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# Production and Characterization of α-Galactosidase by a Multiple Mutant of *Aspergillus niger* in Solid-State Fermentation

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**Summary**

α-Galactosidase is applied in the sugar industry to enhance sugar recovery from sugar beet syrup and to improve nutritional value of the soymilk. In the present investigation, the influence of process variables on the production of this important enzyme has been explored in a newly isolated multiple mutant strain of *Aspergillus niger* in solid-state fermentation (SSF). Defined fermentation parameters include substrate type (pure lactose and by-products of rice and flour mills as prime substrates), nitrogen source, incubation time, initial pH of the medium and incubation temperature. Extracellular α-galactosidase reached the value of 135.4 IU/g of dry substrate (IU/g) after 96 h of fermentation. Supplementation with 2 g of glucose and 3 g of corn steep liquor significantly increased the enzyme production, and maximum value of product yield (318 IU/g) by the mutant strain was significantly higher than that reported by the wild type (this work), or other *A. niger* mutants, recombinants and yeasts reported in literature as producers of elevated levels of α-galactosidase. Among three α-galactosidases, one possessing high subunit molecular mass proteins (99 and 100 kDa) has been characterized in both wild and mutant organisms. Thermal properties of the purified enzymes indicate that the mutation decreased the values of activation energy for the formation of enzyme-substrate (ES) complex, enthalpy, Gibbs free energy demand for substrate binding, and transition state stabilization. A thermodynamic study of irreversible inactivation of enzymes suggests that the mutant–derived enzyme is more thermostable than the native enzyme, which is attributable to amino acids involved in active catalysis. Because of these properties, the mutant organism is a novel organism and may be exploited for bulk production of thermostable α-galactosidase for the above industrial and nutritional applications.

*Key words*: enthalpy, entropy, production of α-galactosidase, Gibbs free energy, kinetics, solid-state fermentation

## Introduction

α-Galactosidase (EC 3.2.1.22) (α-D-galactoside galactohydrolase) catalyzes the hydrolysis of α-1,6-linked α-galactose residues in oligosaccharides and polymeric galactomannan (*1*). It is employed in the sugar industry to enhance sugar recovery by eliminating raffinose from sugar beet syrup (*2*). It hydrolyzes raffinose and sta-

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chyose in soymilk to relieve the problem of flatulence and gastrointestinal distress (*3*). Reducing the cost of enzyme production by optimizing the fermentation medium and process is the goal of basic research for their industrial applications. *Aspergillus niger* is an efficient producer of α-galactosidase (*4*) and can be exploited for mass production of this useful enzyme.

For the production of industrial enzymes, complex media are used, which may result in increased cost of production. It is suggested that for bulk production of enzymes, economical and commercially available nutrients be examined to reduce their production costs. In Pakistan, a total output of agroindustrial wastes, *viz*. rice, wheat bran, rice polishings, bagasse, molasses, corncobs and alike, is estimated over 60 million tonnes per year (*5*) and can be used for production of industrial enzymes to offset the cost of their disposal by municipalities and industries.

Solid-state fermentation (SSF) is an attractive process to produce enzymes economically due to enhanced enzyme yield, enzyme titre, higher product stability, lower catabolite repression and lower capital, operating and recovery costs (*6–8*). Further approach for economizing production is the use of agroindustrial materials as substrates rather than expensive lactose (*9*).

The improvement in yield is easily obtained with genetic or protein engineering (*3*) as has been achieved in other systems (*10*). The mutants of different fungi resistant to deoxy-D-glucose (DG) have been found to hyperproduce hydrolases (*11–12*). Current studies have been performed to isolate a derepressed mutant of *A. niger* for hyperproduction of α-galactosidase in solid-state fermentation using multiple mutagens, and to optimize fermentation variables under which the mutant strain produced enhanced levels of α-Gal. This study also describes the kinetics and thermodynamics of α-Gal purified from locally isolated *A. niger* and its multiple mutant derivative as part of the efforts to deploy indigenous organism and their selected mutants for the production of α-Gal.

## Materials and Methods

### *Organism and reagents*

*Aspergillus niger* NIAB 280, procured from Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan, was used throughout these studies. The strain was maintained on potato-dextrose agar (PDA) plates and slants as described earlier (*13*). For the seed culture, spores of *A. niger* from PDA slants were rinsed with sterilized saline into a suspension of spores, used as inoculum. All reagents were of analytical grade and purchased from Sigma-Aldrich Chemical Co., MI, USA.

### *Substrates and their preparation*

By-products of rice industry, namely rice husk, rice bran and rice polishings, and those of flourmills (wheat bran) were obtained from local sources. The dry powder of rice husk was alkali treated and analyzed as described earlier (*14*). It had 81 % carbohydrates and 4.2 % crude protein. Untreated powder of rice polishings, rice bran and wheat bran had 63.1, 65.5 and 53.6 % total carbohydrates and 12.0, 3.9 and 14.0 % crude protein, respectively.

### *Isolation of mutants*

*A. niger* culture on PDA slant was rinsed with sterilized water into a suspension of spores and placed in 250-mL Erlenmeyer flasks containing 50 mL of Vogel's medium supplemented with yeast extract and glucose grown at 30 °C for 20 h, then centrifuged at 15 000×*g* for 15 min, and suspended in 50 mL of saline (0.9 %) containing 0.01 % yeast extract. The cells were exposed to UV irradiation ($1.2 \cdot 10^6/(m^2 \cdot s)$) for 60 min and mutant derivatives resistant to 0.5 % deoxyglucose (DG) were selected as described previously (*15*). Among 15 variants, the best variant, *A. niger* Raj 1, was regrown as above and suspended in 100 mL of saline containing 0.01 % yeast extract. It was dispensed equally in 30-mL McCartney vials. The exposure of cell suspension ($2 \cdot 10^9$ cells/mL) to nitrosoguanidine (NTG; 150 μg/mL) for 30 min gave approx. 95 % reduction in colony forming units. Afterwards, the treated cells were grown in the presence of 150 μg/mL aspartate (Asp) and 1.0 % DG medium to isolate $Asp^{-1}$ (aspartate-requiring) and simultaneously derepressed mutants as described earlier (*15*). Overall more than 500 different colonies were subsequently replica-plated on lactose and DG (1.0 %, by mass per volume) agar plates with aspartate. Among 150 variants, one mutant strain that produced significantly higher α-Gal was designated *A. niger* M57 and selected for further mutation by γ-rays as described previously for β-xylosidase hyperproduction (*5*). Out of 100 variants, one mutant resistant to 200 μg/mL aspartate and 1.5 % DG (designated *A. niger* AR1) was finally selected for hyperproduction of α-Gal as described previously for β-xylosidase (*5*).

### *α-Galactosidase production*

Experiments were carried out in 500-mL Erlenmeyer flasks containing 4 g of dry substrate (insoluble) or 4 g of lactose using polymeric resin (4 g, 100–500 μm particle size) as inert support material to provide anchorage to fungal cells and 12 mL of salt solution (Vogel's medium) containing (in g/L): trisodium citrate 2.5, $NH_4NO_3$ 2.0, $KH_2PO_4$ 5.0, $(NH_4)_2SO_4$ 4.0, $MgSO_4 \cdot 7H_2O$ 0.2, and yeast extract 2.0 (pH=5.5). After autoclave sterilization at 121 °C for 30 min, the medium was cooled down for inoculation with a 4-mL (by volume per mass, $10^7$ spores/mL) inoculum. The dry matter percentage of the wetted substrate was 25 %. Further experiments were conducted using 6, 8 and 10 g of solid wheat bran in 500-mL flasks and moistened with Vogel's medium (3 mL per g of dry matter) and inoculated at the rate as above. Cultivation in SSF was carried out in a 40-litre incubator where temperature and humidity is controlled automatically. Control temperature was 30 °C and relative humidity was 90 %. During the SSF, three flasks were periodically sampled daily for different analyses. Each unit of data represents the average of the three analyses. The best solid substrate (wheat bran) was selected and used in subsequent experiments.

Various process parameters were optimized by conventional methods (by varying one parameter at a time) for maximal enzyme production as follows: incubation period (0, 24, 48, 72, 96, 120 and 144 h), under previously optimized conditions of moisture content (80 %), inoculum level (1–6 %), fermentation temperature (25–40 °C) and initial pH (pH=4.0 to 9.0). Wheat bran was supplemented with different nitrogen sources (ammonium sulphate, ammonium dihydrogen phosphate, glutamate, urea and corn steep liquor) at 0.08 g of nitrogen per flask. On the basis of the results obtained with all the optimum parameters, the extent of improvement in the optimized medium was evaluated using basal medium as control. The enzyme preparations were analyzed for enzyme activities on periodically collected samples as described earlier.

### *Analytical methods*

For enzyme recovery, the fermented medium was suspended in 50 mL of deionized chilled water containing 1 % (by volume) Tween 80. After vigorous shaking for 30 min on a shaker (200 rpm) at 4 °C, the suspension was filtered through a muslin cloth and the residue was again treated with 50 mL of deionized chilled water in the same manner and filtered. The filtrates were pooled together and centrifuged (10 000 rpm, 30 min at 4 °C). The cell-free supernatant was preserved for enzyme assays. Moisture content was evaluated gravimetrically. The loss in gross mass was calculated as percentage. The pH of the supernatant after centrifugation was measured with a pH meter. The solid material was redispersed in deionized water to make up a 20-mL suspension, then disintegrated at 20 kHz for 10 min and centrifuged at 3000 rpm for 5 min, after which the supernatant was collected. Microbial growth was measured as absorbance (*A*) using a standard curve as described previously (*8*). An *A* reading of 1.0 was equivalent to the cell mass of 0.4 g/L. Relative humidity was measured with hygrometer after a period of equilibrium. The fungal spore number was counted with a haemocytometer. Unless otherwise stated, all tests were conducted in flasks under the aforementioned conditions.

### *Determination of α-Gal activity*

α-Gal activity was determined by adding 100 μL of appropriately diluted α-Gal solution to 1 mL of 1 mM *p*-nitrophenyl-α-D-galactopyranoside (PNPG) and 1.9 mL of McIlvaine buffer (0.15 M, pH=5.5) mixture. The reaction mixture was agitated at 50 °C for 10 min in a shaking water bath. The reaction was terminated by adding 3 mL of sodium carbonate (1 M). The amount of formed *p*-nitrophenol was determined at 410 nm against standard. One unit of enzyme activity was defined as the amount of enzyme which releases 1 μmol of *p*-nitrophenol per mL per min.

In these tests, glucose was determined using Human GmbH (Germany) glucose oxidase/peroxidase kit following the instructions of the suppliers. The protein in the solution was determined by the method of Lowry (*16*) using bovine serum albumin as the standard. Total reducing sugars were determined according to Miller (*17*). All treatments were statistically evaluated using MSTATC software as mentioned previously (*18*).

### *Purification of α-Gal*

The crude extract (500 mL) of both wild and mutant strains was subjected to 80 % ammonium sulphate precipitation at 4 °C and kept overnight at the same temperature. The resulting precipitates collected by centrifugation at 10 000×*g* for 30 min were discarded. More ammonium sulphate was added to make 100 % and left overnight at 4 °C. The precipitates after the centrifugation as above were resuspended in minimum volume of 10 mM phosphate buffer (pH=6.0). The dialysis was carried out at 4 °C against the same buffer in order to remove the salts. The dialyzed samples were loaded onto a HiLoad Q Sepharose column at a flow rate of 2 mL/min employing buffer A (25 mM TrisHCl, pH=7.5) and buffer B (25 mM Tris-HCl, pH=7.5 and 1 M NaCl). Fractions of 2 mL were collected. Volumes of 54–104 mL were pooled. This step was followed by gel filtration chromatography. Pooled fractions from HiLoad anion-exchange column were loaded onto a gel filtration column of Sephadex G-100 (1.5×75 cm) previously equilibrated with 10 mM phosphate buffer (pH=6.0) and eluted with the same buffer. The flow rate was maintained at 0.5 mL/min and fractions of 1 mL were collected. The distribution coefficient was:

$$K_r=(V_e-V_o)/(V_i-V_o) \qquad /1/$$

where $V_e$, $V_o$ and $V_i$ are the retention volumes of the enzyme, Blue Dextran (7.9 mL) and tyrosine (21.2 mL), respectively (*19*). The extent of purification was followed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The native and subunit molecular mass of α-Gal were determined by gel filtration chromatography and by 8 % SDS-PAGE (*20*).

### *SDS-PAGE*

SDS-PAGE was performed using 8 % (by mass per volume) acrylamide gels. The protein bands were visualized using Coomassie Brilliant Blue R-250.

### *Effect of pH*

The effect of pH on α-Gal activities was determined by assaying the enzymes at different pH values from 3 to 10 in various buffers (*20*) at 50 °C. pH stability was checked by incubating the enzymes in the above buffers for 1 h at room temperature, and the remaining activity or time-dependent enzyme activity in different buffers at different temperatures was determined for Dixon plots.

### *Effect of temperature and $E_a$*

α-Gals were assayed at different temperatures ranging from 35 to 65 °C. Activation energy was determined from the Arrhenius plot as described earlier (*19*).

### *Effect of substrate concentrations*

α-Gals from *A. niger* and its mutant were assayed in the reaction mixtures containing different amounts of PNPG (0.025 to 2.5 mM). The data were plotted accord-

ing to the Lineweaver-Burk plot (*13*) to determine the values of kinetic constants ($v_{max}$ and $K_m$).

### *Kinetics of thermal denaturation*

Kinetic and thermodynamic parameters for irreversible thermal denaturation of wild- and mutant-derived α-Gals were estimated by incubating the enzyme in 10 mM phosphate buffer (pH=5) at a particular temperature. Aliquots were withdrawn at different times, cooled in ice for 1 h and assayed for residual enzyme activity (*20*). This procedure was repeated at 7 temperatures. The data were fitted to the first order plots and analyzed as described earlier (*19*,*20*). The thermodynamic parameters for thermostability were calculated by rearranging the Eyring's absolute rate equation as described previously (*13*,*20*).

## Results and Discussion

### *Selection of mutant*

Extensive work has been going on in many laboratories to select a proper organism for the production of α-Gal and efficient inducers for large-scale enzyme production, particularly using biomass wastes in solid-state fermentation. Improvement in enzyme secretion by randomization method for *in vitro* protein evolution was followed as described in Materials and Methods. UV irradiation, followed by chemical mutagenesis and γ-irradiation studies, indicated that well-developed yellow zones on PNPG-agar plates appeared around 100 colonies; final semi-quantitative plate studies revealed that one derivative capable of producing the largest amount of α-Gal was isolated after the third mutation and it was designated *A. niger* AR1 (*5*).

### *Influence of carbon sources*

To avoid the build-up of temperature, pH, moisture, substrate concentration or oxygen gradient during cultivation, which are difficult to control under limited water activity, 4 g of different carbon sources per 500-mL flask were employed to study their effect on the growth and production of extracellular α-Gal from *A. niger* and its mutant derivative (Table 1). The results showed that wheat bran resulted in the highest α-Gal product yield and productivity, followed by rice husk. Lactose took the 5th place among different substrates considered as better inducers. The p-values for carbon sources were <0.05 (with most p-values<0.0003), meaning that they were significantly different with respect to induction power.

The comparison of mean values indicated that mutant strain supported significantly higher (up to >2-fold) values of $Q_p$ and $Y_{p/s}$ as compared to parental strain (Table 1). The influence of organisms on carbon sources for enzyme synthesis was highly significant (p<0.0001), as was between the organisms (p<0.0001). Among different substrates, protein synthesis by both organisms was not significantly different but carbohydrate levels as inducers present in the fermentation flasks (Table 1) at the end of fermentation were significantly different (p<0.0001) and they exerted significantly different inductive effect on the organisms to support different levels of enzyme.

Table 1. Parameters for the growth of parental *A. niger* (P) and its mutant derivative AR1 (M), and α-Gal production on different substrates in solid-state fermentation

| Carbon source | $Y_{P/S}$ IU/g | γ(RS) mg/mL | γ(protein) mg/mL |
|---|---|---|---|
| **Lactose** | | | |
| P | 15.6h | 2.8b | 0.43a |
| M | 43.5f | 2.6c | 0.54a |
| **Rice bran** | | | |
| P | 36.0g | 2.4e | 0.48a |
| M | 61.5d | 1.8i | 0.50a |
| **Rice husk** | | | |
| P | 50.7e | 2.0h | 0.43a |
| M | 99.3b | 2.5d | 0.45a |
| **Rice polishing** | | | |
| P | 48.4ef | 2.3f | 0.47a |
| M | 95.4b | 3.0a | 0.54a |
| **Wheat bran** | | | |
| P | 64.0d | 2.2g | 0.53a |
| M | 135.4a | 2.0h | 0.60a |
| **Mean value** | | | |
| P | 42.9f | 2.5d | 0.47a |
| M | 87.0c | 2.4e | 0.52a |
| **LSD (p≤0.05)** | 5.75 | 0.018 | 0.590 |
| **p** | 0.0001 | 0.0001 | 0.596 |

Experimental conditions: the above substrates are in Vogel's medium (pH=5.5) at temperature of 30 °C in SSF. Each value is the mean of three replicates. Values followed by different letters differ significantly at p≤0.05. RS=reducing sugars in the fermentation mash after 120 h of fermentation

The mutant derivative differed significantly from the wild organism in this attribute and it synthesized significantly (p<0.0001) higher values of product formation parameters.

For the production of α-Gal, wheat bran was the best stimulator of α-Gal production, followed by rice husk. High enzyme production level depends on the magnitude of accessory enzymes, namely endoglucanase (*13*,*21*), exoglucanase (*18*) and cellobiase (*22*) produced along with α-Gal by the test organism(s). The product yield of α-Gal supported by *A. niger* and its mutant is several fold higher than the reported values by other workers on *Aspergillus* spp. and their mutants or some recombinants harbouring heterologous genes for α-Gal (*1*,*3*,*8*,*10*, *23*). Kotwal *et al.* (*1*) also reported maximum α-Gal production in a medium containing 5 % wheat bran extract as a carbon source. Therefore, in all subsequent experiments, wheat bran was used as the substrate for the production of α-Gal. Among different concentrations of wheat bran, 4 g per 500-mL flask was found optimum (results not shown) and was maintained in further studies.

When glucose was used in conjunction with wheat bran, it resulted in enhanced enzyme production in both wild and mutant cultures (results not shown). There was an increase in the enzyme yield (70.4 and 175.0 IU/g in parent and mutant, respectively) even when glucose was

released from the substrate to which 2 g per flask of glucose were added. A high enzyme titre (87 and 289 IU/g) was obtained when the initial moisture level was 70 and not 80 % as used for control experiments. The importance of initial moisture level in SSF media and its influence on the biosynthesis and secretion of enzymes can be attributed to the interference of moisture in the physical properties of the solid particles. An increase in moisture level is believed to reduce the porosity of the solid substrate, thus limiting oxygen and swelling.

### *Effect of nitrogen sources*

Optimization studies of enzyme production using nitrogen sources were also conducted. Ammonium nitrate, ammonium sulphate, corn steep liquor, diammonium phosphate, fish meal and urea were used as nitrogen sources (at 0.16 g/L of nitrogen), and added to the medium containing 8 g/L of wheat bran. Among them, 6 g/L of corn steep liquor, one of the by-products of starch industry and the least expensive nitrogen source, favoured maximum α-Gal (92 and 318 IU/g of wheat bran) production in wild and mutant cultures respectively, followed by fish meal and urea, whereas ammonium sulphate and diammonium hydrogen phosphate (Table 2) were not good sources of nitrogen. Statistical analysis showed that the p-values for all nitrogen sources were <0.05 (with most p<0.0002), indicating that they were significantly different. The influence of both organisms on enzyme production was also significant as indicated by low values of p (p<0.0001). Urea (0.25 % by mass per volume) favoured maximum pectinase production in *Streptomyces* sp. RCK-SC (*24*). When cultures were grown in the presence of corn steep liquor, even lactose induced cells to produce elevated levels of enzyme comparable to those on wheat bran in its absence (Table 1). This may have occurred due to the presence of certain nutrients, vitamins, minerals and carbohydrates in corn steep liquor.

Table 2. α-Gal production parameters of parental *A. niger* (P) and its mutant AR1 (M) from wheat bran medium supplemented with glucose and corn steep liquor moistened with Vogel's medium

| $\gamma$(nitrogen source)=0.16 g/L | $\frac{Y_{P/S}}{IU/g}$ |
|---|---|
| **Ammonium nitrate** | |
| P | 60g |
| M | 200e |
| **Ammonium sulphate** | |
| P | 77fg |
| M | 223d |
| **Corn steep liquor** | |
| P | 92f |
| M | 318a |
| **Diammonium phosphate** | |
| P | 78fg |
| M | 230d |
| **Fish meal** | |
| P | 85f |
| M | 243cd |
| **Urea** | |
| P | 82fg |
| M | 284b |
| **Mean value** | |
| P | 83.2fg |
| M | 259.9c |
| **p≤0.05** | 0.0016 |

Experimental conditions: substrate pH=5.5, temperature 30 °C. Each value is the mean of three independent readings. Standard deviation among replicates varied between 5–10 % of mean values and has not been presented. Values followed by different letters differ significantly from each other at p≤0.05

### *Effect of pH and temperature on enzyme production*

The effect of initial culture medium pH on the production of α-Gal was investigated using wheat bran, urea and glucose as supplements (discussed earlier). Results in Table 3 show that optimal product yield was achieved in a wide pH range (pH=5.5–7.5). These studies indicated that in the absence of pH control, initial pH=5.5 is regarded as optimal for α-galactosidase production in SSF. It was seen that during growth, pH increased from 5.5 to 7.0, which might be due to ammonia production from amino acids and peptides. Therefore, pH=5.5 was used in all further studies. Statistical analysis showed that the influence of pH on product yield was significant. The optimum pH of enzyme productivity was significantly different from that reported for most other α-Gal-producing microorganisms, namely *Aspergillus* sp. (*25*), bacteria and yeast (*9*,*26*).

The temperature of fermentation medium is another critical factor that has a profound influence on the product formation. Maximum production of α-Gal was obtained when the fermentation temperature was maintained at 30 °C (Table 4). The enzyme yield of *A. niger*

Table 3. Regression analysis of the effect of pH on α-Gal production yield ($Y_{P/S}$) by the parent strain and its mutant derivative following the growth on wheat bran medium supplemented with glucose (2 g) and corn steep liquor (3 g) under SSF

| AOV | dF | Sum of squares | Mean square | F-value | p |
|---|---|---|---|---|---|
| pH (A) | 6 | 11340.732 | 1890.122 | 4.3082 | 0.0034 |
| Organisms (B) | 1 | 51083428 | 51084.3 | 1164.3091 | 0.0001 |
| A×B | 6 | 874.198 | 145.7 | 0.3321 | |
| Error | 28 | 12284.427 | 438.738 | | |
| Total | 41 | 535342.784 | | | |

AOV=analysis of variance

Table 4. Regression analysis of the effect of temperature on α-Gal production yield ($Y_{P/S}$) by the parent and its mutant derivative following the growth on wheat bran medium supplemented with glucose (2 g) and corn steep liquor (3 g) under SSF

| AOV | dF | Sum of squares | Mean square | F-value | p |
|---|---|---|---|---|---|
| Temperature/°C (A) | 9 | 33351.483 | 37055.72 | 1.4183 | 0.2136 |
| Organisms (B) | 1 | 429091.267 | 429091.267 | 162.1036 | 0.0001 |
| A×B | 28.55 | n.d. | n.d. | 0.595 | |
| Error | 40 | 104046.00 | 2612.70 | 0.6260 | |
| Total | 59 | 577642.983 | 2621.70 | | |

n.d.=not determined; p-values lower than 0.05 are statistically significant

AR1 was higher than that of its parent at all temperatures (ranging from 30 to 65 °C). Normally, high temperature can cause inactivation of the ribosome, cell wall fluidity and enzymes of the metabolic pathway, while low temperature may not permit the flow of nutrient across the cell membrane, resulting in high demand for maintenance energy (*27*). Similar values of optimum temperature have been reported for *Aspergillus* spp. by other authors as well (*3*,*28*). Statistical analysis of product yield revealed that the treatments had significant effect on enzyme production. The mutant strain was significantly better than its native culture with respect to supporting product formation. However, combined effect of treatments and strains was statistically not significant.

### *Time course production under optimized conditions*

The incubation time for achieving maximum enzyme level is governed by growth rate, initial concentration of the substrate and concentration of metabolites in the cells and media during time course study. The representative kinetics of product formation by the mutant cultures from wheat bran and rice bran (Fig. 1) indicated that both organisms produced high titres of enzymes (92 and 318 IU per g of dry substrate, respectively) at 96 h of incubation in the log phase at initial moisture content of 70 %, pH=5.5, salt concentration of 3 mL per g of solid substrate with glucose (2 g) and corn steep liquor (3 g per flask) as supplements for enzyme production. It was concluded that 1.5- to 2.0-fold increase in enzyme production was achieved under the optimized fermentation conditions, as compared to the basal medium used as control. Future research will focus on using optimized conditions to obtain higher enzyme yields in perforated trays and locally assembled semi-pilot scale SSF bioreactor carrying wheat bran for a low-cost production process.

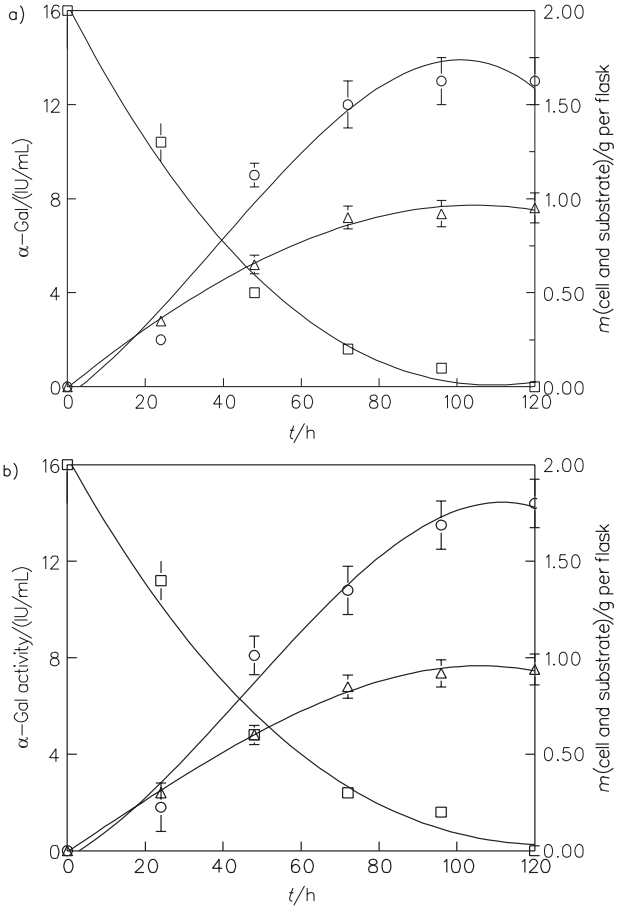


**Fig. 1.** α-Gal (○), and cell mass (Δ) production kinetics of *A. niger* RA1 in fermentation of (a) wheat bran (□) and (b) rice bran (□) in Vogel's medium supplemented with 2 g of glucose and 3 g of corn steep liquor using SSF (initial pH=5.5, temperature 30 °C). Error bars show standard deviation (S.D.) among *N*=3 experiments

### *Thermal effects on α-Gal production*

Thermal effect analysis of the product formation on wheat bran medium supplemented with glucose and corn steep liquor was performed to evaluate the thermostability of the production process. The results (Table 5) indicate that the activation enthalpy of α-Gal formation by the mutant cells ((19.1±2) kJ/mol) was significantly ($p<0.0289$) lower than that by its parental cell and α-Gal production by a thermotolerant yeast and other different production processes (*29*). Activation entropy of α-Gal formation by the mutant (–138.4 J/(mol·K)) was significantly different from its parental culture (–134.3 J/(mol·K); p=0.0001) (Table 5). This suggests that the genetic make up of both organisms is thermodynamically stable during product formation.

Activation enthalpy ($\Delta H_D^*$) of thermal inactivation for the mutated cells ((18.8±2) kJ/mol) and wild cells (42.5 kJ/mol) was also significantly (p=0.0001) different. The entropy value of thermal inactivation of enzyme formation by both cultures was also very low (and had negative sign), and lower than for thermostabilized reactions (*30*). Thus, the mutation further thermostabilized the metabolic network for α-Gal formation. The stabilizing forces may have been provided by the system itself

Table 5. Enthalpy ($\Delta H^*$) and entropy ($\Delta S^*$) of parental *A. niger* (P) and its mutant derivative AR1 (M) for α-Gal production and its inactivation process in SSF using wheat bran supplemented with glucose as a carbon source and corn steep liquor as a nitrogen source

| | $\Delta H^*$/(kJ/mol) | | $\Delta S^*$/(J/(mol·K)) | |
|---|---|---|---|---|
| | Formation | Inactivation | Formation | Inactivation |
| P | 21.6a | 42.5a | –134.3a | –343.8c |
| M | 19.1c | 18.8b | –138.4c | –263.1a |
| Mean | 20.35b | 30.7c | –136.4b | –303.5b |
| F | 9.77 | 80.54 | 325.5 | 300.6 |
| p | 0.0289 | 0.0001 | 0.0001 | 0.0001 |

All experiments were performed up to 120 h. Each value is a mean of three replicates. Values followed by different letters differ significantly at $p \leq 0.05$. Standard deviation among replicates varied between 5–7.5 % of mean values and is not shown

during product formation, most probably by acquiring chaperones under temperature stress or by the effect of mutations, which assisted the folding of protein within cells (*31*). Other authors reported that in SSF, *A. niger* accumulated glycerol, erythritol and arabitol, and three different proteins (32, 28 and 20 kDa) secreted by the fungus were identified only in SSF (*6*,*32*) under temperature stress and may have a role in thermostability.

### *Purification and characterization of purified α-Gal*

α-Gal was purified to electrophoretic homogeneity level by the combination of ammonium sulphate precipitation, ion-exchange and gel filtration chromatography as visualized by a single band on SDS-PAGE (Fig. 2) after purification in case of both enzymes, and it shows

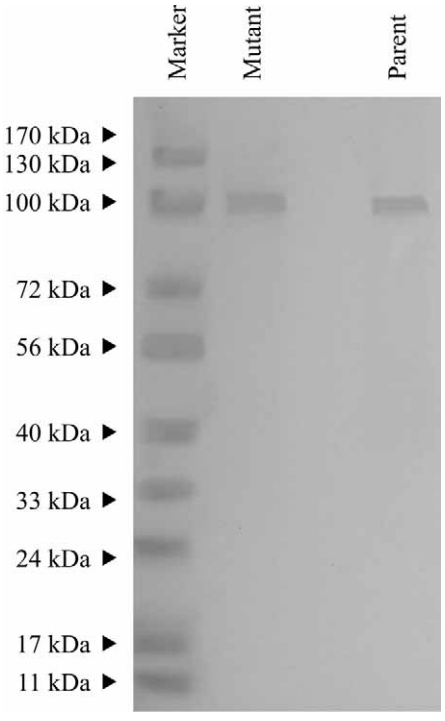


**Fig. 2.** SDS-PAGE electrophoretogram of parent- and mutant-derived α-Gal samples and protein molecular mass markers

that α-Gal was in each case purified to electrophoretic homogeneity. The enzymes were purified 5-fold with 39.1 and 41.2 % recovery of protein. The subunit molecular mass of wild- and mutant-derived enzyme was 99 and 100 kDa, respectively. These enzymes also showed molecular mass of 100 and 103 kDa on gel filtration chromatography, suggesting that both enzymes were monomeric in nature as described earlier (*4*,*27*) but different from those of others (*33*).

### *Effect of pH on the purified α-Gal*

Our enzymes showed broad pH optima (pH=4.5 to pH=6.5) (Fig. 3). It is particularly interesting to have such enzyme in which mutation slightly decreased the pH demand for optimal activity. Dixon's analysis was carried out to evaluate $pK_a$ of ionizable groups of active site residues for substrate hydrolysis at 50 °C (Fig. 3) which were involved in maximum velocity for substrate

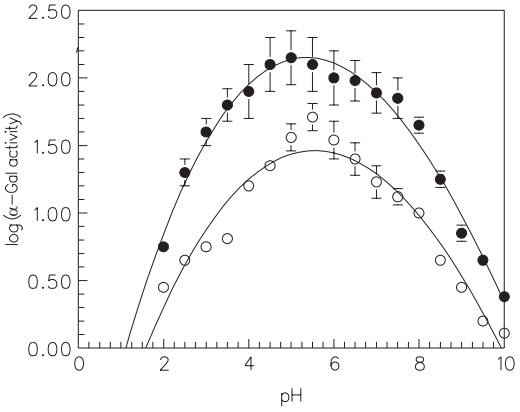


**Fig. 3.** Dixon's plot of (○) *A. niger* and (●) its mutant-derived α-Gal at 50 °C for the determination of $pK_a$ of the active site residues. Error bars show S.D. among $N$=3 experiments

hydrolysis. It was found that both α-Gals at 50 °C involved two types of ionizable groups for hydrolysis of lactose with $pK_{a1}$ and $pK_{a2}$ of 4.0 and 7.4 for the parental α-Gal, respectively, while mutant-derived α-Gal had $pKa_1$ and $pKa_2$ of 3.5 and 7.3, respectively (Table 6) at 50 °C. These $pK_a$ values gave us a rough guide to the nature of the amino acid groups involved in the active site. Actually, one of the two ionizable groups could be ionized and the other protonated (*12*). As suggested earlier, the protonated form of the group with $pK_{a2}$ of 7.4 and 7.3 is required for active catalysis as affinity decreases with the increase in pH in both enzymes (Fig. 3). Mutation did not change the ionizable group of the acidic or basic limb. In both enzymes, glutamate or aspartate was located on the acidic limb, while the $pK_{a2}$ of ionizable groups involved in catalysis as proton receiver were comparable with the imidazole group, suggesting the presence of histidine residues as essential amino acid at the active sites of both enzymes. For maximal activity, this residue is expected to be protonated, as reported earlier by Gote *et al.* (*12*). Recently, circular dichroism spectroscopy (CDS), differential scanning calorimetry (DSC) methods, and fluorescence spectrometry studies on pH-dependent conformational and structural changes (*12*) have

Table 6. Kinetic and thermodynamic properties of α-galactosidase derived from the parental (P) and mutant derivative (M) of *A. niger* for α-Gal hydrolysis at 50 °C

| Kinetic parameter | α-Gal | |
|---|---|---|
| | P | M |
| $v_{max}$/(μmol/(mg·min)) | 59±4.1 | 91±5.2 |
| $K_m$/mM | 0.43±0.1 | 0.28±0.2 |
| $v_{max}/K_m$ | 137.25±21.3 | 325.0±23.1 |
| $E_a$/(kJ/mol) | 64.5±4.0 | 44.6±2.2 |
| $M_r$/kDa | 99.0 | 100.0 |
| Temperature optimum/°C | 45.0±0.2 | 50.0±0.2 |
| pH optimum | 4.0–6.5 | 3.5–6.5 |
| $pK_{a1}$ | 4.0±0.02 | 3.5±0.02 |
| $pK_{a2}$ | 7.4±0.02 | 7.3±0.02 |
| $\Delta G^*$/(kJ/mol) | 67.55±2.1 | 63.71±1.5 |
| $\Delta H^*$/(kJ/mol) | 61.9±1.2 | 41.9±1.2 |
| $\Delta S^*$/(J/(mol·K)) | –68.2±2.3 | –67.52±2.1 |
| $\Delta G^*_{E-T}$/(kJ/mol) | –12.97±0.2 | –15.10±0.25 |
| $\Delta G^*_{E-S}$/(kJ/mol) | –2.20±0.12 | –3.32±0.11 |

Mean values of $N$=3 with standard deviation. Free energy of transition state binding $\Delta G^*_{E-T} = -RT \ln v_{max}/K_m$; free energy of substrate binding $\Delta G^*_{E-S} = -RT \ln K_a$, where $K_a = 1/K_m$

also confirmed the involvement of lysine for substrate binding, while carboxyl groups were involved in catalytic function on the acidic limb and histidyl residues on the basic limb. Further studies are needed on site-directed mutagenesis and crystallographic analysis.

### *Effect of substrate concentration*

A Lineweaver-Burk plot of the data revealed the $K_m$ of 0.284 mM and $v_{max}$ of 91 μmol/(mg·min) for the enzyme from the mutant organism. The specificity constant of α-Gal of the mutant strain showed that it was about 2.4-fold more active than that of its parental strain (Table 6). The kinetic constants, namely $K_m$ and $v_{max}$ can be compared with the $K_m$ (1.89 mM) and $v_{max}$ (12 μmol/(mg·min)) reported by Ozsoy and Berkkan (*28*) and with different enzyme preparations (*4*,*30*). Enzymes also catalyzed stachybiose, melibiose and D-raffinose with $K_m$ values of 2.3, 1.7 and 1.90 mM, respectively (results not shown).

The specificity constant of mutant-derived enzyme was significantly altered by mutation as compared with parental enzyme (Table 6). This could be due to the ionized state of the mutated carboxyls, whose charges have been reversed in such a manner that the substrate binds more strongly to the active site than the transition state of the substrate. As reported earlier (*21*), this case corresponds to the enzyme-substrate complementarity in which the binding energy of enzyme-substrate complex is greater than that of enzyme-transition state, resulting in the decreased $K_m$ and increase in $v_{max}$ at 50 °C (Table 6).

### *Effect of temperature on α-Gal catalysis*

Temperature affects enzymatic reaction rate significantly. From the Arrhenius plot, activation energy for catalysis of PNPG hydrolysis by mutant-derived enzyme was 44.6 kJ/mol (Table 6), which is significantly lower than that required by the parental enzyme (64.5 kJ/mol). The results of thermodynamics of the hydrolysis of PNPG by α-Gal showed that the Gibbs free energy, enthalpy of activation ($\Delta H^*$) and entropy of activation ($\Delta S^*$) demands for substrate hydrolysis at 50 °C by mutant-derived enzyme were lower compared to those for parental enzyme at 50 °C, indicating that the disorder in ES*-complex formation is minimum and the conversion of reactant to product is spontaneous in mutant-derived enzyme. The free energy for the transition state binding ($\Delta G_{E-T}^*$) was lower in the mutant and presented an evidence that the ability to form the transition complex was high for mutant as compared to parent-derived α-Gal. Similarly, native enzyme required higher amount of transition state formation energy ($\Delta G^*_{E-S}$) as compared to mutant-derived enzyme (Table 6). It was not possible to make a comparison of our data with those of other authors as no such information is available for catalysis of α-Gal by other sources in literature.

### *Thermal inactivation of α-Gal*

Thermal stability of α-Gal of *A. niger* and its mutant AR1 was investigated in the temperature range of 35–65 °C. Enzyme samples were pre-incubated at 35, 40, 45, 50, 55, 60 and 65 °C for 15, 30, 45, 60, 75, 90 and 120 min, and the residual activity was determined after cooling on ice at 45 and 50 °C. Natural logarithm of percentage residual activity was plotted against $1/T$ to determine specific rate of inactivation ($k_d$), which is presented in Table 7. The energy of activation for thermal denaturation ($E_{a(d)}$) was determined by applying Arrhenius plot (Fig. 4a) using $k_d$ values at different temperatures (Table 7). Corresponding values were 69.0 and 78.3 kJ/mol for the mutant and parental *A. niger* α-Gal, respectively. Along with the increase in temperature, inactivation of enzyme increased from 35 to 60 °C. Half-life of mutant-derived enzyme was significantly higher (1317.5 and 266.5 min at 45 and 60 °C, respectively) than that of the wild organism (1001 and 147.4 min at 45 and 60 °C, respectively; Table 7). Thus the mutation improved the thermostability by 1.80 times at 60 °C.

When an enzyme is exposed to temperatures higher than its optimum, denaturation takes place and polypeptide may need more energy to keep the protein ensemble in a folded form. Several events occur during thermal denaturation of enzymes including the disruption of non-covalent linkages, hydrophobic interactions, with concomitant increase in the enthalpy of activation demand (*30*,*34*). This aspect can be described by usual thermodynamic values calculated from the formulae given previously (*20*). The values thus calculated may provide inherent information to the mechanism involved in these reactions. This mechanism is generally presented in the form of two-state unfolding mechanism (*19*) and has been applied to various reactions (*20*).

According to this denaturation phenomenon, the values of $k_d$ were processed at different reaction temperatures (Table 7) and presented in Fig. 4. Unfolding of the enzyme structure is accompanied by an increase in the disorder, randomness or entropy of activation (*34*,*35*). The values of thermodynamic parameters were calcu-

Table 7. Kinetics and thermodynamics of irreversible thermal denaturation of α-Gal from parental (P) and mutant (M) strain of *Aspergillus niger*

| Treatment/°C | $k_d$/min$^{-1}$ | $t_{½}$/min | $\Delta H^*$/(kJ/mol) | $\Delta G^*$/(kJ/mol) | $\Delta S^*$/(J/(mol·K)) |
|---|---|---|---|---|---|
| **45** | | | | | |
| P | 0.000689a | 1001b | 113.0a | 97.3a | 0.049a |
| M | 0.00053a | 1317a | 66.36b | 98.0a | –0.099b |
| **50** | | | | | |
| P | 0.00218a | 330e | 112.91a | 95.8a | 0.053a |
| M | 0.00091a | 760c | 66.31b | 98.12a | –0.098b |
| **55** | | | | | |
| P | 0.00309a | 224.3e | 112.87a | 96.353a | 0.050a |
| M | 0.0014a | 495d | 66.27b | 98.51a | –0.098b |
| **60** | | | | | |
| P | 0.00475a | 147.4gh | 112.83a | 96.67a | 0.049a |
| M | 0.0026a | 266.5ef | 66.23b | 98.34a | –0.096b |
| **65** | | | | | |
| P | 0.0089a | 77.9h | 112.79a | 96.4a | 0.049a |
| M | 0.0044a | 157.5fgh | 66.19b | 98.38a | –0.095b |
| **F-value** | 288.81 | 157.5 | 9.600 | 0.0051 | 15.5 |
| **p** | 0.0001 | 0.0001 | n.s. | 0.256 | 0.0006 |
| **LSD** | 0.018 | 109.6 | 14.97 | 16.01 | 0.018 |

Mean values of $N$=3. Values followed by different letters in each column differ significantly at $p \leq 0.05$. Standard deviation in some cases was very small, therefore not presented. $t_{½}$=half life calculated using the relation: ln 2/$k_d$, where $k_d$ is the rate of denaturation obtained from Fig. 4a. $E_{a(d)}$=slope×$R$=115.6 and 69.0 kJ/mol for the parent- and mutant-derived α-Gal, respectively (calculated from Fig. 4a). n.s.=not significant

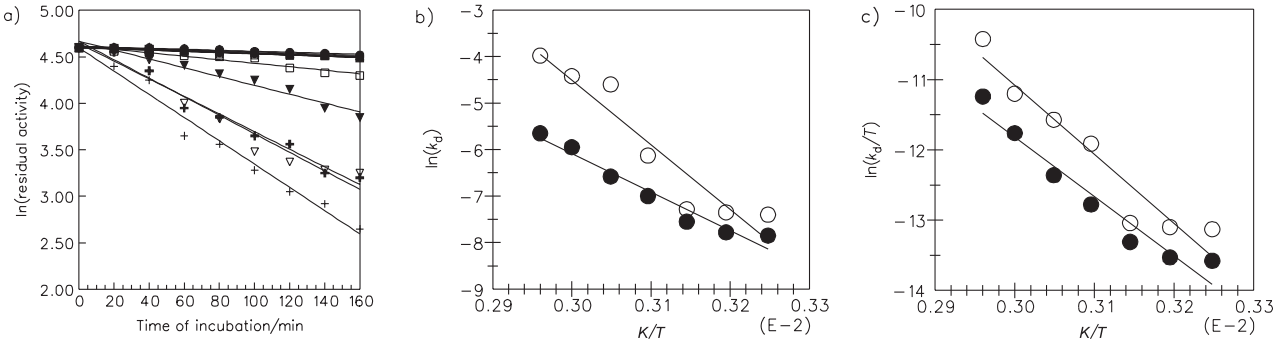


**Fig. 4.** Determination of thermodynamic parameters of α-Gal derived from wild and mutant organisms: (a) first order plot for thermal denaturation of α-Gal derived from wild organism and from the mutant, respectively, at 35 (○,●), 40 (△,▲), 45 (□,■), 50 (◊,♦), 55 (▽,▼) and 60 °C (+,+) and at pH=4.5 to calculate the specific rate of denaturation ($k_d$) of α-Gal; (b) Arrhenius plot to calculate activation energy $E_{a(d)}$ for irreversible thermal inactivation of α-Gal derived from wild organism (○), and from the mutant (●): error bars of standard deviation among three observations are too small to be visible; (c) Arrhenius plot for irreversible thermal denaturation of: α-Gal derived from wild (○) and mutant strain (●) of *A. niger*: error bars of standard deviation among three observations are too small to be visible

lated from Fig. 4c. The enzyme derived from the mutant required 69.6 kJ/mol and –87.3 J/(mol·K) values for $\Delta H^*$ (enthalpy of deactivation) and $\Delta S^*$ (entropy of deactivation), respectively. These values are significantly lower than those required by the native enzyme (78.3 kJ/mol and –72.0 J/(mol·K), respectively) and that reported for β-glucosidase from a thermophilic strain of *Aspergillus wentii* (125 kJ/mol and 65 J/(mol·K), respectively) (*36*) and chemically modified carboxymethyl cellulase (*13*) and β-glucosidase (*19*).

When enthalpy and entropy values for inactivation were calculated at each temperature, $\Delta S^*$ demanded by the mutant-derived α-Gal was lower than that needed by the native enzyme (Table 7). This suggests that triple mutation significantly reduced the unfolding of mutant-derived enzyme when exposed to the studied temperature range and behaved just like α-glucosidase from a thermophilic culture of *A. wentii* (*36*), which was found to be more ordered as revealed by its low values of $\Delta H^*$ (enthalpy of deactivation) and $\Delta S^*$ (entropy of deactivation).

## Conclusion

Triple mutation gave a stable and viable mutant for hyperproduction of α-galactosidase; the productivity was >2.0-fold more than that of the wild strain and may be exploited for bulk production of α-galactosidase using inexpensive agroindustrial substrates abundantly available in the country. Mutation made the enzyme production system more active and thermostable. Mutations (single, multiple or site-directed) had also been used previously to enhance thermostability (*29*,*35*). The enzyme derived from the mutant organism had aspartate on the acidic limb and histidine on the basic limb, which imparted higher efficiency of substrate binding with significantly minute substrate binding energy demand and could have academic and industrial application. This work provided insight into the inherent properties of thermostabilization by multiple mutations in protein ensemble. The mutation altered the values of both entropy and enthalpy of irreversible inactivation of enzymes as observed for thermostable or thermostabilized enzymes (*35*,*37*).

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## References

1. S.M. Kotwal, M.M. Gote, S.R. Sainkar, M.I. Khan, J.M. Khire, Production of α-galactosidase by thermophilic fungus *Humicola* sp. in solid-state fermentation and its application in soyamilk hydrolysis, *Process Biochem. 33* (1998) 337–343.
2. F. Jin, Y. Li, C. Zhang, H. Yu, Thermostable α-amylase and α-galactosidase production from the thermophilic and aerobic *Bacillus* sp.JF strain, *Process Biochem. 36* (2001) 559–564.
3. K. Kondoh, K. Morisaki, W.D. Kim, G.G. Park, S. Kaneko, H. Kobayashi, Cloning and expression of the gene encoding *Streptomyces coelicolor* A3(2) α-galactosidase belonging to family 36, *Biotechnol. Lett. 27* (2005) 641–647.
4. P. Ademark, M. Larsson, F. Tjerneld, H. Stålbrand, Multiple α-galactosidases from *Aspergillus niger*: Purification, characterization and substrate specificity, *Enzyme Microb. Technol. 29* (2001) 441–448.
5. M.I. Rajoka, A. Bashir, K.A. Malik, Mutagenesis of *Cellulomonas biazotea* for enhanced production of xylanases, *Bioresour. Technol. 62* (1997) 99–108.
6. F. Hölker, M. Höfer, J. Lenz, Biotechnological advantages of laboratory-scale solid-state fermentation with fungi, *Appl. Microbiol. Biotechnol. 64* (2004) 175–186.
7. G.T. Tsao, L. Xia, N.Cao, C.S. Gong, Solid-state fermentation with *Aspergillus niger* for cellobiase production, *Appl. Biochem. Biotechnol. 84-86* (2000) 743–749.
8. C.L. Wang, D.F. Li, W.Q. Lu, Y.H. Wang, C.H. Lai, Influence of cultivating conditions on the α-galactosidase biosynthesis from a novel strain of *Penicillium* sp. in solid-state fermentation, *Lett. Appl. Microbiol. 39* (2004) 369–375.
9. S.A. Furlan, A.L.S. Schneider, R. Merkle, M.F. Carvalho-Jonas, R. Jonas, Formulation of a lactose-free, low-cost culture medium for the production of β-D-galactosidase by *Kluyveromyces marxianus*, *Biotechnol. Lett. 22* (2000) 589–593.
10. O. Fridjonsson, H. Watzlawick, R. Mattes, Thermoadaptation of α-galactosidase AgaB1 in *Thermus thermophilus*, *J. Bacteriol. 184* (2002) 3385–3391.
11. K.E. Allen, M.T. McNally, H.S. Lowendorf, C.W. Slayman, S.J. Free, Deoxyglucose-resistant mutants of *Neurospora crassa*: Isolation, mapping, and biochemical characterization, *J. Bacteriol. 171* (1989) 53–58.
12. M.M. Gote, M.I. Khan, J.M. Khire, Active site directed chemical modification of α-galactosidase from *Bacillus stearothermophilus* (NCIM 5146): Involvement of lysine, tryptophan and carboxylate residues in catalytic site, *Enzyme Microb. Technol. 40* (2007) 1312–1320.
13. K.S. Siddiqui, A.A.N. Saqib, M.H. Rashid, M.I. Rajoka, Carboxyl group modification significantly altered the kinetic properties of purified carboxy methylcellulase from *Apergillus niger, Enzyme Microb. Technol. 27* (2000) 467–474.
14. F. Latif, M.I. Rajoka, K.A. Malik, Saccharification of *Leptochloa fusca* (kallar grass straw) by thermostable cellulases, *Bioresour. Technol. 50* (1994) 107–111.
15. I.U. Haq, S. Khurshaid, S. Ali, H. Ashraf, M.A. Qadeer, M.I. Rajoka, Mutation of *Aspergillus niger* for hyperproduction of citric acid from black strap molasses, *World J. Microbiol. Biotechnol. 17* (2001) 35–37.
16. O.H. Lowry, N.J. Roseborough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, *J. Biol. Chem. 193* (1951) 265–271.
17. G.L. Miller, Use of dinitrosalicylic acid (DNS) for determination of reducing sugars, *Anal. Chem. 31* (1959) 426–428.
18. A. Hanif, A. Yasmeen, M.I. Rajoka, Induction, production, repression and de-repression of exoglucanase synthesis in *Aspergillus niger*, *Bioresour. Technol. 94* (2004) 311–319.
19. M.H. Rashid, K.S. Siddiqui, Thermodynamic and kinetic study of stability of the native and chemically modified β-glucosidases from *Aspergillus niger, Process Biochem. 33* (1998) 109–115.
20. A.J. Afzal, S. Ali, F. Latif, M.I. Rajoka, K.S. Siddiqui, Innovative kinetic and thermodynamic analysis of a purified superactive xylanase from *Scopulariopsis* sp., *Appl. Biochem. Biotechnol. 120* (2005) 51–70.
21. K.S. Siddiqui, M.H. Rashid, M.I. Rajoka, Kinetic analysis of the active site of an intracellular β-glucosidase from *Cellulomonas biazote, Folia Microbiol. 42* (1997) 53–58.
22. M.I. Rajoka, M.W. Akhtar, A. Hanif, A.M. Khalid, Production and characterization of a highly active cellobiase from *Aspergillus niger* grown in solid state fermentation, *World J. Microbiol. Biotechnol. 22* (2006) 991–998.
23. S.K. Shankar, V.H. Mulimani, Alpha-galactosidase production by *Aspergillus oryzae* in solid-state fermentation, *Bioresour Technol. 98* (2007) 958–961.
24. R.C. Kuhad, M. Kapoor, R. Rustagi, Enhanced production of an alkaline pectinase from *Streptomyces* sp. RCK-SC by whole cell immobilization and solid-state cultivation, *World J. Microbiol. Biotechnol. 20* (2004) 257–263.
25. R.P. de Vries, H.C. van den Broeck, E. Dekkers, P. Manzanares, L.H. de Graaff, Differential expression of three α-galactosidase genes and a single β-galactosidase gene from *Aspergillus niger*, *Appl. Environ. Microbiol. 65* (1999) 2453–2460.
26. A.M. Elshafei, M.S. Foda, E. Abdel-Mobde, N.H. Ali, Optimization of α-galactosidase production in *Streptomyces erythrus*, *Acta Microbiol. Pol. 50* (2001) 53–63.
27. S. Aiba, A.E. Humphrey, N.F. Millis: *Biochemical Engineering*, Academic Press, New York, USA (1973) pp. 92–127.

*28.* N. Ozsoy, H. Berkkan, Production and characterization of α-galactosidase from *Aspergillus flavipes*, *Cell Biochem. Funct. 21* (2003) 387–389.

*29.* A. Converti, J.M. Domínguez, Influence of temperature and pH on xylitol production from xylose by *Debaryomyces hansenii*, *Biotechnol. Bioeng. 75* (2001) 39–45.

*30.* C. Vieille, J.G. Zeikus, Thermozymes: Identifying molecular determinants of protein structural and functional stability, *Trends Biotechnol. 14* (1996) 183–190.

*31.* J.C. Borges, C.H. Ramos, Protein folding assisted by chaperones, *Protein Pept. Lett. 12* (2005) 257–261.

*32.* H. Ishida, Y. Hata, A. Kawato, K. Abe, K. Suginami, S. Imayasu, Identification of functional elements that regulate the glucoamylase-encoding gene (*glaB*) expressed in solid-state culture of *Aspergillus oryzae*, *Curr. Genet. 37* (2000) 373–379.

*33.* T. Sripuan, K. Aoki, K. Yamamoto, D. Tongkao, H. Kumagai, Purification and characterization of thermostable α-galactosidase from *Ganoderma lucidum*, *Biosci. Biotechnol. Biochem. 67* (2003) 1485–1491.

*34.* M. Violet, J.C. Meunier, Kinetic study of the irreversible thermal denaturation of *Bacillus licheniformis* α-amylase, *Biochem. J. 26* (1989) 665–670.

*35.* J. Chen, W.E. Stites, Higher-order packing interactions in triple and quadruple mutants of staphylococcal nuclease, *Biochemistry, 40* (2001) 14012–14019.

*36.* G.I. Kvesitadze, R.S. Svanidze, V.L. Tsuprun, D.N. Nizharadze, L.T. Chirgadze, T.H. Buachidze, Quaternary structure and properties of β-glucosidase isolated from a thermophilic culture of *Aspergillus wentii*, *Bioorg. Khim. 16* (1990) 881–888.

*37.* N. Declerck, M. Machius, P. Joyet, G. Wiegand, R. Huber, C. Gaillardin, Hyperthermostabilization of *Bacillus licheniformis* alpha-amylase and modulation of its stability over a 50 °C temperature range, *Protein Eng. 16* (2003) 287–293.