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PURIFICATION AND CHARACTERIZATION OF TWO INVERTASES FROM MUTANT STRAIN OF *SACCHAROMYCES CEREVISIAE*

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Abstract

In the present study, a mutant strain of *Saccharomyces cerevisae* EMS-42 was used for the biosynthesis of invertase (E.C.3.2.1.26). Both types of invertases i.e., extracellular and intracellular invertase are present in *S. cerevisae.* An extracellular invertase was purified to homogeneity by two step purification i.e., ammonium sulfate precipitation and DEAE-Sephadex A-50. The enzyme was present in the supernatant of 85% saturation being glycoprotein in nature. DEAE column chromatography eluted enzyme as single active fraction at 0.2 M NaCI. The enzyme was purified by 15 fold with recovery of 38%. The molecular mass of 110 kDa was determined after SDS-PAGE. The carbohydrate content was found to be 48%. The intracellular invertase contains both forms of glycosylated (large) and non-glycosylated (small). The similar above procedure was applied for glycosylated intracellular invertase (L-form) while three steps for non-glycosylated invertase (S-form). The L-form was purified by 19 fold with recovery of 32%. Like extracellular invertase, the molecular weight was (110 kDa) for L-form. Ammonium sulfate precipitation separated the enzyme (S-form) as insoluble fraction. The enzyme was eluted at 0.3 M NaCl using DEAE-Sephadex. A single band of molecular weight (55 kDa) was estimated after Sephadex G-50 with purification (16 fold) and recovery of 17%. Both types of invertases were isolated as monomeric protein. The optimum pH, temperature, $MnCl_2$ and the values of the K_m and V_{max} for non-glycosylated and glycosylated were found to be as 5, 50 and 60ºC, (109 and 111%), (1.2 mM and 909 U/ml/min, 1.8 mM and 1429 U/ml/min), respectively.

Introduction

The yeast *Saccharomyces cerevisiae* is a rich source of both intracellular and extracellular invertase (β.D. fructofuranosidase, E.C. 3.2.1.26) catalyzes the reaction of detachment of the terminal nonreducing β.D. fructofuranoside residue in β.D. fructofuranosides (Amin *et al*., 2010). Its preferred substrate is saccharose but invertase is also able to catalyze the hydrolysis of raffinose, stachyose and inulin (Belcarz *et al.,* 2002; Gore *et al*., 2009). There are many industrial uses of invertase in the production of invert syrup, non- crystallizing creams, jams, artificial honey, lactic acid and ethanol, confectionary, in digestive aid tablets, powder milk for infants and other infant foods etc. (Haq and Ali, 2007; Acosta *et al*., 2000; Phadtare *et al.,* 2004; Safarik *et al.,* 2009).

Invertase exists in two forms, glycosylated periplasmic protein and cytosolic non-glycosylated protein (Vitolo *et al.,* 1995; Rashad *et al*., 2006). The secretion of enzyme located intracellularly which corresponds to repressed forms of invertase and extracellular one, containing nine or ten N-glycosidically linked oligosaccharides which corresponds to the de-repressed form of the enzyme (Huffaker & Robins, 1983) are regulated by catabolic repression. The high concentration of glucose in the culture medium completely repressed production of the enzyme, whereas the use of sucrose or raffinose as carbon source allowed derepression of invertase synthesis. It is known that both of these enzymes are synthesized on the matrix of the same structural gene and their protein moieties have a molecular mass of 60 kDa (Moreno *et al*., 1990).

Sonication is one of the most commonly employed methods for cell disruption (James *et al.,* 1972). Ultrasound has been used to extract and release intracellular enzymes such as invertase from *S. cerevisae* being secreted to the periplasmic space (Balasundaram & Pandit, 2001).

For the purification of proteins different precipitants such as ammonium sulphate, acetone, ethanol etc have been used as initial purifying agents (Pimpa 2004). The column chromatography has been a popular technique for isolation and quantifying the components from mixture of the compounds. For purification of invertase, mostly anion exchange chromatography and gel filtration techniques are used (Guimaraes *et al*., 2007; Uma *et al*., 2010).

Materials and Methods

Microorganism: *Saccharomyces cerevisiae* IS-66 was isolated from different fruits and soil samples and identified by Wickerham (1951) and Lodder & Kreger-Van (1952). It was chemically mutated by ethyl methane sulfonate (EMS). One mutant EMS-42 was achieved after treating with EMS concentration of 100 µl/ml at 15 min exposure time and selected as hyperproducer for invertase. It was maintained on YPSA medium containing (g/l), Yeast extract 3, Peptone 6, Sucrose 15 and Agar 20.

2-deoxy D-glucose resistance: The potential mutant strains were cultured overnight on the YPS agar medium, harvested during the exponential phase of growth (1×10^7 cells/ml), washed with sterilized distilled water and plated on the 2dg-YPR agar medium containing (mg/ml): yeast extract 3, peptone 5, raffinose 20, agar 20 and 2-deoxy-Dglucose (0.02-0.10). The concentration of 0.04 mg/ml was found optimal, as at this level EMS-42 gave consistent invertase production. Colonies exhibiting the most vigorous growth were tested for stability in invertase production by shake flask fermentation. The master culture was preserved in sterilized 20 % (v/v) glycerol at -80°C.

Preparation of inoculum: Fifty millilitre of the YPS medium was transferred to the individual 250 ml Erlenmeyer flasks. Cell suspension was prepared from a

2-3 day old slant culture. One millilitre of the cell suspension was aseptically transferred into the flask and incubated at 30°C in a rotary shaking incubator (Model: 10X400.XX2.C, SANYO Gallenkamp, PLC, UK) at 200 rpm for 18 h.

Invertase production in stirred fermentor: Production of invertase was carried out in a laboratory scale stirred fermentor of 7.5 L capacity with working volume of 5 L (New Brunswick Scientific Bioflo 110, USA). The inoculum was transferred into YPS broth medium at level of 7.5%, v/v. The cultural conditions were maintained as agitation speed (240 rpm), aeration rate (1 vvm), dissolved oxygen (10%) at 30°C for 24 h.

Isolation of extracellular invertase: The cell free broth was used for two step purification of extracellular invertase by ammonium sulfate precipitation and DEAE-column chromatography.

Isolation of intratracellular invertase: The harvested cells from stirred fermentor were washed once with acetate buffer, pH 5.0. resuspend in the same buffer with 40 µM phenylmethylsufonylfluride (PMSF). The cell suspension (pH 5) was sonicated with 0.5 duty cycle of impulses at amplitude (40%) for 60 min, using probe (Horn H22 D) immersed 2.5 cm in the suspension. The crude extract after sonication was spun at 12,000×*g* for 15 min at 4°C.

Purification of invertase: Both enzymes (extracellular and intracellular) from *S. cerevisiae* EMS-42 were purified to homogeneity by following purification steps.

Ammonium sulfate precipitation: The ammonium sulfate was added at varying amounts (20-85%) to cell free broth contained extracellular invertase and supernatant crude extract having intracellular invertase. They were spun at 18,000×*g* for 30 min to get the precipitates of all fractions. The resultant precipitates and supernatant were dissolved in 0.5 M Tris-HCl buffer, pH 7.5 and dialyzed against same buffer.

Anion-exchange chromatography: For the purpose of ion exchange chromatography, 1 g DEAE-Sephadex A-50 (Sigma, USA) was swollen in 100 ml of the 0.05 M Tris-HCl buffer, pH 7.5 in a boiling water bath for 2 hours. After cooling poured it into the column and made final bed volume (1.5 × 15.0 cm). The dialyzed enzyme solution was applied to column that pre-equilibrated with five column volumes of the 0.05 M Tris-HCl buffer, pH 7.5. A linear NaCl gradient from 0 to 1 M in 150 ml of the same buffer was applied. Fractions of 3 ml were collected at a flow rate 0.5 ml/min. The collected fractions were assayed for protein at 280 nm and invertase activity by performing enzyme assay. The fractions containing enzyme activity were pooled, dialyzed and analyzed on SDS-PAGE.

Gel filtration: Sephadex G-50 (Phamacia Fine Chemical), 2 g was swollen in 50 ml of 0.05 M Tris-HCl buffer, pH 7.5 in a boiling water bath for 2 h. Poured the gel slurry along the side of tilted column by taking care that no air bubble was entrapped. The column (1.5 × 20 cm) was equilibrated with five column volumes of the 0.05 M Tris-HCl buffer, pH 7.5 in order to stabilize the bed. The enzyme sample (3 ml) was eluted with the same buffer; adjusting flow rate at 0.5 ml/min. The collected fractions were assayed for protein and invertase activity. The active enzyme fractions were pooled, dialyzed and used for enzyme characterization.

Dialysis: The salts were removed from precipitates and pooled samples by using 12,000 molecular weight cut off dialyzing bag, which was placed in one liter of the 0.05 M Tris-HCl buffer (pH 7.5) for 5-6 h at 4ºC. The process was repeated 4-5 times until all salts were removed from the enzyme solution.

Electrophoresis: At each step of purification, sodium dodecyle sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Hames (1990).

Protein marker: The molecular weight of the invertase was estimated by SDS- polyacrylamide gel with protein marker (BIORAD, Catalog #161-0363).

Carbohydrate content: The total carbohydrate content was detected by the phenol sulfuric acid method (Dubois *et al*., 1956) with mannose as standard.

Characterization of purified invertase

Effect of pH and temperature on stability of invertase: It was observed by taking hundreds microlitres of appropriately diluted enzyme solution incubated in 0.05 M citrate/ 0.05 M acetate buffer at different pH values ranging for 2-8 at 40°C for 15 min. At optimal pH, the invertase activity was tested at varying temperature values (20-80°C). For this, the reaction mixture was incubated for 15 min at different temperatures and residual activity in both parameters was determined under standard conditions (Akgol *et al.,* 2001).

Effect of additives on enzyme activity: Different chemicals and metal ions such as NaCl, KCl, $MnCl_2$, EDTA, $BaCl_2$, $MgCl_2$, $CuSO_4$, $HgCl_2$, $CoCl_2$, $FeSO_4$, $CaCl_2$ and $ZnSO_4$ preincubated with the purified enzyme at 1 mM at 30°C for 30 min. before determination of the enzyme activity. Blank was taken showing relative activity (100 %) before adding the metals.

Determination of kinetic constant (K_m): The K_m value of the invertase is determined using sucrose as a substrate and using Lineweaver-Burk plot (Lineweaver & Burk, 1934) by following conditions of sucrose (10-100 mM) in 0.05 M acetate buffer (pH 4.5), incubation time (15 min) and temperature (35°C). The amount of liberated reducing sugars was measured by Miller, (1959).

Determination of maximum velocity (V_{max}): The maximum velocity (V_{max}) of sucrose hydrolysis of invertase under same optimal conditions of kinetic constant was calculated.

Results and Discussion

An extracellular invertase from *S. cerevisiae* EMS-42 was purified through successive steps of ammonium sulfate (40-85%) precipitation and DEAE-Sephadex A-50 (Table 1). The key step involved a fractionation of insoluble and soluble forms of invertase apparently due to differences in carbohydrate content of the enzymes. The ammonium sulfate at the 85% saturation level separates the external (glycosylated) invertase as soluble fraction while giving insoluble in precipitated form. Gascon & Lampen (1968) separated the external invertase from the internal by ammonium sulfate precipitation method. Both precipitated and soluble fractions (85% ammonium sulfate supernatant) were dialyzed and run on SDS-PAGE as shown in Fig. 1. The one major peak as shown in Fig. 2 was eluted by using 0.2 M NaCI. When this peak was tested by electrophoresis, only one broader band (Fig. 3) was found with molecular mass of 110 kDa. Milintawisamai *et al*., (2007) found extracellular invertase from *C. humicolus* as the result from 60-100% ammonium sulfate saturation followed by DEAE column chromatography and eluted extracellular protein fraction using 0.3-0.35 M NaCl with molecular mass of 110 kDa as a single active fraction. The purified extracellular was glycoprotein in nature with 48% carbohydrate content.

Table 1. Purification steps of extracelluar invertase.

Purification steps	Volume (ml)	Total activity (U)	Total Protein (mg/ml)	Specific activity (U/mg)	Fold Purification	Activity recovery (%)
Crude broth	1000	53120	404	131	-	100
Freeze dried ammonium sulfate supernatant (85%)	200	34016	210	162	1.2	64
DEAE-Sephadex	8	20110	10.5	1915	15	38

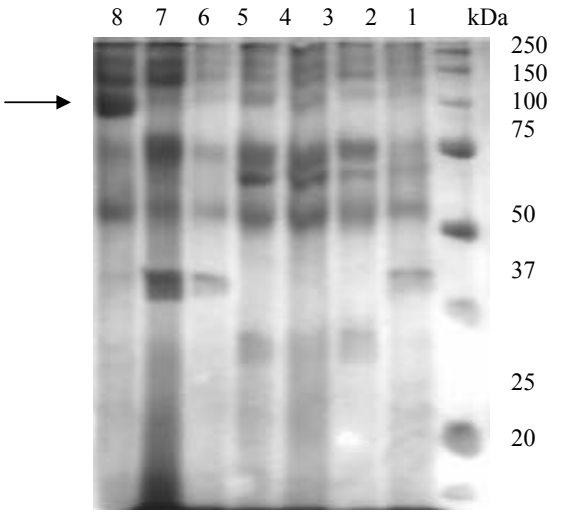

Fig. 1. The SDS-PAGE after ammonium sulfate treatment for extracellular invertase.
*Lane 1, Protein marker
Lanes 2-7, 60-70% ammonium sulfate precipitation fractions
Lane 8, concentrated fraction of 85% supernatant

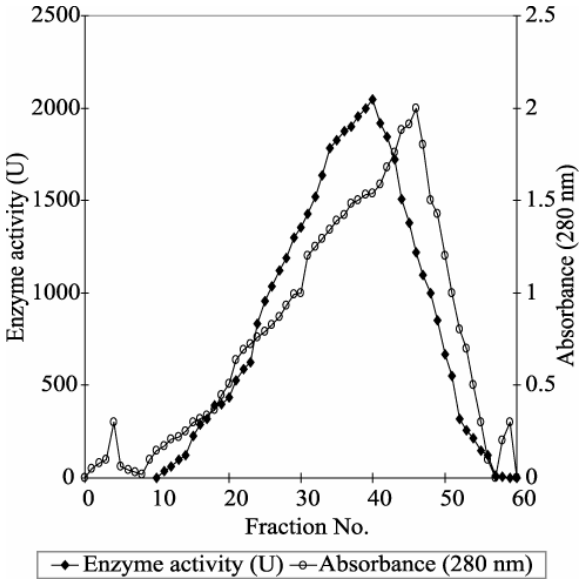

Fig. 2. Elution pattern on DEAE-Sephadex for extracellular glycosylated invertase.

The specific activity of the purified extracellular invertase was estimated to be 1915 U/mg, which is about 15 fold than that of the crude enzyme with final activity recovery of 38%. The optimum pH and temperature were found to be as 5 and 60°C, respectively (Figs. 7 & 8). Chavez *et al*., (1997) obtained invertase from *Candida utilis* with molecular weight of 150 kDa at pH of 5.5 temperature of 60-75°C. Table 3 depicts the data on the effect of additives such as NaCl, KCl, $MnCl_2$, EDTA, $BaCl_2$, $MgCl_2$, $CuSO_4$, $HgCl_2$, $CoCl_2$, $CuCl_2$, $FeSO_4$, $CaCl_2$ and $ZnSO_4$ at the concentration of 1 mM on glycosylated invertase. Of the all, $MgCl_2$, $MnCl_2$ and $CoCl_2$ was found to be as slightly stimulatory with relative activity from 102-111 % while remaining caused reduction in relative activity. The addition of $HgCl_2$, $CuSO_4$ and $CuCl_2$ completely inhibited the enzyme. By using Lineweaver-burk plot, the K_m and V_{max} values were observed to be as 1.8 mM and 1429 U/ml/min, respectively (Fig. 9). Hernalsteens & Maugeri (2008) gave the K_m (13.4 g/l) and V_{max} (21 µmol/ml/min) for sucrose by invertase in *Candida* sp.

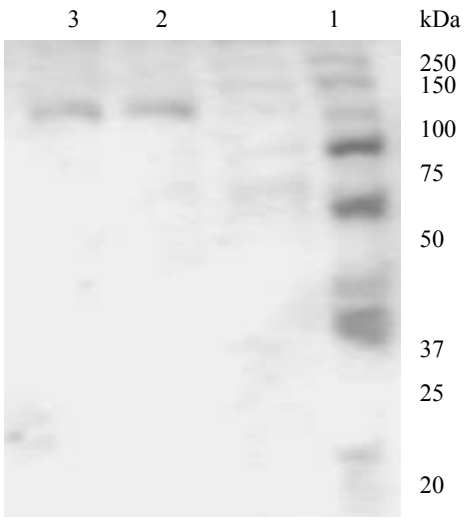

Fig. 3. The SDS-PAGE of purified extracellular invertase.
*Lane 1 Protein marker. Lanes 2-3, purified extracellular invertase

The intracellular invertase from mutant strain was purified successively through three step i.e., ammonium sulfate (20-85%) saturation, DEAE-Sephadex A-50 and Sephadex G-50 (Table 2). The procedure of ammonium sulfate was also used for the separation of two forms of intracellular invertase, one in small amount (S-form) being non-glycosylated was recovered from (20-85%) saturation in precipitated form. On the other hand, the second form in large amount (L-form) was found from supernatant of 85% ammonium sulfate saturation being glycoprotein in nature. It means that extracellular invertase isolated from the cell free broth was secretory periplasmic enzyme. Carlson *et al*., (1983) and Batista *et al*., (2004) reported that two forms of invertase (secreted and non-secreted) are exist in *S. cerevisiae*. It can be extracted from the cells in the same glycosylated form as shown in Fig. 1 (lane 8, arrow indicated) & Fig. 4 (lanes 2-3). In both cases the bands were broad having same molecular weight of 110 kDa. The further purification of L-form invertase was obtained in the same manner as extracellular invertase. For the purification of S-form invertase, the collected precipitates were dialyzed and then loaded on DEAE-Sephadex column. After anion-exchange, out of four protein peaks one peak was eluted at 0.3 M NaCl showing enzyme activity as shown in Fig. 5. Kern *et al*., (1992) found intracellular invertase in yeast at NaCl (0.15-0.3 M) by using Q-Sepharose and found it of 115 kDa. Similar finding by Trimble & Maley (1977) who obtained carbohydrate free invertase composed of two 60 kDa subunits in *S. cerevisiae*. After pooling active fractions, the concentrated protein was further purified on Sephadex G-50 column. As that result only one protein peak showing invertase activity was obtained (Fig. 6). After SDS-PAGE only one protein band was found having approximately molecular weight of 55 kDa as shown in (Fig. 4, lane 5). After Phenol-Sulfuric test the L-invertase was found to be glycoprotein and S-invertase as carbohydrate-free protein. Both glycosylated and non-glycosylated invertase proteins were found to be monomeric being single bands. In contrary it was reported as dimer, tetramers, hexamers, octamer, (Trimble & Maley 1977; Chu *et al*., 1983; Rodriguez *et al*., 1995).

Table 2. Purification steps of intracelluar invertase (S and L forms).

Foms of intracellular invertase	Purification steps	Volume (ml)	Total activity (U)	Total protein (mg/ml)	Specific activity (U/mg)	Fold purification	Activity recovery (%)
S-form invertase	Crude extract	500	29700	287	103	-	100
	Ammonium sulfate (20-80%)	100	10346	95	109	1.1	35
	DEAE-Sephadex	28	7300	28	260	2.5	25
	Sephadex G-50	1.5	5011	03	1670	16	17
	Freeze dried ammonium sulfate supernatant (85%)	100	14221	72	98	1.9	46
L-form invertase	DEAE-Sephadex	2.5	9820	05	1964	19	32

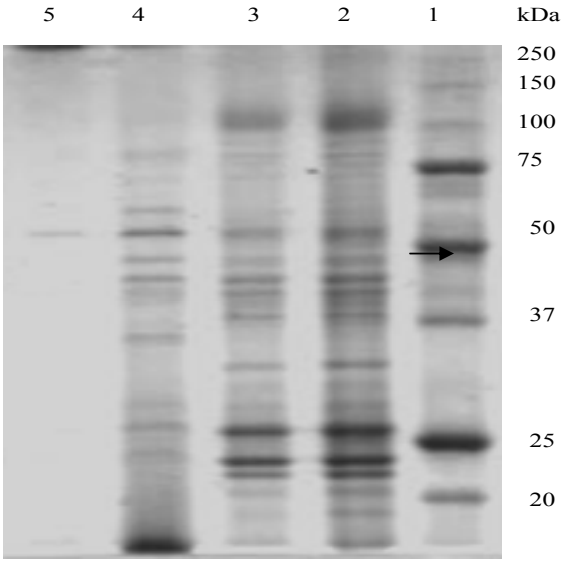

Fig. 4. The SDS-PAGE after ammonium sulfate and chromatography for intracellular invertase.
* Lane 1, protein marker
Lanes 2-3, intracellular crude extract
Lane 4, 60% ammonium sulfate precipitate
Lane 5, purified non-glycosylated intracellular S-invertase

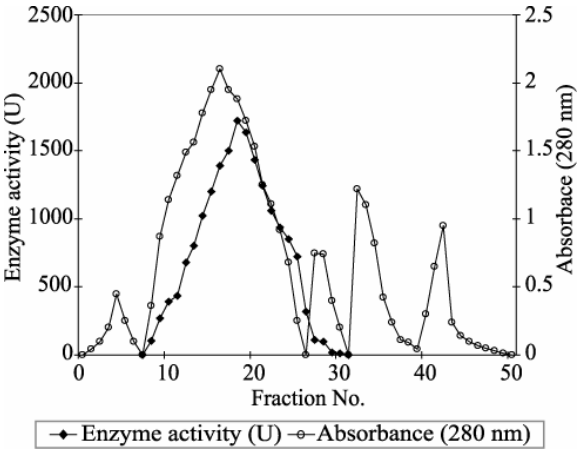

Fig. 5. Elution Pattern on DEAE-Sephadex for intracellular non-glycosylated invertase.

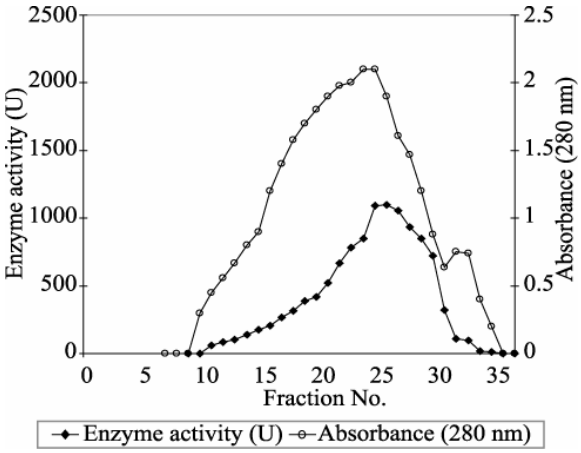

Fig. 6. Elution Pattern on Sephadex G-50 for intracellular non-glycosylated invertase.

The specific activity of the purified intracellular S-invertase and L-invertase were estimated to be as 1670 U/mg, 1964 U/mg and fold purification of 16, 19 with recovery of 17% and 32%, respectively as shown in Table 2. The optimum pH (5) and temperature (50°C) of non-glycosylated invertase was found to be observed (Figs. 7 & 8). The effect of chemicals and metal ions such as NaCl, KCl, $MnCl_2$, EDTA, $BaCl_2$, $MgCl_2$, $CuSO_4$, $HgCl_2$, $CoCl_2$, $CuCl_2$, FeSO4, $CaCl_2$ and $ZnSO_4$ on non-glycosylated invertase was also investigated (Table 3). The findings after adding all additives was almost similar to glycosylated invertase but with slight decrease of relative activity. In contrast with glycosylated, the nonglycosylated internal form of invertase is extremely sensitive to proteolysis (Williams *et al.,* 1985). From Lineweaver-burk plot, the K_m and V_{max} values for intracellular non-glycosylated were found to be as 1.2 mM and 909 U/ml/min, respectively (Fig. 10). Similar findings by Belcarz *et al*., (2002) were obtained K_m values against sucrose for S (slow) and F (fast) forms of invertase in *Candida utilis* as 2 and 1.5 mM, respectively.

Table 3. Effect of additives on stability of purified glycosylated and non-glycosylated invertase.

Additives (1 mM)	Relative activity (%)	
	Non- glycosylated	Glycosylated
Control	100	100
NaCl	93 ± 0.2	96 ± 0.5
KCl	96 ± 1.0	96 ± 1.1
$MnCl_2$	109 ± 0.04	111 ± 0.09
EDTA	102 ± 1.5	103 ± 2.5
$BaCl_2$	97 ± 0.3	98 ± 1.3
$MgCl_2$	105 ± 1.4	107 ± 0.9
$CuSO_4$	17 ± 1.5	17 ± 1.0
$HgCl_2$,	2.9 ± 2.0	3.0 ± 1.9
$CoCl_2$	102 ± 1.0	104 ± 0.8
$FeSO_4$	86 ± 2.2	89 ± 1.3
$CaCl_2$	78 ± 1.8	80 ± 1.6
$CuCl_2$	19 ± 1.2	21 ± 0.8
$ZnSO_4$	88 ± 2.4	90 ± 2.5

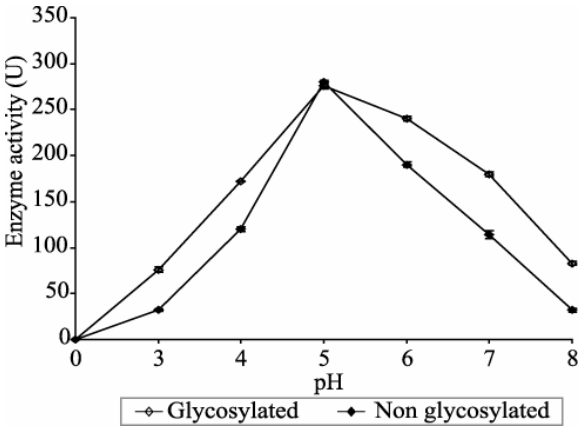

Fig. 7. Effect of pH on stability of purified glycosylated and non-glycosylated invertase.
*Hundreds microlitres of each enzyme solution was incubated in 0.05 M citrate/ 0.05 M acetate buffer at pH values ranging for 2-8 at 40 °C for 15 min. The residual activity was determined under standard conditions.

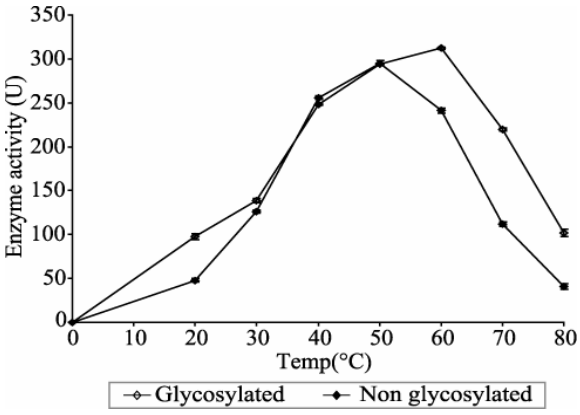

Fig. 8. Effect of temperature on stability of purified glycosylated and non-glycosylated invertase.
*The enzyme activity was measured in the temperature range of 20-80°C. The reaction mixture (pH 5.0) was incubated for 15 min and residual activity was determined under standard conditions.

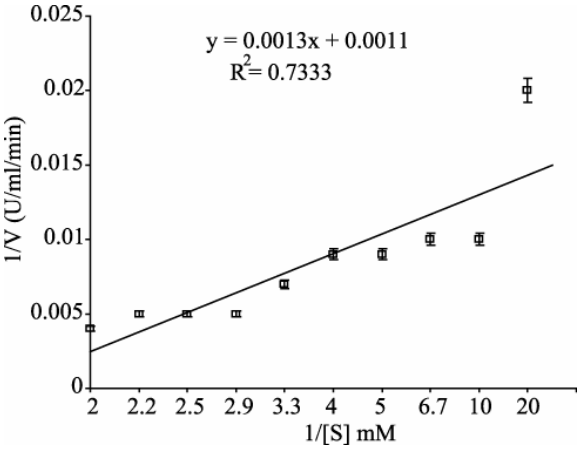

Fig. 9. Lineweaver-Burk plot for extracellular glycosylated invertase
The intercept on the y-axis corresponding to $1/V_{max}$ = 0.0011, Slope = 0.0013.

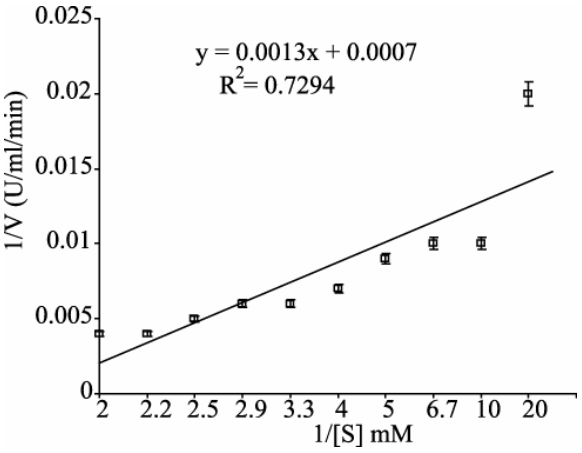

Fig. 10. Lineweaver-Burk plot for intracellular non-glycosylated invertase
The intercept on the y-axis corresponding to $1/V_{max}$ = 0.0007, Slope = 0.0013.
The mean difference is significant at $p \leq 0.05$. Y bars indicate the standard deviation (±sd) among the three parallel replicates.

Conclusion

Saccharomyces cerevisae was selected because it contained both types of invertases. It was previously mutagenized chemically by EMS for the production of extracellular invertase as major secretory enzyme. A 15 fold purification of extracellular invertase with recovery of 38% was achieved. The molecular weight (110 kD) of glycosylated invertase having 48% carbohydrate content and intracellular non-glycosylated (55 kD) as monomeric proteins were noticed after SDS-PAGE.

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