ISSN 1330-9862
(FTB-2479)

original scientific paper

Hyperproduction and Thermal Characterization of a Novel Invertase from a Double Mutant Derivative of *Kluyveromyces marxianus*

Shaheen Aziz[1,2], *Fatima Jalal*[2], *Muhammad Nawaz*[2,3]*, *Bushra Niaz*[2,3], *Farman Ali Shah*[1,2], *Muhammad Hafeez-ur-Rahman Memon*[1], *Farooq Latif*[2], *Shahid Nadeem*[3] *and Muhammad Ibrahim Rajoka*[2,3]

[1]Department of Chemical Engineering, Mehran University of Engineering and Technology (MUET), Jamshoro, Sindh, PK-76062 Pakistan

[2]Industrial Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, PK-38000 Pakistan

[3]Government College University, Allama Iqbal Road, Faisalabad, PK-38000 Pakistan

Received: March 16, 2010
Accepted: December 8, 2010

Summary

Kinetics of intracellular invertase production employing a double mutant derivative of *Kluyveromyces marxianus* was optimized by varying different process variables in a 23-litre fermentor. The maximum volumetric rate (Q_P) and invertase yield ($Y_{P/S}$) by M15 mutant were 1222 U/(L·h) and 160 U/g of substrate utilized, respectively (2-fold more than those of parental strain) at 50 °C on the molasses (150 g/L of total fermentable sugars) at pH=5.5. Glucose or sucrose (100, 150 or 170 g/L) did not repress invertase catabolically under the optimized fermentation conditions, contrary to the previous reports on other yeasts and filamentous fungi, where catabolite repression of sugars was predominant. Invertases derived by the wild (I_W) and mutant (I_M) strains were purified employing ammonium sulphate precipitation, and then characterized by column chromatographic techniques both kinetically and thermodynamically. The acidic limb of invertases was missing and collation of pK_a and the heat of ionization values indicated that carboxyl groups were involved in proton transfer during active catalysis. Ratios of K_{cat}/K_m and v_{max}/K_m indicated that I_M was significantly more specific for sucrose hydrolysis. The I_M exhibited stability in different buffers at pH=3.0–10.0 and temperature of 50–70 °C, as reflected by long half-lives. I_M showed significantly lower values of enthalpy of activation (ΔH^*) and entropy of activation (ΔS^*), while Gibbs free energy (ΔG^*) was significantly increased at higher temperatures, making the I_M thermodynamically more thermostable. Thus I_M could be used as a catabolite-resistant invertase for the production of fructose syrup or high gravity ethanol.

Key words: enthalpy/entropy, invertase, *Kluyveromyces marxianus*, medium optimization, purification, thermostability

Introduction

Sugarcane molasses has D-sucrose and inulin as the major forms of carbohydrates. The breakdown of sucrose is catalyzed by invertases, also known as β-fructofuranosidases (*1*). Invertases (EC 3.2.1.26) belong to glycoside hydrolase family 32 (GH32) and during hydrolysis of sucrose they produce equimolar mixture of D-glucose and D-fructose at concentrations of sucrose lower than 10 %. This mixture is called invert sugar syrup and it

*Corresponding author; Phone: ++92 41 9201 553/374; Fax: ++92 41 920 1419; E-mail: m.nawaz9@gmail.com

has several commercial applications (*2–4*) including as a nutrient for honeybee rearing. In addition to a higher sweetening capacity, fructose is beneficial for diabetics, and it potentiates iron absorption in children (*5*).

Invertases also hydrolyze β-D-fructuranosides (raffinose and stachyose) whose hydrolyzates are useful in the production of confectionary and as an aid in fermentation of cane molasses. They are produced by both filamentous fungi and yeasts (*2,4,6,7*) but are subject to repression by glucose, fructose and sucrose. *Saccharomyces cerevisiae* strains can ferment molasses at high concentrations but invertase yield is low and the fermentation process is prolonged. Cultural conditions and nutritional demands for the enhanced production of invertase by *S. cerevisiae* in batch culture have been optimized using catabolite resistant mutant derivatives (*7*) but both Q_p (0.723 U/(L·h)) and $Y_{P/S}$ (2.036 U/g) were very low. *K. marxianus* strains produce invertase mainly intracellularly and inulinase extracellularly (*8*). They are noteworthy for their high ethanol formation rate, and broad substrate specificity, which makes them attractive candidates for industrial ethanol production in the summer when daytime temperature rises to 45–50 °C in different countries including Pakistan (*9*). Ethanol produced by *Kluyveromyces* strains is comparable to that produced by *S. cerevisiae* at industrial scale (*10*).

Combined effect of high sucrose content in molasses and high fermentation temperature is detrimental for final ethanol yield due to reduced activity of invertase (*9*). Commercial interest in invertase production is aimed at making such forms that produce more saccharified sugar mixtures without the loss of their enzyme activities. In order to exploit the potential of invertases in ethanol production, it is important to screen new microbes, optimize their production by mutation and/or regulation of process variables and to understand the structure-stability relationship of this enzyme.

The main objective of the current investigation is to develop a derepressed and thermotolerant mutant strain of *K. marxianus* capable of producing invertase from molasses at 50 °C in batch cultures using high sugar concentrations at which *S. cerevisiae* does not produce high invertase activity (*7*). The enzymatic activity of invertase from plants (*11*), and microorganisms including *S. cerevisiae* (*12*), *Candida utilis* (*13*), *Thermomyces lanuginosus* (*6*) and *Aspergillus ochraceus* (*2*) has been characterized. Detailed thermal properties of the purified enzymes (I_W and I_M) were determined to understand their thermostable nature. To our knowledge, this is the first detailed report on the characterization of intracellular invertase of *K. marxianus* strains. The application of such invertases will enhance the rate of sucrose hydrolysis, making this process economically more attractive.

Materials and Methods

Organism and reagents

Kluyveromyces marxianus strain was obtained from the Shakkar Gunj Distillery Ltd., Jhang, Pakistan. This culture was maintained on YPDA (yeast extract 1 %, peptone 2 %, glucose 8 % and agar 2.5 %) medium plates and slants as described earlier (*14*). For liquid culture studies, the seed culture developed in YPD (the same as YPDA but without agar) liquid medium was used. In fermentor studies, *K. marxianus* was grown on yeast salt medium containing (in g/L): glucose, sucrose or molasses (100, 150 or 170), KH_2PO_4 5.0, $(NH_4)_2SO_4$ 5.0, $MgSO_4 \cdot 7H_2O$ 0.20, and yeast extract 2.0 as a cheaper medium (*14*). The initial medium pH=5.5 was adjusted with 1 M H_3PO_4. All chemicals used were of analytical grade and were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany).

Isolation of mutants

K. marxianus cells were cultured in YPD liquid medium at 40 °C for 20 h, centrifuged at 15 000×*g* for 15 min, and suspended in 50 mL of saline containing 0.02 % yeast extract. The cell suspension (containing $3 \cdot 10^9$ mL^{-1}) was dispensed equally in 30-mL McCartney vials and exposed to 800 Gy of γ-rays in a Co-60 irradiator. The survivors were grown in the presence of 12 % sucrose, 1.5 % deoxyglucose (DG) and 8 % ethanol in yeast extract-peptone (YP; yeast extract 1 %, peptone 2 %) liquid medium (pH=5.5) at 50 °C for 48 h to isolate derepressed mutants, as described earlier (*15,16*). Among several putative mutants, one faster growing variant was selected and designated M-Shahraj1. This culture was regrown as above and exposed to 1200 Gy of γ-rays and the survivors from this exposure were grown in the presence of 12 % sucrose, 1.5 % DG-YP and 10 % ethanol medium at 60 °C for 72 h. Selection on solid medium was done as above. This procedure was repeated three more times to get stable mutants as described earlier (*15,16*). One mutant strain produced substantially higher invertase at 60 °C in the presence of ethanol in liquid culture and was designated *K. marxianus* M15 for further studies.

Batch culture studies

Fermentor studies were carried out in a microprocessor-controlled 23-litre stainless steel fermentor (Biostat C5, Braun Biotechnology, Melsungen, Germany) containing 15 L of culture medium as described earlier (*16*). The medium pH=5.5 (found optimum) was adjusted with H_3PO_4 and steam sterilized *in situ* for 1 h. When glucose or sucrose was used, it was added to the yeast salt medium after filter sterilization. The inoculum was prepared in 1000-mL conical flasks containing glucose (10 g/L) in YPD medium (pH=5.5) grown at 50 °C on an orbital shaker (150 rpm for 24 h). Ten percent of this freshly prepared inoculum was aseptically transferred to the fermentation medium (pH=5.5), which was controlled automatically using KOH during all fermentation experiments at 50 °C. Aeration was carried out through a sparger at 1 vvm for 8 h to enhance biomass yield before switching over to 0.25 vvm for additional 40 h. Foaming was controlled by adding pre-autoclaved silicone oil. Time course measurements were done in duplicate.

Extraction of intracellular invertases

During batch culture studies, 100 mL of culture were harvested by centrifugation at 4 °C (15 min, 15 000 rpm) in triplicate. The cell pellet was used as a source of intracellular invertase in each case separately. This pellet

was suspended in a buffer with vigorous vortexing to remove colouring compounds from the surface of the yeast and incubated for 1 h at 30 °C in 10 mL of enzyme extraction buffer containing phenylmethylsulphonyl fluoride at 5 mM (to inhibit proteases) as described earlier (*8*). This suspension was sonicated at 4 °C using a Soniprep 150 (MSE Ltd, Crawley, Sussex, UK) disintegrator for 5 min. Cell debris was removed by centrifugation at 4 °C as above. Enzyme activities present in the supernatant were preserved for assays and purification purpose. Extracellular fraction possessed small but measurable invertase activity, which was ignored.

Enzyme assays

To one mL of 0.16 M sucrose and 1 mL of sodium acetate/acetic acid buffer (100 mM, pH=5.5) mixture, 100 μL of appropriately diluted invertase solution were added. The reaction mixture was agitated at 50 °C for 30 min in a shaking water bath. The reaction mixture of 50 μL was added to 950 μL of distilled water and boiled for 10 min to inactivate the enzyme. The amount of formed glucose was determined using glucose oxidase kit (Bio-Con Valley®, Rostock, Germany). The assay and calculations were done as described by the manufacturer. One unit of enzyme activity is defined as the amount of enzyme which releases 1 μmol of glucose equivalent per mL per min. Inulin activity was determined using inulin from dahlia tubers. Assays consisting of 0.025 mL of properly diluted enzyme solution in 0.05 M citrate buffer (pH=5.0), and 10 mL of 1 % inulin were incubated at 50 °C for 30 min and the released sugars were quantified using DNS method, as described earlier (*8*).

Growth was measured spectrophotometrically as dry cell mass by measuring the absorbance at 620 nm using a standard curve for absorbance *vs.* cell mass. Difference of an absorbance unit was equal to 0.085 g per 100 mL. Glucose, fructose, sucrose and other products present in the culture supernatant were analysed by HPLC (PerkinElmer, Waltham, MA, USA) using column HPX-87H (300×78 mm) (Bio-Rad, Hercules, CA, USA) maintained at 45 °C in a column oven. Sulphuric acid (0.00025 M) in HPLC grade water was used as a mobile phase at 0.6 mL/min. The samples were detected by refractive index detector and quantified using Turbochron 4 software by PerkinElmer (*17*).

Determination of kinetic parameters

All kinetic parameters for batch fermentation were calculated as described previously (*18*). The product yield coefficient ($Y_{P/S}$) was determined by using the following relationship:

$$Y_{P/S}=\mathrm{d}P/\mathrm{d}S \qquad /1/$$

Volumetric rate of invertase formation (Q_p) was determined from the relationship:

$$Q_p=\mathrm{d}P/\mathrm{d}t \qquad /2/$$

The specific rate constant for invertase formation (q_p) was calculated using the relationship:

$$q_p=(1/X)\cdot\mathrm{d}P/\mathrm{d}t \qquad /3/$$

Purification of invertases

The crude extract (500 mL) from both wild and mutant cultures grown on 150 g of total sugars per L in molasses medium in batch fermentors was obtained, subjected to precipitation with 25 % ammonium sulphate at 0 °C and left overnight at 4 °C. The resulting precipitates collected by centrifugation at 10 000 rpm for 30 min were discarded and more ammonium sulphate was added to the supernatant to 80 % saturation and left overnight at 4 °C. The resulting precipitates were collected by centrifugation at 10 000 rpm for 30 min and dissolved in minimum volume of 10 mM phosphate buffer (pH=6.0). The dialysis was carried out against the same buffer in order to remove the salts. This enzyme sample was fractionated on diethylaminoethyl (DEAE) cellulose column (1.6×10 cm) equilibrated with Tris-HCl buffer (pH=8.5) at a flow rate of 1.0 mL per min. The bound proteins were eluted by a linear gradient of 0–1.5 M NaCl in Tris-HCl buffer (pH=8.5). Fractions (2 mL) were collected using a fraction collector FRAC-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) and assayed for protein and invertase activity. Active fractions containing invertase activity were pooled, dialyzed and freeze dried for further experiments.

The enzyme sample from the above step was dissolved in one tenth of the original volume of distilled water and applied to Sephadex G-100 column (1.6×60 cm) pre-equilibrated with 0.05 M phosphate buffer (pH=7.0) and eluted with the same buffer. The flow rate was maintained up to 0.5 mL/min and fractions of 1 mL were collected. The active fractions were combined and dialyzed against the same buffer and this dialysate was used as the final preparation for further experiments. The homogeneity and subunit molecular mass of this final preparation were checked by 10 % SDS-PAGE (*11*). The protein bands were visualized using Coomassie brilliant blue R-250 (0.25 %).

Protein estimation

All dialysates were preserved for enzyme activity measurements and determination of total protein. Total protein mass was estimated as described by Bradford (*19*) with bovine serum albumin (BSA) as a standard.

Characterization of invertases

pH stability and its effect on invertase activity

I_W and I_M were assayed at 50 °C and pH=3.0–11.0, as described earlier (*11*). pH stability data were obtained by incubating the enzymes in different buffers at 50 °C for different time intervals (0–120 min with 20-min increments), after which the remaining activity was determined. Dixon plots of enzyme activity in the above buffers at different temperatures were drawn to calculate the pK_a1 and pK_a2 of the acidic and basic limbs of enzymes and heat of ionization (*11*,*20*) in order to predict the most probable amino acids involved in sucrose binding and active catalysis (*11*). Natural log of the remaining activity (in %) was plotted against time to calculate the half-life of enzymes (*2*).

Effect of temperature on enzyme activity and determination of E_a

Both I_W and I_M were assayed at different temperatures ranging from 30 to 70 °C. The values of enzyme activity at each temperature were used to calculate the optimum temperature and activation energy demand for sucrose hydrolysis using Arrhenius relationship (*11*).

Kinetics of sucrose hydrolysis by invertases

I_W and I_M were assayed at 50 °C in the reaction mixtures (pH=5.0) containing different amounts of sucrose, and inulin (0.05 to 100.0 mM). The data were plotted according to the Lineweaver-Burk plot to determine the values of kinetic constants (v_{max} and K_m, K_{cat}, and K_{cat}/K_m) as described previously (*11*).

Thermodynamics of sucrose hydrolysis

The thermodynamic parameters, namely ΔH^* (enthalpy of activation), E_a (activation energy), ΔG^* (Gibbs free energy of activation), ΔS^* (entropy of activation), ΔG^*_{E-S} (Gibbs free energy of substrate binding) and ΔG^*_{E-T} (free energy of transition state formation) during sucrose hydrolysis were calculated as described previously (*11*,*20*).

Thermodynamics of enzyme stability

Thermodynamic parameters for irreversible thermal stability of I_W and I_M were estimated by incubating the enzyme in 100 mM acetate buffer (pH=5.0) in the absence of sucrose at six different temperatures ranging from 50 to 70 °C. Aliquots were withdrawn at different time intervals, cooled in ice for 3 h and assayed at 50 °C for enzyme activity. The data were fitted to the first order plots and analyzed as described earlier (*20*). The thermodynamic parameters for irreversible thermostability were calculated by rearranging the Eyring's absolute rate equation as described by Hussain *et al.* (*11*) using the following equation:

$$K_d=(k_bT/h)\ e^{(-\Delta H^*/RT)}\ e^{(\Delta S^*/R)} \qquad /4/$$

Statistical analysis

The effects of treatment were compared using M-STAT-C software (MSTAT, East Lansing, MI, USA). Duncan's multiple range tests were applied using two-factor factorial design. When two treatments were compared, Welch correction t-test was used applying GraphPad In-Stat 3.0 software (GraphPad Software, Inc, La Jolla, CA, USA). Significance is presented in the form of probability values, of which $p<0.05$ was considered significant.

Results and Discussion

Improved production of invertase by a double mutant of Kluyveromyces marxianus

By double mutation with γ-rays, a variant that could grow at 60 °C in the presence of 1.5 % deoxyglucose, 12 % sucrose and 10 % ethanol was evolved and designated mutant 15. It was found to be derepressed for invertase production (*12*,*15*). This mutant was checked for stability and it yielded consistent intracellular invertase. Extracellular invertase production was too small to be of any practical significance and was intentionally ignored. The biosynthesis of invertase was strongly dependent on the sugar used as the main carbon source. The highest levels of intracellular invertase activity were obtained in yeast salt culture medium supplemented with glucose (150 g/L) or molasses (150 g of total sugars per L), followed by sucrose (150 g/L) but not inulin. This enzyme preparation exhibited low activity on inulin and raffinose and confirmed the work of earlier researchers (*8*).

Representative time course of invertase production from 150 g of total sugars per L in molasses, or equivalent amount of glucose and sucrose (Fig. 1) indicated

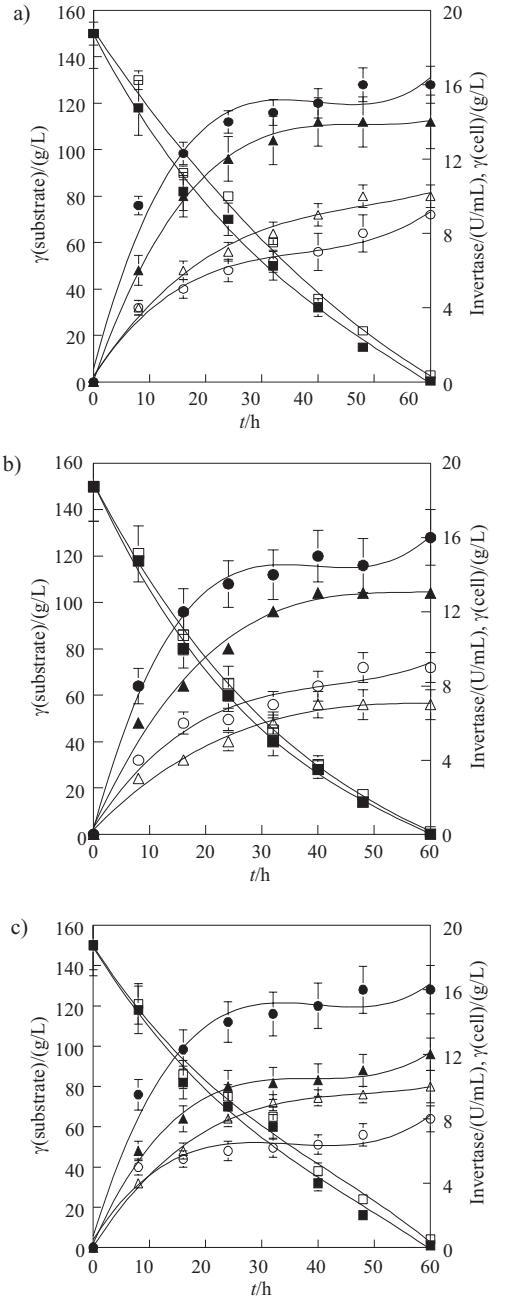

Fig. 1. Time course of the production of intracellular invertase by wild (○) and mutant (●) strains following the growth of wild (△) and mutant (▲) strains at 50 °C on 15 % of total sugars in: a) molasses, b) 15 % glucose and c) 15 % sucrose in yeast salt medium. Error bars show standard error between two experiments; □ and ■ stand for respective substrate concentrations in culture medium

that maximum invertase synthesis occurred after 40 h in continuously stirred 23-litre fermentor with a working volume of 15 L. The effect of substrate concentrations (Table 1) on kinetic parameters of mutant derivative revealed that 150 g of glucose per L and 150 g of total sugars per L in molasses medium (pH=5.5) inoculated with 10 % (by volume) inoculum at 50 °C supported significantly (p=0.001) higher values of invertase production (148 IU per g substrate). Among glucose, sucrose and molasses, 150 g of glucose or sucrose per L in the fermentation medium did not cause catabolite repression of intracellular invertase synthesis (Table 1), while in earlier studies with *Rhodotorula glutinis* and *S. cerevisiae*, glucose caused substantial catabolite repression of invertase synthesis (*3,5,12*). In some other DG-resistant mutant derivatives, glucose caused lower but measurable catabolite repression of invertase synthesis (*12*). The Q_P of intracellular invertase (1222 U/(L·h)) by the mutant derivative is several-fold higher than the values reported by other workers on *Aspergillus* spp., *S. cerevisiae* and their mutants (*2,7,12,13*). Recently, other authors (*7*) have isolated a triple mutant UME-2 of *S. cerevisiae* and optimized process parameters (incubation time 48 h, sucrose concentration 5.0 g per L, initial pH=6.0, inoculum size 2.0 %, by volume), and got Q_P of 0.723 U/(L·h), $Y_{P/S}$ of 2.036 U/g and q_P of 0.091 U per g of cells per h, which are quite negligible compared to our values (Table 1).

Table 1. Fermentation kinetic parameters of *K. marxianus* (W) and its mutant strain M15 (M) for invertase formation using yeast grown with ammonium sulphate (1.0 %) supplement (pH=5.5) on different carbon sources at 50 °C

w(carbon source) %		Strain	Q_P U/(L·h)	q_P U/(g·h)	$Y_{P/S}$ IU/g
glucose	10	W	505^{e}	135^{h}	65^{k}
		M	1025^{c}	287^{e}	120^{f}
	15	W	615^{d}	158^{g}	70^{j}
		M	1224^{a}	331^{a}	150^{a}
	17	W	516^{d}	170^{f}	85^{h}
		M	1124^{b}	323^{bc}	147^{c}
sucrose	10	W	602^{d}	124^{i}	64^{l}
		M	1022^{c}	273^{f}	118^{g}
	15	W	613^{d}	158^{g}	72^{j}
		M	1020^{c}	286^{e}	135^{d}
	17	W	612^{d}	169^{f}	74^{j}
		M	1122^{b}	302^{d}	147^{c}
molasses	10	W	604^{d}	224^{i}	74^{l}
		M	1024^{c}	286^{e}	120^{f}
	15	W	615^{d}	259^{g}	80^{j}
		M	1220^{a}	332^{a}	148^{b}
	17	W	615^{d}	269^{f}	115^{h}
		M	1122^{b}	324^{b}	140^{b}
LSD values (p≤0.05)			18.0	7.6	0.23
		p	<0.001	<0.001	<0.001

Each value is a mean of two experiments. Means in each column followed by different letters in superscript differ significantly at p≤0.05 using MSTAT-C software

Effect of temperature on enzyme production

Fermentation temperature strongly influenced fermentative ability of the mutant and wild strains. There was highly significant (p=0.001) impact of temperature (Table 2) on all kinetic parameters of invertase synthesis and invertase yield by the mutant was significantly (2-fold) improved at 50 °C and 4-fold improved at 60 °C compared to the wild-type culture. As temperature increased above 40–45 °C, the production rate and yield by the wild-type decreased rapidly but all parameters of the mutant strain did not change so abruptly. During the production process at different temperatures, stabilizing forces of metabolic network are supposedly provided by the system itself by assisting the folding of proteins of the metabolic network (*17*) most probably by acquiring chaperones (*16*). Other authors reported that under heat stress conditions, organisms accumulated glycerol or erythritol to survive (*18*).

Table 2. Fermentation kinetic parameters of *K. marxianus* (W) and its mutant strain (M) for invertase formation using ammonium sulphate (1.0 %), pH=5.5, in molasses medium (15 % sugars) at different temperatures

Temperature/°C	Strain	Q_P/(IU/(L·h))	$Y_{P/S}$/(IU/g)
35	W	848^{d}	86de
	M	960^{cd}	97^{cd}
40	W	859^{d}	110^{bc}
	M	986^{bcd}	122^{bc}
45	W	855^{d}	100^{bc}
	M	1112^{b}	143^{b}
50	W	512^{e}	77^{cd}
	M	1222^{a}	160^{a}
55	W	450^{f}	65^{e}
	M	1020^{bc}	135^{b}
60	W	240^{g}	35^{f}
	M	952^{cd}	100de
65	W	54^{h}	22^{g}
	M	654^{e}	84^{cd}
LSD values (p≤0.05)		125.5	14.23
	p	<0.001	<0.001

Each value is a mean of two experiments. Values in each column followed by different letters in superscript differ significantly at p≤0.05

Purification of invertases

Crude and dialyzed invertases from both strains of *K. marxianus* were purified applying ammonium sulphate precipitation, anion exchange and gel filtration chromatography. This three-step purification procedure of the I_W and I_M from *K. marxianus* resulted in 8.1- and 9.6-fold purification, respectively, while their recovery was 20.2 and 25.6 %, respectively (Table 3). These values are significantly higher than those reported previously (*2,3,8*).

Table 3. Purification of invertases from wild (I_W) and deoxyglucose-resistant mutant derivative (I_M) of *K. marxianus*

Treatment	Invertase	Total activity / IU	*m*(total protein) / mg	Specific activity / IU/mg	Purification factor	Recovery / %
crude	I_W	6200	400	15.5	1	100
	I_M	7800	450	17.3	1	100
$(NH_4)_2SO_4$	I_W	5015	300	16.7	1.10	80.9
precipitation	I_M	6525	325	20.1	1.17	83.7
anion exchange	I_W	3550	80	44.37	2.86	57.3
chromatography	I_M	4625	85	54.41	3.15	59.3
gel filtration	I_W	1250	10	125.0	8.1	20.2
chromatography	I_M	2000	12	166.7	9.6	25.6

All quoted values were taken after dialysis against distilled water

Catalytic and thermodynamic characterization of I_W and I_M

The subunit molecular masses of I_W and I_M were determined using 10 % SDS-PAGE (Fig. 2). They both had the same subunit molecular mass of 56 kDa, comparable with those of *K. marxianus* var. *bulgaricus* and *A. ochraceus* (*2*). Invertases of high subunit mass are produced by *Candida utilis*, *S. cerevisiae*, *Aspergillus* spp. and *Xanthophylomyces dendrorhous* (*2,13,21*).

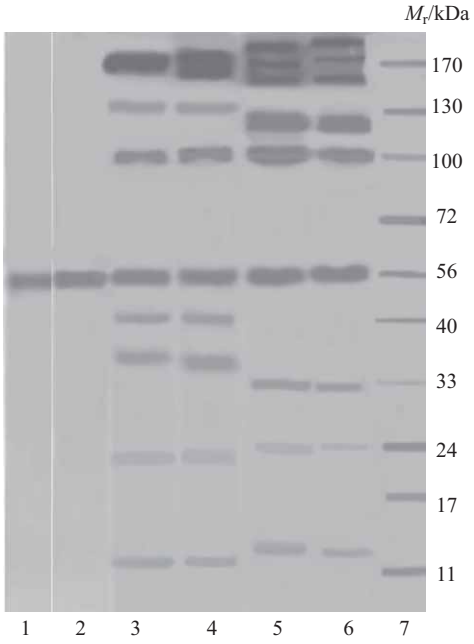

Fig. 2. SDS-PAGE of I_W (lanes 1, 3 and 4) and I_M (lanes 2, 5 and 6) with protein molecular mass markers (lane 7). Lanes 1 and 2 are purified I_W and I_M, respectively

Mutation did not change pH optima of I_M (Table 4). Dixon's analysis for pH-dependent invertase activity evaluated the pK_a of ionizable groups of amino acids present on acidic and basic limbs to control v_{max} for sucrose hydrolysis (Fig. 3). Ionization of active site residues is mainly dependent on the hydrogen ion concentration and temperature of the reaction mixture. Acid limb was missing in both I_W and I_M, while pK_a2 values were 6.2 and 7.4 for the I_M and I_W respectively (Table 4). It is interesting to note that the pK_a of the basic limb of I_M was slightly decreased. Comparison of the pK_a values along with heat of ionization (ΔH_i) (9.7 and 4.6 kJ/mol for I_W and I_M respectively) of the ionizable groups of amino acids (Table 4) on the basic limbs revealed the involvement of histidine and cysteine on I_M and I_W, respectively (Fig. 3). Mutation did not change the ionizable group of the acidic limb because the acidic limb was missing from both enzymes and most probably aspartate group was involved in both enzymes as a proton donor because pH optima were in the acidic range, as proposed by Le Roy *et al.* (*22*). The above properties of invertases suggest that they can be used for the investigation of highly thermostable invertase derived from catabolite-resistant mutant

Table 4. Physicochemical properties of invertases from parent (I_W) and deoxyglucose-resistant mutant derivative (I_M) of *K. marxianus*

Parameters	I_W	I_M	p[a]
Temperature optimum/°C	50–55	50–55	0.999
E_a/(kJ/mol)	39±2	24±1	0.0015
pH optimum	2.0–5.5	2.0–5.5	n.d.
$t_{1/2}$/min at pH=3	887±5	1086±10	0.0011
$t_{1/2}$/min at pH=10	42±2	78±3	0.0004
pK_a1	missing	missing	
pK_a2	6.2±0.2	7.4±0.3	0.0093
ΔH_i*/(kJ/mol)	9.7±0.2	4.6±0.1	0.0006
v_{max}/(U/mg·min) on sucrose	325±10	564±15	0.0002
v_{max}/(U/mg·min) on inulin	12±1	18±1	0.0018
K_m/mM	2.5±0.1	2.0±0.1	0.0036
v_{max}/K_m	130±11	282±15	0.0008
K_{cat}/s^{-1}	301±10	522±12	0.0001
ΔG*/(kJ/mol)	64±2	63±1	0.410
ΔH*/(kJ/mol)	36±1	21±1	0.0001
ΔS*/(J/(mol·K)	–86±10	–127±11	0.0172
ΔG^*_{E-S}/(kJ/mol)	2.46±0.03	1.86±0.025	0.0018
ΔG^*_{E-T}/(kJ/mol)	–12.9±0.5	–15±1	0.0009

n.d. – not determined

All values are mean±S.D. of three experiments; [a]p>0.05 statistically significant, calculated using Welch correction t-test with Graph Pad InStat v. 3.0 software (*www.graphpad.com*); *all values were calculated as described previously (*11*)

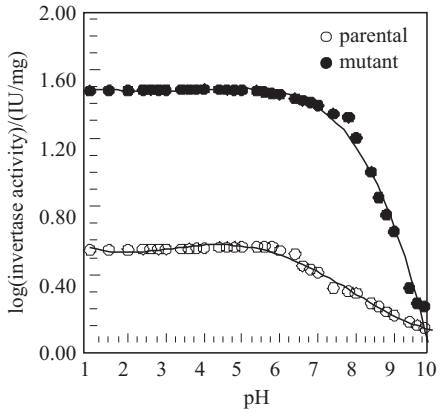

Fig. 3. Dixon plot of *K. marxianus* I_W (○) and its mutant derivative I_M (●) at 50 °C for the determination of pK_a of active site residues of ionizable amino acids

in order to improve sucrose hydrolysis in high gravity sucrose-based fermentations or for hyperproduction of invert sugar from high concentration of sucrose.

Invertase activities were quite stable in different buffers up to 120 min of incubation and exhibited different half-lives. Half-life of I_W preparations decreased from 887 to 42 min as the pH increased from 3.0 to 10, and it was 1086 min at pH=3.0 and 78 min at pH=10.0 in the case of I_M (Table 4). The I_M was more stable in all buffers than the I_W (results not shown). I_W and I_M from *K. marxianus* showed the same acidic pH optima (pH=2.0-5.5) (Table 4). Optimal pH from 2.6–6.5 has been reported for invertases from different yeasts and filamentous fungi (*6,13*).

The temperature optimum for I_W and I_M was 50–55 °C (Table 4) and was comparable to other microbial invertases, giving maximum activity at 55 °C (*2,8*). The E_a for I_M was 24 kJ/mol and was 1.6-fold lower (p=0.0015) than that needed by I_W (39 kJ/mol) for sucrose hydrolysis (Table 4). Invertase from *K. marxianus* var. *bulgaricus* needed 34.9 kJ/mol for sucrose hydrolysis. Similarly, invertases from mesophilic organisms required 38.7 kJ/mol of E_a (*2*), while commercial invertase and invertases from *S. cerevisiae* needed 73.96 kJ/mol to form activated complex with sucrose (*13*). Acidic invertase from sugarcane juice needed 55.3 kJ/mol for sucrose hydrolysis (*11*).

The I_W and I_M from *K. marxianus* were assayed at 50 °C using different sucrose concentrations for the determination of Michaelis-Menten kinetic constants (v_{max} and K_m) for which Lineweaver-Burk plot (figure not shown) was applied (Table 4). The I_M had significantly (p=0.0036) lower value of K_m as compared to I_W. This indicated that I_M had higher affinity towards binding sucrose to the active site. The turnover number (K_{cat}) (p=0.0001) and specificity constant (K_{cat}/K_m) values (p=0.0036) of both enzymes (Table 4) showed that the activity of I_M was significantly better compared to I_W.

The I_M was about 1.74-fold more active (p=0.0002) than I_W because their v_{max} values were 564 and 325 U per mg of protein per min, respectively. Furthermore, specificity constant (v_{max}/K_m) again confirmed that I_M was twofold more (p=0.0008) specific for sucrose as compared to I_W (Table 4). Intracellular invertase from *Aspergillus niveus* showed K_m value of 5.78 mM and v_{max} of 28.46 U per mg of protein per min (*2*). Invertases from *Candida utilis* and *S. cerevisiae* exhibited K_m values of 11 and 25 mM (*13*), respectively.

Thermodynamics of sucrose hydrolysis by I_W and I_M showed that the Gibbs free energy (ΔG^*) (p=0.410), and enthalpy of activation (ΔH^*) for sucrose hydrolysis was significantly (p=0.0001) lower in the case of I_M as compared to I_W at 50 °C (Table 4). The entropy of activation (ΔS^*) for substrate hydrolysis was also significantly (p=0.0172) lower in the case of I_M indicating that the formation of ES* complex and the conversion of reactant to product were both more spontaneous with I_M. The free energy for the formation of transition state complex (ΔG_{E-T}^*) for I_M was significantly (p=0.00009) lower and presented an evidence that the ability to form the transition complex was significantly higher for I_M as compared to I_W, as described earlier (*11*).

Irreversible thermostability of invertases

Irreversible thermostability represents the capability of enzyme molecules to resist thermal unfolding in the absence of substrate (*11,20,23*). Representative pseudo-first order plot of irreversible thermal denaturation of both enzymes is shown in Fig. 4a. I_M exhibited maximum half-life of 235 min at 55 °C and was 1.69-fold higher than that of I_W, and also higher than that of the acidic invertase of sugarcane juice (*11*). When half-lives of I_M were compared with those of I_W at each temperature (calculated from Fig. 4a), the I_M exhibited higher values (Table 5). With the increase in time-dependent temperature of reaction (55–70 °C), I_M showed significantly (p=0.0001) higher half-lives, which were 1.69- to 3.6-fold higher than those shown by the I_W.

In order to determine the thermodynamic parameters for irreversible thermal stability, the energy of activation for thermal denaturation, $E_{a(d)}$, was determined by applying Arrhenius plot (Fig. 4b). The activation energy ($E_{a(d)}$) for unfolding the transition state for I_W was significantly (p=0.0278) higher (83.5 kJ/mol) than that of I_M (66.94 kJ/mol). The values of ΔH^* (enthalpy) and ΔS^* (entropy) for irreversible stability (Fig. 4c) of I_M were 63.22 kJ/mol and –99.02 J/(mol·K), respectively. The respective magnitudes for I_W were 82.6 kJ/mol and –22.7 J/(mol·K). Therefore, up to 70 °C, both magnitudes of ΔH^* (p=0.0167) and ΔS^* (p=0.0001) for irreversible inactivation of I_M were significantly lower than those for I_W. Thus, double mutation made I_M significantly more thermostable than I_W. These values are also significantly lower than those reported for invertase from sugarcane juice (*11*) and other invertases (*24*), and comparable to those for xylanase derived from thermophilic mutant derivative of *Thermomyces lanuginosus* (*23*).

When enthalpy and entropy values for irreversible inactivation were calculated at each temperature, I_M again exhibited lower values of ΔH^* for the formation of transition state and they were significantly (p>0.01) lower than those needed by I_W (Table 5). This trend indicated that double mutation significantly (p=0.001) altered the conformation of I_M to protect it from thermal inactivation. Magnitudes of ΔG^* for the activation of unfolding of transition state were increased from 105.77 to 108.55 kJ/mol in the case of I_M at 70 °C, indicating that the I_M exhibited more resistance against thermal unfolding/denaturation. Moreover, ΔS^* magnitudes for thermal un-

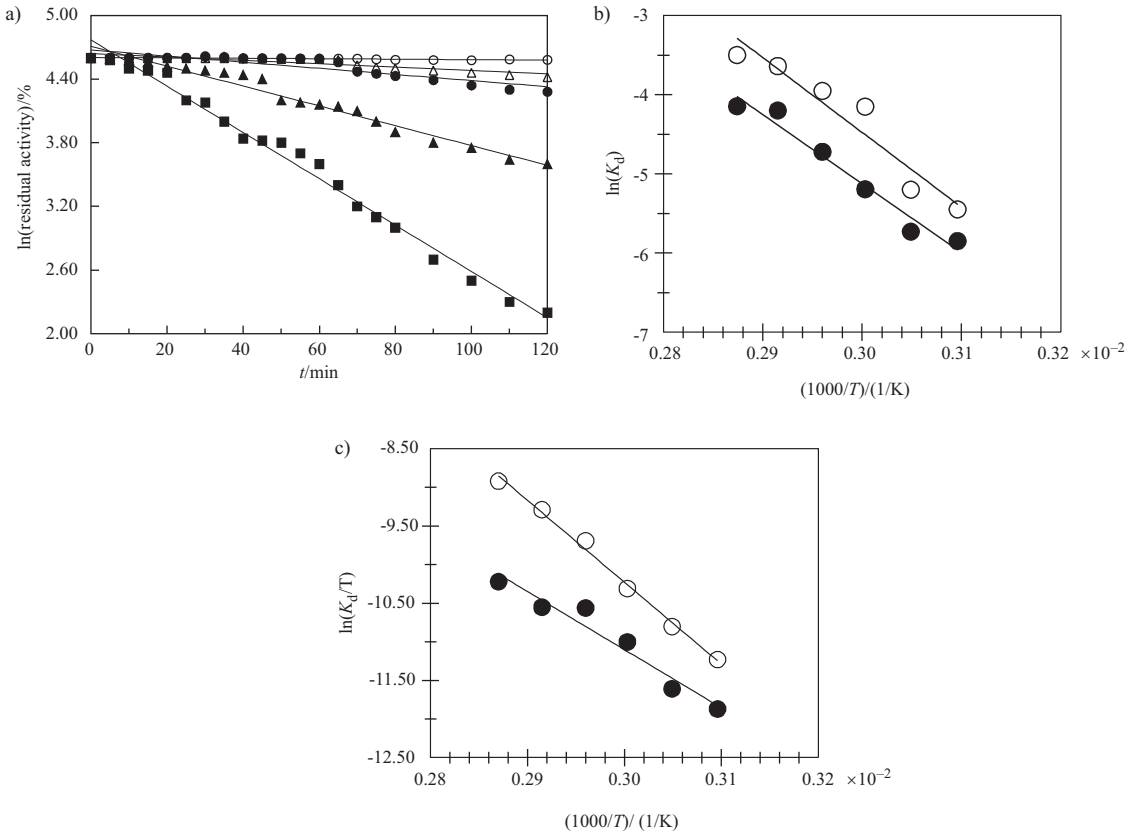

Fig. 4. Determination of thermodynamic parameters for thermal stability and irreversible inactivation: a) representative pseudo-first order plot for determination of specific rate of inactivation of I_M at 50 (○), 55 (△), 60 (▲), 65 (●) and 70 °C (■) respectively; b) determination of activation energy for inactivation ($E_{a(d)}$=slope·R/(kJ/mol)) of I_W (○) and I_M (●) at the above temperatures; c) determination of enthalpy and entropy of activation for irreversible thermal stability of I_W (○) and I_M (●) at the above temperatures

Table 5. Thermodynamics of irreversible thermal stability of I_W and I_M

Temperature		Invertase	K_d/min^{-1}	$t_{½}$/min	ΔH^*/(kJ/mol)	ΔG^*/(kJ/mol)	ΔS^*/(J/(mol·K))
°C	K						
45	318	I_W	0.0018	385^{d}	80.50^{a}	105.59^{i}	-78.90^{a}
		I_M	0.00168	412^{d}	64.30^{b}	105.77^{g}	-130.41^{b}
50	323	I_W	0.00264	263^{ef}	80.45^{a}	106.26^{f}	-79.91^{a}
		I_M	0.00224	309^{e}	64.25^{b}	106.70^{c}	-131.42^{b}
55	328	I_W	0.0050	139^{g}	80.41^{a}	106.20^{j}	-78.63^{a}
		I_M	0.00295	235^{f}	64.21^{b}	107.64^{d}	-132.41^{b}
60	333	I_W	0.0111	62.4^{ij}	80.37^{a}	105.66^{h}	-75.95^{a}
		I_M	0.0055	126^{gh}	64.17^{b}	107.60^{e}	-130.42^{b}
65	338	I_W	0.021	33^{ij}	80.33^{a}	105.49^{g}	-74.44^{a}
		I_M	0.0088	78.8^{hi}	64.13^{b}	107.91^{b}	-129.53^{b}
70	343	I_W	0.045	15^{j}	80.29^{a}	104.92^{l}	-71.81^{a}
		I_M	0.0126	55^{ij}	64.09^{b}	108.55^{a}	-129.62^{b}
	LSD		n.d	53.1	7.33	0.02	n.d.

$t_{½}$ – half-life calculated using the relation: ln $2/K_d$, where K_d is the specific rate of denaturation obtained from Fig. 4a

folding of transition state were significantly (p=0.001) lower in the case of I_M as well. These parameters suggested that there was negligible defolding of both enzymes when exposed to temperature range studied and both were significantly thermostable and performed sucrose hydrolysis more spontaneously than other invertases (*11,24*).

Thermostabilization is normally accompanied by a decrease in ΔS^* and an increase in ΔG^* (*11*). This behaviour has been noted only in the case of thermophilic enzymes (*11,20*). Thus both invertases reported here are apparently thermostable and more suited for commercial manipulation.

Conclusion

A thermostable, derepressed and stable mutant for invertase synthesis was developed. This mutant (named M15) exhibited significantly higher values of all kinetic parameters for invertase production under all fermentation conditions in a 23-litre fermentor. M15 produced maximum invertase at 50 °C, while the wild strain exhibited maximum production at 40–45 °C thus indicating that the mutation stabilized the metabolic network for invertase biosynthesis in the case of M15. Half-lives of enzymes in different buffers (pH=3.0–10.0), at different temperatures (50–70 °C) and magnitudes of thermodynamic parameters reflected that I_M showed values comparable to those for thermophilic enzymes and may have academic and industrial applications.

Acknowledgements

This work was supported by Higher Education Commission (HEC), Government of Pakistan, and Pakistan Atomic Energy Commission. We thank the Director of NIBGE for providing the research facilities. MUET permitted Shaheen Aziz and Farman Ali Shah to work at NIBGE with financial support of HEC. Technical assistance of Ali Ahmed is gratefully appreciated.

References

1. F. Alberto, C. Bignon, G. Sulzenbacher, B. Henrissat, M. Czjzek, The three-dimensional structure of invertase (β-fructosidase) from *Thermotoga maritima* reveals a bimodular arrangement and an evolutionary relationship between retaining and inverting glycosidases, *J. Biol. Chem. 279* (2004) 18903–18910.
2. L.H.S. Guimarães, A.F. Somera, H.F. Terenzi, M.L.T.M. Polizeli, J.A. Jorge, Production of β-fructofuranosidases by *Aspergillus niveus* using agroindustrial residues as carbon sources: Characterization of an intracellular enzyme accumulated in the presence of glucose, *Process Biochem. 44* (2009) 237–241.
3. M.C. Rubio, R. Runco, A.R. Navarro, Invertase from a strain of *Rhodotorula glutinis*, *Phytochemistry, 61* (2002) 605–609.
4. S.M. Kotwal, V. Shankar, Immobilized invertase, *Biotech. Adv. 27* (2009) 311–322.
5. C. Herwig, C. Doerries, I. Marison, U. von Stockar, Quantitative analysis of the regulation scheme of invertase expression in *Saccharomyces cerevisae, Biotechnol. Bioeng. 76* (2001) 247–258.
6. A. Chaudhuri, R. Maheshwari, A novel invertase from a thermophilic fungus *Thermomyces lanuginosus:* Its requirement of thiol and protein for activation, *Arch. Biochem. Biophys. 327* (1996) 98–106.
7. I. ul-Haq, S. Ali, A. Aslam, M.A. Qadeer, Characterization of a *Saccharomyces cerevisiae* mutant with enhanced production of β-D-fructofuranosidase, *Bioresour. Technol. 99* (2008) 7–12.
8. R.J. Rouwenhorst, W.S. Ritmeester, W.A. Scheffers, J.P. Dijken, Localization of inulinase and invertase in *Kluyveromyces* species, *Appl. Environ. Microbiol. 56* (1990) 3329–3336.
9. S. Gough, O. Flynn, C.J. Hack, R. Marchant, Fermentation of molasses using a thermotolerant yeast, *Kluyveromyces marxianus* IMB3: Simplex optimisation of media supplements, *Appl. Microbiol. Biotechnol. 46* (1996) 187–190.
10. W.R. Abdel-Fattah, M. Fadil, S.N. Nigam, I. Banat, Isolation of thermotolerant ethanologenic yeasts and use of selected strains in industrial scale fermentation in an Egyptian distillery, *Biotechnol. Bioeng. 68* (2000) 531–535.
11. A. Hussain, M.H. Rashid, R. Perveen, M. Ashraf, Purification, kinetics and thermodynamic characterization of soluble acid invertase from sugarcane (*Saccharum officinarum* L.), *Plant Physiol. Biochem. 47* (2009) 188–194.
12. A.M. Rincón, A.C. Codón, F. Castrejón, T. Benítez, Improved properties of baker's yeast mutants resistant to 2-deoxy-D-glucose, *Appl. Environ. Microbiol. 67* (2001) 4279–4285.
13. F.P. Chávez, L. Rodriguez, J. Díaz, J.M. Delgado, J.A. Cremata, Purification and characterization of an invertase from *Candida utilis*: Comparison with natural and recombinant yeast invertases, *J. Biotechnol. 53* (1997) 67–74.
14. M.I. Rajoka, F. Latif, S. Khan, R. Shahid, Kinetics of improved productivity of β-galactosidase by a cycloheximide-resistant mutant of *Kluyveromyces marxianus, Biotechnol. Lett. 26* (2004) 714–746.
15. A. Mizuno, H. Tabei, M. Iwahuti, Characterization of low-acetic-acid-producing yeast isolated from 2-deoxyglucose-resistant mutants and its application to high-gravity brewing, *J. Biosci. Bioeng. 101* (2006) 31–37.
16. M.I. Rajoka, M. Ferhan, A.M. Khalid, Kinetics and thermodynamics of ethanol production by a thermotolerant mutant of *Saccharomyces cerevisiae* in a microprocessor-controlled bioreactor, *Lett. Appl. Microbiol. 40* (2005) 316–321.
17. J.C. Borges, C.H.I. Ramos, Protein folding assisted by chaperones, *Protein Pep. Lett. 12* (2005) 257–261.
18. S. Aiba, A.E. Humphrey, N.F. Millis: *Biochemical Engineering*, Academic Press, New York, NY, USA (1973) p. 92.
19. M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem. 72* (1976) 248–254.
20. K.S. Siddiqui, A.A. Najmus-Saqib, M.H. Rashid, M.I. Rajoka, Carboxyl group modification significantly altered the kinetic properties of purified carboxymethylcellulase from *Aspergillus niger*, *Enzyme Microb. Technol. 27* (2000) 467–474.
21. D. Linde, I. Macias, L. Fernández-Arrojo, F.J. Plou, A. Jiménez, M. Fernández-Lobatto, Molecular and biochemical characterization of a beta-fructofuranosidase from *Xanthophyllomyces dendrorhous*, *Appl. Environ. Microbiol. 75* (2009) 1066–1073.
22. K. Le Roy, W. Lammens, M. Verhaest, B. De Coninck, A. Rabijns, A. Van Laere, W. Van den Ende, Unraveling the difference between invertases and fructan exohydrolases: A single amino acid (Asp-239) substitution transforms *Arabidopsis* cell wall invertase 1 into a fructan 1-exohydrolase, *Plant Physiol. 145* (2007) 616–625.
23. S.A.I. Bokhari, F. Latif, M.I. Rajoka, Purification and characterization of xylanases from *Thermomyces lanuginosus* and its mutant derivative possessing novel kinetic and thermodynamic properties, *World J. Microbiol. Biotechnol. 25* (2009) 493–502.
24. L. Zhang, C. Zhao, D. Zhu, Y. Ohta, Y. Wang, Purification and characterization of inulinase from *Aspergillus niger* AF 10 expressed in *Pichia pastoris*, *Protein Expr. Purif. 35* (2004) 272–275.