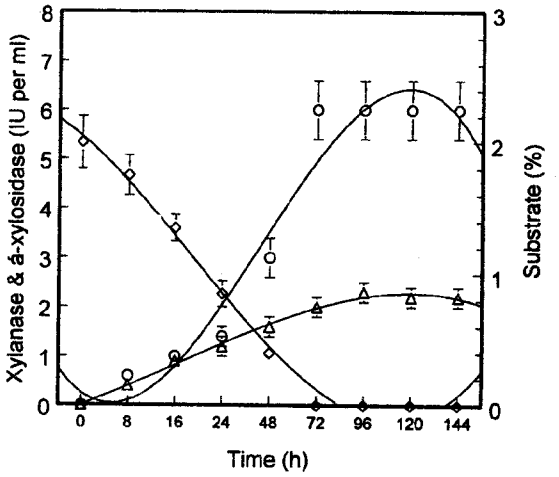

Fig. 1: Kinetics of endo-xylanase and β-xylosidase production in shaken cultures of one representative substrate namely xylose using Vogel's basal medium. The initial pH of the medium, inoculum size and temperature were 6.5, 5 mL g^{-1} substrate and 45 °C respectively. (○)= Endo-xylanase, (Δ) = β-xylosidase and ◊ = substrate.

Table-1: Comparative yield ($Y_{P/S}$=IU IU g^{-1} substrate utilized) of endo-xylanase and β-xylosidase by *H. lanuginosa* following growth on different substrates in shaken culture studies at 45 °C in Vogel's basal salt medium.

C. Source	Yield of Endo-xylanase Yield (IU g^{-1})	β-Xylosidase Yield (IU g^{-1})	Specific growth rate (μ) and R.S μ (h^{-1})	R.S. ($\mu g\ ml^{-1}$)
Arabinose	35±1.0	6.5±0.20	0.21±0.01	0±0
Fructose	20±0.6	7.5±0.22	0.19±0.01	0±0
Galactose	20±0.7	7.5±0.23	0.21±0.01	0±0
Glucose	10±0.3	7.5±0.24	0.23±0.01	0±0
Xylose	150±4.5	50±1.5	0.21±0.01	100±3.0
Lactose	148±4.2	21±0.63	0.21±0.01	100±3.0
Maltose	130±3.9	13±0.40	0.21± 0.01	150±4.5
Sucrose	100±3.0	15±0.45	0.23±0.01	100±3.0
α-Cellulose	22±0.67	50±1.50	0.21±0.01	300±8.5
Bagasse	220±6.0	25±0.80	0.23±0.01	400±11.0
Corn cobs	268±8.0	46±1.38	0.21±0.01	350±10.0
CMC	160±4.8	10±0.30	0.18± 0.01	800±22.0
Wheat bran	750±22.0	82±2.46	0.23±0.01	400±12.0
Wheat straw	335±9.6	55±1.65	0.21±0.01	250±7.0
Xylan	850±25.0	85±2.60	0.23±0.01	150±4.6

Each value is a mean of three replicates. ± Stands for standard deviation among replicates R.S = reducing sugars. In all experiments, initial pH of the medium and inoculum size was 6.5 and 10 % (v/v).

added at equimolar concentration to medium containing xylan (2 % w/v), CSL favoured maximum endo-xylanase and β-xylosidase production, followed by sodium glutamate in the absence of pH control

(results not presented). Urea (0.25 % w/v) favoured maximum pectinase production in *Streptomyces* sp. RCK-SC [14]. When *H. lanuginosa* was cultured on different substrates in the presence (Fig. 2) of corn steep liquor in Vogel's medium containing mono-saccharides, disaccharides, sugarcane bagasse, corncobs, wheat bran and xylan, there was significant increase in enzyme yields and rate of product formation; even mono-saccharides induced cells to produce elevated level of both enzymes comparable to that on corncobs in basal medium (Table-2). Molecular mechanism of this unusual phenomenon is not known (and needs further study) but was distinctly different from previous findings. This may have occurred due to the up regulation of global nitrogen metabolism regulator, AreA [15] in corn steep liquor culture medium.

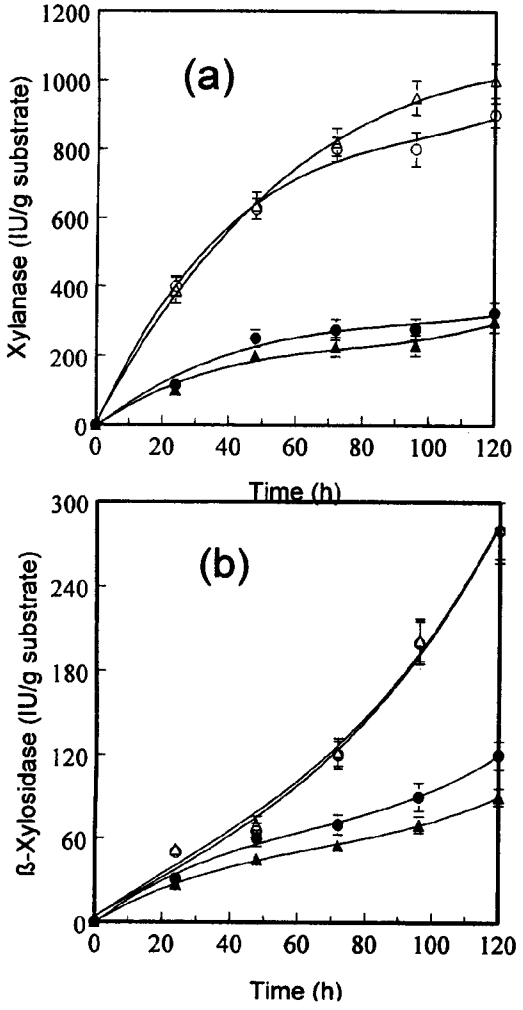

Fig. 2: Kinetics of production process in shaken culture fermentation of wheat bran (○), xylan (Δ), corn cobs (●) and sugarcane bagasse (▲) for synthesis of xylanase (Fig. 2a) and β-xylosidase (Fig. 2b) in the presence of corn steep liquor. The initial pH of the medium, inoculum size and temperature were 6.5, 5 ml g^{-1} substrate and 45 °C respectively.

Table-2: Comparative yield (Y= $Y_{P/S}$ = IU IU g^{-1}) and product formation rate (R = Q_P) of endo-xylanase and β-xylosidase by *H. lanuginosa* following growth on different substrates in shaken cultures at 45 °C in Vogel's salt medium fortified with corn steep liquor (replacing nitrogen salts in basal medium)

	Yield and product formation rate of Endo-xylanases		β-Xylosidase	
C. Source	Y (IU g^{-1})	R (IU l^{-1} h^{-1})	Y (IU g^{-1})	R (IU l^{-1} h^{-1})
Arabinose	220±8.8	140±4.2	90±2.7	35±1.0
Fructose	175±5.3	120±3.6	45±1.3	30±1.0
Galactose	160±4.8	148±4.4	35±1.0	37±1.0
Glucose	125±3.7	74±2.1	40±1.2	19±0.6
Xylose	280±8.4	125±3.6	145±4.3	32±0.9
Bagasse	470±14.0	244±7.3	119±3.5	62±1.6
Lactose	194±5.8	115±3.4	45±1.3	30±0.9
Maltose	180±5.4	105±3.1	35±1.0	26±0.8
Sucrose	260±7/8	229±6.8	28±0.8	52±1.5
α-Cellulose	550±16.0	254±7.6	200±6.0	80±2.4
Corn cobs	900±27.0	580±16.0	200±6.0	150±4.5
CMC	450±13.0	240±7.4	175±5.2	60±1.8
Wheat bran	1650±45.0	860±24.0	380±10.0	215±6.5
Wheat straw	1375±40.0	800±23.6	300±9.0	200±6.0
Xylan	1726±50.2	1040±30.0	400±12.0	270±5.1

Each value is a mean of three replicates. ± Stands for standard deviation among replicates. Y= Product yield and R= Volumetric rate of product formation.

In all experiments, initial pH of the medium was 6.5 and all media were inoculated with 10 % (v/v) inoculum. Ammonium nitrate and ammonium sulphate were replaced by corn steep liquor containing 1.2 g l^{-1} nitrogen (8.3 ml CSL 100 mL^{-1} medium).

After extensive screening and optimization of the process variables namely particle size (0.5 mm), inoculum concentration (10 %), pH of Vogel's medium (6.5), effect of temperature (45°C), substrate and addition of nitrogen sources, experiments were conducted under optimal cultural conditions. The organism had strong propensity of substrate utilization and that xylan supported more values of product formation than those by other carbon sources (Table-2). Product yield and product formation rates of both endoxylanase and β-xylosidase of *H. lanuginosa* are significantly higher than the values reported for *E. coli* and *Saccharomyces cerevisiae*

recombinants harboring heterologous β-xylosidase gene [2, 16], *Thielavia terrestris* and *Thermoascus crustaceus* [8], *Thermoascus aurantiacus* (500 IU g^{-1} bagasse) [17] and *Humicola* sp.[9, 12]. However, *T. lanuginosus* [10] supported higher values of xylanase. Though the highest values of xylanase and β-xylosidase synthesis were obtained in culture medium containing xylan (Table-2), however, the cost precludes the use of wheat bran for industrial fermentation.

Effect of Temperature

The organism was capable of rapid fermentation at temperature up to 45 °C with significantly higher specific growth rate (Fig. 3a) better than those of other strains of *H. lanuginosa* grown at 45 °C [9, 12]. Maximum specific productivity of endo-xylanase and β-xylosidase occurred at fermentation temperature of 45-50°C (Fig. 3b). The optimum temperature was in good agreement to the values on many thermophilic fungi [1, 6, 10] and *Humicola* sp.[1, 9, 12]. Requirement of lower energy of activation (E_a) for growth and product formation, endpoint for inactivation of product may be considered potential indices for thermo-stability of cultures during production processes as they (parameters) are considered for thermo-stable enzymes [8, 11]. On wheat bran, the culture required lower activation energy for growth, which was lower than that required by other thermo-stable cells [18]. Midpoint inactivation temperatures were 55 and 62.5 °C for β-xylosidase and endo-xylanase respectively. These values are normally for those organisms whose endogenous metabolism is thermo-stable [18]. The values of activation enthalpy and entropy for product formation (results not presented) indicated that the activation enthalpy (33±4 k J mol^{-1}) and activation entropy of formation of xylanases (-306.5 $Jmol^{-1}$) is lower than those for mesophilic organism [19] and thermo-tolerant *Kluyveromyces marxianus* [20]. The enzyme preparation was used as bio-bleaching agent in Century Paper and Pulp Industry at the rate 4 mL per g pulp; the enzyme preparation improved brightness and reduced kappa number and may find practical application in bio-bleaching.

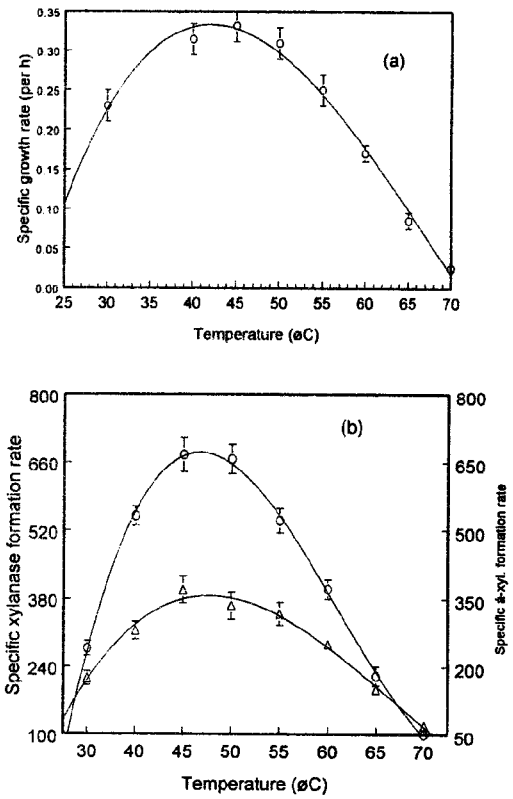

Fig. 3: Effect of fermentation temperature on specific growth rate (a) and specific product formation rate (IU g^{-1} cells h^{-1}) of endo-xylanase (○) and β-xylosidase, β-xyl (Δ) from wheat bran (b) and corn steep liquor medium.

Experimental

Microorganism

Strain of *H. lanuginosa* NIBGE F220 isolated from putrefied biomass in saline soil was maintained on potato-dextrose agar plates and slants [21]. For inoculum preparation, Vogel's medium (containing 0.5 % trisodium citrate, 0.2 % NH_4NO_3, 0.5 % KH_2PO_4, 4 % $(NH_4)_2$ SO_4, 0.02 % $MgSO_4.7H_2O$, 1 % glucose 0.1 % yeast extract) 100 ml (pH 5.5) HCl-washed glass beads (20 in number), was made up in 500 ml flasks. The flasks were inoculated with spores of *H. lanuginosa* and incubated overnight at 45 °C on a gyratory shaker (150 rpm). Non-induced and washed conidial suspensions (10 mL) at 3 g L^{-1} (at absorbance of 0.3x10) were used to inoculate the enzyme production medium. Two grams of solid substrates (Table-1) (100-500 μm particle size) or 2 g soluble substrate (used filter-sterilized) were added to Vogel's medium (pH 6.5) in 500 ml Erlenmeyer's flasks in triplicate. The whole contents were sterilized at 121°C for 30 min. After cooling to ambient temperature, the contents were inoculated with 10 ml of a cell suspension of the culture as

above. The inoculated flasks were shaken at 45 ± 2.0 °C for 120-144 h. After each 24 h, flasks (in triplicate) were harvested and whole contents were centrifuged (4000 x g for 10 min) and substrate free enzyme extract was used for following the enzyme activities. Substrate was washed thoroughly and dried at 95 °C to a constant weight. The moisture content was determined from the weight loss after drying the sample at 80 °C for 2 days.

Enzyme Assays

Endo 1, 4-β-D-xylanase activity was assayed according to Bailey *et al.* [22] by incubating the diluted enzyme solution (1 ml) at 60 °C in reciprocal shaker at 60 rpm for 5 min using a substrate solution (1 mL) of 1 % (w/v) crystalline oat-spelt xylan in 0.05 M acetate buffer, pH 5.0. After the incubation time, 3 mL of dinitro-salisylate reagent (DNS) was added and mixture was placed in boiling water bath for 5 min. A control was run parallel as above but the enzyme was added after addition of DNS reagent. Diluted enzyme from *Aspergillus oryzae* recombinant harboring xylanase gene from *Thermomyces lanuginosus* (Sigma-Aldrich chemicals, cat. #2753) was used as internal standard. The absorbance of the mixture in each test tube was taken with the help of spectrophotometer at 550 nm. A zero point absorbance was adjusted by blank containing 2 mL of distilled water and 3 mL of DNS reagent.

β-Xylosidase activity was determined using *p*-nitrophenyl β-D-xylopyranoside as substrate [7]. Commercial β-xylosidase (Sigma-Aldrich Chemicals, cat. X 3501) was used as positive control and autoclaved distilled water as negative control. One IU of endo-xylanase and β-xylosidase has been defined as the amount of enzyme that releases 1 μmol xylose or *para*-nitrophenol equivalents per ml per min.

Saccharide Determination

In these tests, reducing sugars were estimated calorimetrically with 3, 5-dinitrosalicylic acid according to Miller [23] using xylose as standards. Glucose was determined using Human (Germany) glucose kit following instructions of the suppliers. Solid material in the fermentation broth was determined gravimetrically. Cellulose and hemi-cellulose in the dry material were determined as described previously [21].

Mycelial Biomass

Biomass was determined indirectly from measurement of protein contents [24]. Equivalent amount of biomass from liquid culture on glucose was used to standardize the assay.

Determination of Kinetic Parameters

All kinetic parameters were determined as described previously [18]. Volumetric rate of production (Q_p) was determined from a plot between enzyme (IU l^{-1}) and time of fermentation, process product yield ($Y_{P/S}$) was determined from $dP.dS^{-1}$, specific product yield ($Y_{P/X}$, $IU.g^{-1}$ cells) was determined using relationship $dP.dX^{-1}$ and specific rate of product formation (q_P) was determined using $Y_{P/X} \times \mu$ (specific growth rate). Specific growth rate was determined as slope of a straight line between ln X (X = cell mass, $g.l^{-1}$) and time of fermentation (h) using relationship μ t = ln X/X_0, t = time of fermentation (h).

Conclusion

A strain of *H. lanuginosa*, which also produces β-glucosidase and other glycosidases [10] was also found to have clearly improved xylanase and β-xylosidase enzyme production. Presence of reducing sugars in the fermentation medium was essential for induction of xylanases (Table-1) as rapid uptake of mono-saccharides repressed enzyme synthesis as discussed earlier. That substrate that released sugars slowly and for longer period was a better inducer. The possibility of using locally available substrates (Table-2) for enzyme production was promising in that induction on wheat bran and wheat straw yielded xylanases to a level greater than 0.84^{th} and 0.75^{th} of that induced by xylan. Corn steep liquor was essential for rapid uptake of substrates and microbial activity. Low values of thermo-dynamical parameters indicated that the intrinsic metabolic network of the organism was like that of thermophilic organisms and is due to the presence of certain macromolecules which impart thermo-stability [8, 11, 18, 19]. The organism is prone to mutagenesis and will be more suitable organism for its application in futuristic bulk production of xylanases for paper and pulp industry in Pakistan.

References

1. S. Singh, A. M. Madlala and B. A. Prior, *FEMS Microbiol. Rev.*, **27**, 3 (2003).
2. J. A. Perez-Gonazalez, N. N. van Peij, A. Bezoen, A. P. MacCabe, D. Ramon and L. H. deGraff, *Appl. Environ. Microbiol.*, **64**, 1412 (1998).

3. I. Viikari, A. Kantelinen, J. Sundquist and M. Linko M, *FEMS Microbiol. Rev.*, **13**, 335 (1994).
4. A. Bohm and W. Boos, *Curr. Opin. Microbiol.*, *7*, 151 (2004).
5. M. J. de Groot, P. J. van de Vondervoort, R. P. de Vries , P. A. vanKuyk , G. J. Ruijter and J. Visser, *Microbiology*, **149**, 1183 (2003).
6. M. M. Gilbert, D. C. M, Yaguchi, K. K. Y. Watson, KY, C. W. Breuil and J. N. Saddler *Appl. Microbiol. Biotechnol.*, **40**, 508 (1993).
7. M. I. Rajoka, A. Bashir and K. A. Malik, *Bioresour. Technol.*, **62**, 99 (1997).
8. M. L. T. M. Polizeli, A. C. S. Rizzatti, R. Monti, H. F. Terenzi, J. A. Jorge and D. S. Amorim. *Appl. Microbiol. Biotechnol.*, **67**, 577 (2005).
9. L. Anand and P. J. Vithayathil, *J. Ferment. Bioeng.*, **81**, 511 (1996).
10. B. S. Chadha, K. Jaswinder, K. Rubinder, H. S. Saini and S. Singh, *World J. Microbiol. Biotechnol.*, **15**, 195 (1999).
11. D. A. Cowan, *Biochem Soc Symp.*, **58**, 149 (1992).
12. R. Da Silava., D. K. Yin and K. K Park, *J. Ferment. Bioeng.*, **77**, 109 (1994).
13. M. Hrmova, E. Petrakova and P. Biely, *J. Gen. Microbiol.*, **137**, 541 (1991).
14. R. C Kuhad, M. Kapoor and R. Rustagi, *World J. Microbiol. Biotechnol.*, **20**, 35 (2004).
15. R. P. de Vries, J. Visser and L. H de Graaff, *Res. Microbiol.*, **150**, 281(1999).
16. D. C. La-Grange, I. S. Pretoriusm, M. Claeyssens, W. H. van-Zyl, *Appl. Environ. Microbiol.*, **67**, 5512 (2001).
17. E. dos Santos, T. Piovan, I. C. Roberto, A. M. Milagres, *Biotechnol. Lett.*, **25**, 13 (2003).
18. S. Aiba, A. E. Humphrey and N. F. Millis, Biochemical Engineering. 2nd Edition, New York: Academic Press. pp. 92 127. ISBN : 0-12-045052-6 (1973).
19. A. Converti and J. M. Dominguez, *Biotechnol. Bioeng.*, **75**, 39 (2001).
20. M. I. Rajoka, F. Latif, S. Khan and R. Shahid, *Biotechnol Lett.*, **26**, 741 (2004).
21. F. Latif, M.I. Rajoka and K.A. Malik, *World J. Microbiol. Biotechnol .*, **11**, 347 (1995).
22. M. J. Bailey, P. Biely, and K. Poutanen, *J. Biotechnol.*, **23**, 257-270(1992).
23. G. L. Miller, *Anal. Chem.*, **31**, 426 (1959).
24. C. Lopez, A. Blanco and F. I. J. Pastor, *Biotechnol Lett.*, **20**, 243 (1998).