Pak. J. Bot., 48(1): 357-262, 2015.

KINETIC EVIDENCE OF A NOVEL INVERTASE IN AN ETHYL METHANE SULPLONATE DEREPRESSED MUTANT OF *YARROWIA LIPOLYTICA*

SIKANDER ALI[1*] AND MUHAMMAD UMAR HAYYAT[2]

[1]*Institute of Industrial Biotechnology, GC University Lahore, Pakistan*
[2]*Sustainable Development Study Centre, GC University Lahore, Pakistan*
[*]*Corresponding author e-mail: alisbiotech@yahoo.com*

Abstract

In the present study, we report on the kinetics of an extracellular invertase production by *Yarrowia lipolytica* in defined medium i.e., sucrose peptone agar yeast extract, pH 6 (SAPY). The wild-type IIB-II was treated with ethyl methane sulphonate (EMS) as a chemical mutagen. Among the six mutants isolated, EMS-IV was found to be the best enzyme producing mutant strain (51±2.4[a] U/ml). The maximum enzyme production (73±3.1[a] U/ml) occurred at 48 h of incubation (67±2.7[a] mg/ml protein). The potential mutant was stabilized at low levels of 2-deoxy-D-glucose (2dg) and the viable mutants were further optimized both culturally and nutritionally. The sucrose concentration, incubation period and pH were optimized to be 30 g/l, 28°C and 6.5, respectively. EMS-IV exhibited an improvement of over 10 folds in enzyme production when 5 g/l ammonium sulphate was used as a nitrogen source. Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) analysis showed that optimal enzyme activity caused a higher hydrolysis rate of sucrose into monosaccharides (α-D-glucose and β-D-fructose). The values for Q_p (1.7±0.12[c] U/ml/h) and $Y_{p/s}$ (3.7±1.24[b] U/g) of the mutant were considerably higher in comparison to the wild-type or all other yeast strains. The mutant could be used for enzyme production over the temperature range of 26-34°C, which is highly significant (LSD 0.048, *HS*).

Key words: *Yarrowia lipolytica*, Invertase, Kinetic study, Microbial fermentation, Batch culture, EMS mutagenesis.

Introduction

The enzyme invertase (*EC 3.2.1.26*) cleaves α-1,4 glycosidic bonds between α-D-glucose and β-D-fructose moiety in sucrose to release monosaccharide units. It has industrial applications particularly soft-centre confectionary production and ethanol fermentation of molasses (Nakano *et al*., 2004). Although *Saccharomyces cerevisiae* has been a superior organism for enzyme manufacture because of its typical sucrose fermenting capability, however the pursuit of future strain selection should be based on factors such as strain stability, yield, initial pH and fermentation period. Other factors notably tolerance to temperature, oxygen supply and shear stress are also worth-mentioning (Sanchez *et al*., 2001; Djegui *et al*., 2014). Sucrose has been a useful carbon source for enzyme synthesis because glucose availability for yeast culture is largely dependent on its efficient hydrolysis. The optimization of nature and type of a potential source could provide a basis for an efficient batch-culture process (Iraqi *et al*., 2005; Abdullah *et al.,* 2014). The process efficiency of yeast strains at relatively higher incubation temperature (>35°C) remains low because of the increased membrane fluidity, which changes the type, chain lengths and composition of fatty acids. Temperature rise generally supports the synthesis of some heat-shock proteins which are implicated in thermal cross tolerance of different organisms' prominently non-filamentous fungi like yeasts (Stowers *et al*., 2008).

In the past, only a few reports have appeared which studied the mechanics of catabolic repression. Although enzyme expression was regulated in various yeast cultures, however a hyper-producing viable strain with consistent activity was not being produced (Schnierda *et al*., 2014; Ahmed *et al.,* 2015). The classical strain improvement methods like ultraviolet (UV) irradiation or treatment with alkylating agents such as N-nitroso guanidine (NG) to obtain positive mutants in terms of net enzyme productivity have demonstrated some success. The selection and screening of better survivors was also found critical (Barratt *et al*., 2009; Aslam *et al.,* 2013). More work is required to increase enzyme activity which could end-up in an economical commercial process. The use of mild chemical mutagens like ethyl methane sulphonate (EMS) for enhanced mutation frequency has so far been neglected. Therefore efforts are still needed to develop a potent yeast mutant having increased substrate utilization and concomitant enzyme biosynthesis rates. In this report, we attempted to isolate a mutant of *Y. lipolytica* IIB-II with improved enzyme production after randomly induced mutagenesis.

Materials and Methods

The chemicals and reagents used were procured from Sigma Chemicals (USA).

Organism and EMS induced mutagenesis: *Yarrowia lipolytica* strain IIB-II, previously isolated from *Phoenix dactylifera* was subjected to induced mutagenesis through ethyl methane sulphonate (EMS) treatment. Five millilitre of EMS (50-300 µg/ml) was added to the centrifuge tubes having 5 ml of the yeast cell suspension. Afterwards, the tubes were placed at ambient temperature (25ºC) for various intervals (15-90 min). The treated suspension was centrifuged at 5,500×*g* for 20 min. The upper layer containing the mutagen was removed from the cells. Then 6 ml of sterile saline water containing NaCl 0.085%, yeast extract 0.05% and polypeptone 0.025% were dispensed in all of the tubes as reported by Das & Nandi (1969). The treated suspension (0.1 ml) was spread on the plates having 10 g/l sucrose, 15 g/l agar, 2.5 g/l peptone along with 2 g/l yeast extract, pH 6 (also known as SAPY medium). Bromocresol green dye (40 ml/l of 3% dye in 70% ethanol) was added and the plates were kept at 30ºC. Yeast colonies appeared in 24-36 h with pink-colour zones showing sucrose hydrolysis. These were compared to the control plates (without EMS), run in parallel. Later, colonies were aseptically picked and inoculated to the agar slants of the same medium.

Development of 2-deoxy-D-glucose (2dg) resistance in viable mutant strains: The treated cells of selected mutant strains were collected during the log phase. The cells were twice washed with the sterile distilled water before plating on the SAPY medium. Various levels of 2-deoxy-D-glucose (0.01-0.1 mg/ml) were added before incubation. However, sucrose was replaced with stachyose. The plates were incubation for 16-24 h, and frequently sub-cultured. The vigorous growth of the yeast mutants was checked for stability during batch culture. Samples were taken periodically. Inoculation and plating was accomplished on the medium having 2dg levels to choose resistant strains. The mutant cultures was screened and preserved in paraffin oil at 4°C.

Inoculum preparation: The inoculum was grown in 50 ml of SAPY medium (without agar) in a 250 ml Erlenmeyer flask. The medium was sterilized at 15 psi (121°C) for 15 min and seeded with a loopful of the yeast cells. The culture medium was placed in a rotary shaking incubator (4043-Gallenkamp-JB, London, UK) at 28°C for 16-24 h (160 rpm). The suspension contained 1.25×10^7 CFU/ml after haemocytometer count (OD~1 at 590 nm).

Fermentation procedure: Enzyme production was carried out with shaking using 250 ml Erlenmeyer flasks. The SAPY medium (without agar), pH 6 was prepared in the flasks and cotton-plugged. The medium was sterilized in an autoclave. After cooling to about 20-25°C, 1 ml of the cell suspension was aseptically seeded to the medium of each flask. The flasks were incubated at 200 rpm at 30°C for 48 h. The batch-cultures were run in triplicate.

Analysis of biomass development and sugar consumption: The dry biomass of selected yeast culture was determined after centrifugation, and then by transferring the broth at 6,250×*g* in pre-weighed tubes at 20ºC. The tubes were oven dried at 105°C for 2 h. Residual sugar was assayed by DNS method after Miller *et al*. (1959) at 546 nm wavelength using a UV/Vis scanning spectrophotometer (MVP153-CECIL-700, London, England).

Invertase assay: The enzymatic activity was measured following the procedure described by Myers *et al*. (1997). "One unit is defined as the amount of enzyme, which releases 1 mg of inverted sugar in 5 min at 20°C, pH 4.5". Tubes having 2.5 ml acetate buffer at pH 5.5 (50 mM) along with 0.1 ml of sucrose (300 mM) were allowed to pre-incubate at 32°C for a period of 5 min. Then 0.1 ml of an appropriately diluted enzyme mixture was added and incubated for another 5 min. Later, the assay mixture was kept in a water bath at 90°C for 5 min and cooled to room temperature. A control tube was also run in parallel by replacing enzyme solution with same quantity of distilled water. One millilitre of DNS reagent was dispensed to 1 ml of the mixture. The tubes were again placed in boiling water for another 5 min. After cooling the reaction mixture to about 25°C, the final volume was made up to 10 ml. The %T was read at 546 nm. The protein content was measured after Bradford (1976).

Chromatographic analysis of enzyme preparation: Thin layer chromatography (TLC) was employed for the qualitative analysis of invertase hydrolysis products. Pre-coated TLC plates (E-Merck) were spotted on the origins with enzyme samples in duplicate. The plates were developed in acetone: diethyl ether: water (7:3:1 by volume). The plates were sprayed thoroughly with 0.2 % ninhydrin and allowed to dry at 70ºC for 20 min (Dastager *et al*., 2006). The resulting sugars were quantitatively analysed by a double beam HPLC (Perkins Elmer, USA). The universal column (C18, HPX-87H ion exchange, 400×118 mm) was maintained at 25ºC. The mobile phase (ethanol-butanol 2:1) was adjusted at a flow-rate of 0.42 ml/min. The samples were identified using a refractive index detector (Turbochron-4 software).

Kinetic study: The kinetic parameters were computed following the procedures as described by Pirt (1975). The volumetric rate for enzyme production (Q_p) was calculated from the plot of enzyme (U/ml) and ΔT (h), the product yield coefficient ($Y_{p/s}$) from dP/dS, the specific product yield coefficient ($Y_{p/x}$, U/g cells) from dP/dX and the specific rate for product formation (q_P) from $Y_{p/x}\times\mu$. The specific growth rate (μ) was determined from the slope between ln X/X_0 (X=cell mass, g/l) and ΔT (h). The growth yield coefficient ($Y_{x/s}$) was calculated as g cells formed/g [S] utilized. The cell biomass formation (Q_x, g X/l/h), and substrate consumption rates were determined from g dry weight of yeast cells or [S]/l vs. ΔT (h).

Statistical analysis: The treatment effects were equated after Snedecor & Cochran (1980). The significance has been presented as Duncan's multiples as probability (≤p>) values.

Results and Discussion

In this study, *Y. lipolytica* wild-type IIB-II was mutated after treatment with ethyl methane sulphonate (EMS), which is a potent chemical mutagen. The mutant variant EMS-IV was found to be a better invertase yielding strain (51±2.4 U/ml) when compared to the wild-type (17±1.8 U/ml). This mutant was grown on the medium containing low levels of 2-deoxy D-glucose (2dg). The stability for enzyme production was monitored at various 2dg levels. When initially compared, high enzyme producing colonies were isolated at 0.02 mg/ml; however, the potential cultures lost their stability after approximately two weeks as reported by Fernandez *et al*. (2007). This instability was correlated with the possible development of resistance in growing yeast cells. Only a few mutants thrived for several generations. To eliminate this problem, these mutant strains were again cultured on the medium containing some other 2dg concentrations. A concentration of 0.05 mg/ml was optimized, as EMS-IV gave consistent enzyme production. The selected mutant revealed an above average values for growth yield coefficients, while lower specific growth rate. IIB-II (wild-type) and the chemically derepressed mutant (EMS-IV) of were again compared on time course basis (Figs. 1-3). Biomass level revealed a general growth pattern by both of the cultures; still EMS-IV exhibited a much faster growth rate as compared to IIB-II. The optimum sugar consumption was recorded 22±1.12g/l by EMS-IV in comparison to 16±0.81 g/l for IIB-II. A notable improved enzyme production by EMS-IV was seen over the wild-

type and all the other mutant variants. In a similar study, Weber & Roitsch (2003) improved another yeast mutant, which yielded over 2 folds better enzyme productivity than the wild-type. During the course of incubation, enzyme production initiated after a lag phase of approximately 8-12 h and maximised just before the onset of stationery phase. The enzyme biosynthesis degenerated afterwards, probably due to decreased nutrient availability and catabolic repression. Enzyme expression in yeast was adversely hampered by monosaccharides particularly glucose or fructose (Lazar *et al*., 2013).

A comparison of invertase yield coefficients and productivity rates of wild-type IIB-II and the mutant EMS-IV during batch culture was made at different cultural conditions. The sugar concentration (25, 30 & 35 g/l), temperature (22, 24, 26, 28, 30, 32, 34 & 36ºC) and preliminary pH (6, 6.5 & 7) were determined (Table 1). Maximum enzyme production (67±2.5[a] U/ml) was achieved at 30 g/l sucrose in 48 h by EMS-IV following incubation and subsequent growth. A higher than the optimal sucrose level caused an increase in the sugar consumption and hence dry biomass formation rate; however, there was not an increase in the enzyme productivity as reported by Ahmed *et al.* (2015). Among the incubation temperatures, the best enzyme activity (70±2.2[a] U/ml) was observed at 28°C, which was over 3.5 folds higher than the wild-type. Biochemically, it was interesting that the mutant strain was quite capable of growing between 34-36°C or even higher temperatures; however the enzyme activity decreased abruptly. The optimal enzyme synthesis by EMS-IV (74±3.1[a] U/ml) was recorded at an initial pH of 6.5. The wild-type was found to be much less thermo-tolerant and it produced the enzyme only in a narrow temperature range (30-32°C), revealing no preference within the pH optima as reported by Haq & Ali (2007).

The sugar concentration, temperature and initial pH directly affect fungal growth and hence influence the secretion of primary and secondary metabolites (Persike *et al*., 2002). The hyper-secretive mutant EMS-IV exhibited lower rates for biomass formation and much higher rates for sucrose fermentation-ability compared to the wild-type. A greater than the optimal (30 g/l) inverted sucrose concentration in the production medium resulted in a faster glucose-induced repression of the enzyme (Vitolo *et al*., 1995). The mutant was quite capable of growing up to 36°C (pH 6.5), a much higher temperature than its wild-type. The final pH was found proportional to the enzyme production rate. The possible reason for this relation is that enzyme production complements secretion of various anions along with some basic proteins, or to the selective uptake of certain cations (Haq *et al.,* 2003; Madhan *et al*., 2010). Although the wild-type IIB-II achieved an upper value of $Y_{x/s}$ (0.15±0.03[b] g yeast cells/g) larger than its mutant EMS-IV, the later demonstrated an improvement in the volumetric rate of product development. In addition to these findings, when the cultures were equated for specific rate constant, EMS-IV revealed much higher values of q_p (over >15-20 folds improvement). It is noteworthy that EMS-IV showed several folds improved values of Q_p, $Y_{p/x}$, $Y_{p/s}$ and q_p when compared to the wild-type (LSD 0.048).

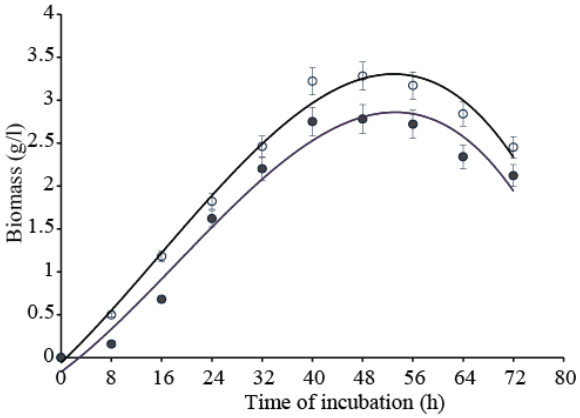

Fig. 1. Comparison of biomass formation by wild-type (IIB-II) and mutant (EMS-IV) strains of *Y. lipolytica* in batch culture (IIB-II -●-, EMS-IV -o-). Sucrose concentration 30 g/l, temperature 28°C, initial pH 6.5, agitation rate 200 rpm. Y-error bars indicate standard deviation among the three parallel replicates.

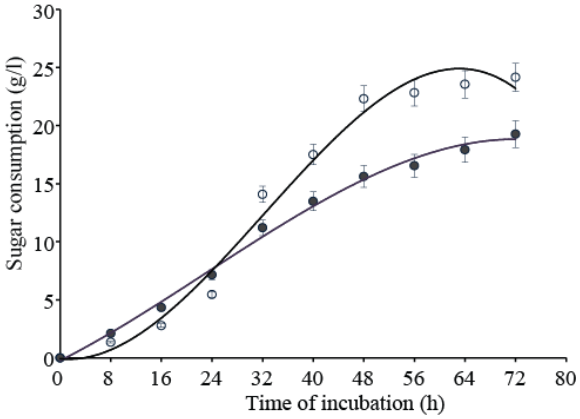

Fig. 2. Comparison of sugar consumption by wild-type (IIB-II) and mutant (EMS-IV) strains of *Y. lipolytica* in batch culture (IIB-II -●-, EMS-IV -o-). Temperature 28°C, initial pH 6.5, agitation rate 200 rpm. Y-error bars indicate standard deviation among the three parallel replicates.

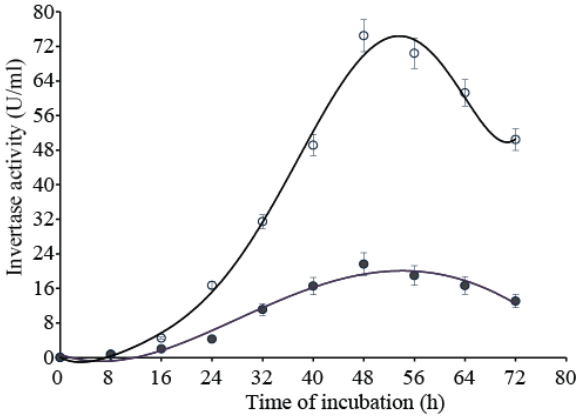

Fig. 3. Time of incubation of invertase production by wild-type (IIB-II) and mutant (EMS-IV) strains of *Y. lipolytica* in batch culture (IIB-II -●-, EMS-IV -o-). Sucrose concentration 30 g/l, temperature 28°C, initial pH 6.5, agitation rate 200 rpm. Y-error bars indicate standard deviation among the three parallel replicates.

Table 1. Comparisons of yield coefficients for invertase production by wild-type and mutant cultures of *Y. lipolytica*.

Cultivation conditions	Mutant invertase production (U/ml	Yield coefficients					
		Product yield coefficients				Growth yield coefficients	
		$Y_{p/x}$ (Enzyme units/g cells formed)		$Y_{p/s}$ (Enzyme units/g substrate utilized)		$Y_{x/s}$ (g cells formed/g substrate utilized)	
		IIB-II	EMS-IV	IIB-II	EMS-IV	IIB-II	EMS-IV
Sugar conc. (g/l)							
25	44.56 ± 1.6^{c}	7.81 ± 1.1^{a}	34.63 ± 2.1^{b}	9.15 ± 0.2^{a}	2.33 ± 1.1^{a}	0.18 ± 0.02^{a}	0.03 ± 0.01^{c}
30	67.45 ± 2.5^{a}	4.55 ± 1.5^{d}	41.07 ± 2.3^{a}	1.07 ± 0.1^{b}	2.69 ± 1.3^{a}	0.15 ± 0.03^{b}	0.07 ± 0.01^{a}
35	55.82 ± 2.1^{b}	4.02 ± 0.7^{de}	33.85 ± 3.5^{b}	0.74 ± 0.2^{c}	1.48 ± 0.4^{b}	0.12 ± 0.02^{c}	0.04 ± 0.02^{b}
Incubation temp. (°C)							
22	22.99 ± 1.3^{fg}	5.61 ± 1.7^{d}	20.34 ± 1.6^{c}	$0.12 \pm 0.2_{e}$	0.98 ± 0.2^{d}	0.09 ± 0.02^{b}	0.04 ± 0.02^{f}
24	34.12 ± 2.2^{de}	6.72 ± 1.6^{c}	26.15 ± 2.5^{c}	0.79 ± 0.1^{c}	1.64 ± 0.4^{c}	0.11 ± 0.01^{a}	0.04 ± 0.01^{f}
26	61.48 ± 3.4^{ab}	7.36 ± 2.2^{b}	34.12 ± 2.4^{b}	1.02 ± 0.2^{b}	1.86 ± 0.2^{b}	0.13 ± 0.03^{a}	0.05 ± 0.01^{e}
28	69.78 ± 2.2^{a}	8.91 ± 2.3^{a}	44.36 ± 2.1^{a}	1.34 ± 0.3^{a}	2.57 ± 0.3^{a}	0.14 ± 0.02^{a}	0.06 ± 0.02^{d}
30	67.55 ± 3.5^{ab}	7.82 ± 1.8^{b}	33.81 ± 2.1^{b}	1.05 ± 0.1^{b}	2.91 ± 0.9^{a}	0.12 ± 0.03^{a}	0.09 ± 0.01^{a}
32	64.74 ± 2.6^{ab}	7.08 ± 2.2^{b}	27.42 ± 2.4^{c}	1.02 ± 0.6^{b}	2.28 ± 0.2^{a}	0.11 ± 0.02^{a}	0.08 ± 0.02^{b}
34	60.65 ± 1.8^{ab}	6.72 ± 2.4^{c}	23.52 ± 3.2^{c}	0.82 ± 0.3^{c}	1.95 ± 0.5^{b}	0.10 ± 0.01^{a}	0.07 ± 0.01^{c}
36	56.26 ± 2.4^{bc}	5.72 ± 2.2^{d}	21.05 ± 1.5^{c}	0.13 ± 0.1^{e}	1.34 ± 0.2^{c}	0.08 ± 0.05^{b}	0.03 ± 0.01^{g}
Initial pH							
6	65.65 ± 1.5^{ab}	9.16 ± 2.4^{a}	37.44 ± 1.2^{b}	1.16 ± 0.4^{a}	2.42 ± 1.2^{b}	0.07 ± 0.02^{c}	0.05 ± 0.01^{b}
6.5	73.78 ± 3.1^{a}	7.81 ± 2.3^{bc}	45.49 ± 2.7^{a}	0.92 ± 0.2^{b}	3.15 ± 1.5^{a}	0.09 ± 0.03^{b}	0.08 ± 0.01^{a}
7	61.52 ± 2.6^{ab}	6.77 ± 1.1^{cd}	45.24 ± 2.4^{a}	0.75 ± 0.1^{c}	3.08 ± 1.6^{a}	0.14 ± 0.01^{a}	0.04 ± 0.02^{c}

± Indicates standard deviation among the three parallel replicates. The values designated by different letters in each set differ significantly from each other at $p \leq 0.05$

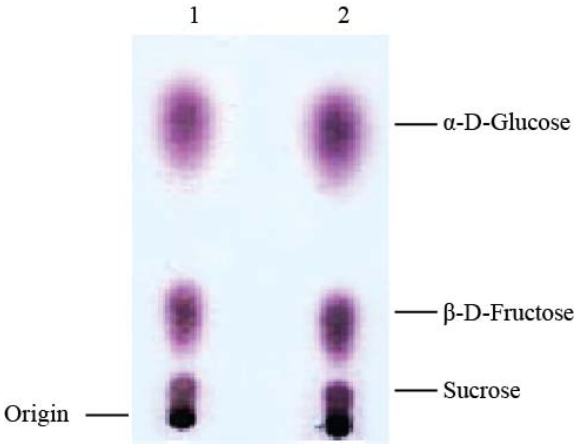

Fig. 4a. Thin layer chromatography (TLC) for the separation of invertase hydrolysis sugars (α-D-Glucose, β-D-Fructose, Sucrose) in duplicate samples of the reaction broth at optimal conditions.

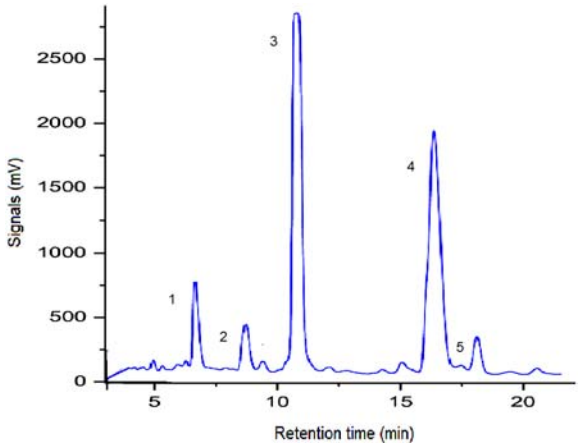

Fig. 4b. HPLC chromatograph of invertase activity by mutant strain of *Y. lipolytica* EMS-IV at optimal conditions (1. Raffinose, 2. Stachyose, 3. α-D-Glucose, 4. β-D-Fructose, 5. Sucrose).

The qualitative analysis through thin layer chromatography (TLC) demonstrated the invertase hydrolysis sugar products viz. α-D-glucose, β-D-fructose and sucrose. Image 1 is one of the TLC chromatograms run for the identification of sugars (Fig. 4a). The spot analysis and comparison of the values of relative front (Rf values) confirmed the hydrolysis sugars when run against their ultrapure sugar counter-parts suggesting complete separation as reported by Dastager *et al*. (2006). HPLC analysis depicted the separate and well-organised peaks for α-D-glucose, β-D-fructose and sucrose, thus highlighting the product stability in the mixture form (Fig. 4b). Raffinose and stachyose were also secreted into the reaction broth but their highest productivities remained very low i.e., 0.011 and 0.0007 mg/ml, respectively (Silveira *et al*., 2000).

The influence of cultural parameters on product formation was computed by biomass formation, specific production rate, substrate conversion rate, synthetic activity and the product breakdown (Elorza *et al*., 1977; Cadena *et al*., 2011). The values for $Y_{p/x}$ (enzyme units/g cells formed) and $Y_{p/s}$ (enzyme units/g substrate consumed) were found to be 45 ± 2.7^{a} U/g cells and 3 ± 1.5^{a} U/g substrate, respectively. Ammonium sulphate (5, 7.5 g/l) and tryptone (2.5, 5 g/l) were added into the production medium (Table 2). The values for $Y_{p/s}$, $Y_{x/s}$, and $Y_{p/x}$ at various ammonium sulphate concentrations were considerably improved (LSD 0.048, *HS*, $p \leq 0.05$) as compared to the control (tryptone + yeast extract) or sole peptone supplementation. This steady increase could be attributed to ammonium sulphate which facilitates the release of periplasmic enzyme by making the yeast cell walls relatively more permeable as reported earlier by Pirt (1975). The volumetric rate (Q_x) was marginally different at 5-7.5 g/l ammonium sulphate in 48 h. Similar kinds of findings were reported earlier by Gomez *et al*. (2000); however, the present enzyme production is over 4.5 times higher than the previous reports (Egorov *et al*., 2000). When the mutant strain was compared for Q_p and q_s, significant enhancement ($p \leq 0.05$) was noted at 5 g/l ammonium sulphate when compared to other ammonium sulphate concentrations or even other nitrogen sources.

Table 2. Comparison of kinetic parameters for invertase production by wild-type and mutant cultures of *Y. lipolytica.*

Kinetic parameters	Control		Ammonium sulphate (g/l)				Tryptone (g/l)			
			5		7.5		2.5		5	
	IIB-II	EMS-IV	IIB-II	EMS-IV	IIB-II	EMS-IV	IIB-II	EMS-IV	IIB-II	EMS-IV
Substrate consumption parameters										
$Y_{x/s}$ (g cells/g)	0.123±0.01$_c$	0.067±0.01^c	0.124±0.03^c	0.078±0.02^c	0.157±0.03^b	0.212±0.03^b	0.134±0.02^c	0.095±0.01^c	0.125±0.02^b	0.111±0.07^c
Q_s (g/l/h)	0.325±0.05^a	0.436±0.03^a	0.545±0.05^a	0.452±0.02^a	0.464±0.02^a	0.648±0.04^a	0.244±0.02^a	0.288±0.02^a	0.316±0.02^a	0.542±0.03^a
q_s (g/g cells/h)	0.172±0.02$_b$	0.297±0.04^b	0.172±0.04^b	0.205±0.05^b	0.126±0.02^c	0.196±0.02^c	0.154±0.01^b	0.179±0.03^b	0.103±0.02^c	0.198±0.02^b
Q_x (g cells/l/h)	0.048±0.04^d	0.038±0.01^d	0.076±0.02^d	0.058±0.01^d	0.108±0.02^d	0.067±0.01^d	0.086±0.02^d	0.064±0.02^d	0.059±0.02^d	0.038±0.02^d
Enzyme formation parameters										
Q_p (U/ml/h)	0.275±0.05^c	0.724±0.01^c	0.455±0.05^c	1.682±0.12^c	0.564±0.02^c	1.186±0.22^c	0.257±0.03^c	1.138±0.42^c	0.355±0.01^c	1.665±0.045^d
$Y_{p/s}$ (U/g)	0.672±0.02^b	1.542±0.08^b	1.238±0.24^b	3.416±1.12^b	1.314±0.62^b	3.732±1.24^b	0.709±0.03^b	2.685±1.51^b	0.906±0.02^b	2.892±1.024^b
$Y_{p/x}$ (U/g cells)	4.241±0.17^a	16.621±2.4^a	7.831±1.11^a	31.28±4.32^a	7.702±2.82^a	29.67±3.31^a	4.625±1.55^a	30.66±1.62^a	7.285±1.85^a	29.336±2.22^a
q_p (U/g cells/h)	0.108±0.02^d	0.341±0.26^d	0.198±0.01^d	0.795±0.35^d	0.192±0.01^d	0.796±0.02^d	0.216±0.02^d	0.704±0.12^d	0.134±0.02^d	0.176±0.02^c
LSD	0.006	0.015	0.026	0.048	0.020	0.035	0.018	0.031	0.014	0.027
Significance level	-	-	-	*HS*	-	*S*	-	*S*	-	-

± Indicates standard deviation among replicates. LSD for least significant difference, HS denotes highly significant and S for significant values. The values designated by different letters in each set differ significantly from each other at p≤0.05

Conclusion

In the present study, a locally developed *Y. lipolytica* mutant (EMS-IV) exhibited a substantial improvement (p≤0.05) in invertase production when 5 g/l ammonium sulphate was added as an organic nitrogen ingredient directly into the SAPY medium, pH 6.5. Many folds improvement in volumetric enzyme productivity was recorded by the mutant at all rates examined. The TLC and HPLC studies showed an effective sucrose hydrolysis process by the super-active enzyme preparation, which may be highly useful for inverted-syrup production on a larger-scale. The potential mutant could be exploited for enzyme production at a wider temperature range (26-34°C), exhibiting the potential for its larger scale exploitation.

Acknowledgements

Vice Chancellor, GC University Lahore is acknowledged for his services to promote research in the University.

References

Abdullah, R., N. Shaheen, M. Iqtedar, S. Naz and T. Iftikhar. 2014. Optimization of cultural conditions for the production of alpha amylase by *Aspergillus niger* (BTM-26) in solid state fermentation. *Pak. J. Bot.,* 46(3): 1071-1078.

Ahmed K., E.E. Valeem and Q. Ul-Haq. 2015. Biosynthesis, purification and characterization of commercial enzyme by *Penicillium expansum*. *Pak. J. Bot.,* 47(4): 1521-1526.

Aslam, A., I. Haq and S. Ali. 2013. Purification and characterization of two invertases from mutant strain of *Saccharomyces cerevisiae*. *Pak. J. Bot.*, 45: 285-291.

Barratt, D.H., P. Derbyshire, K. Findlay, M. Pike, N. Wellner, J. Lunn, R. Feil, C. Simpson, A.J. Maule and A.M. Smith. 2009. Normal growth of *Arabidopsis* requires cytosolic invertase but not sucrose synthase. *Proc. Natl. Acad. Sci. USA,* 106: 13124-13129.

Bradford, M.M. 1976. A rapid method for the quantification of program quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*., 72: 248-254.

Cadena, P.G., F.N. Wiggers, R.A. Silva, J.L. Lima Filho and M.C. Pimentel. 2011. Kinetics and bioreactor studies of immobilized invertase on polyurethane rigid adhesive foam. *Bioresour. Technol.,* 102: 513-518.

Das, A. and P. Nandi. 1969. Improved production of citric acid from *Aspergillus niger* by mutagenic treatment. *Ind. J. Exp. Biol.,* 7: 275-276.

Dastager, S.G., W.J. Li, A. Dayanand, S.K. Tang, X.P. Tain, Y.Z. Zhi, L.H. Xu and C.L. Jiang. 2006. Separation, identification and analysis of pigment (melanin) production in *Streptomyces*. *Afr. J. Biotechnol.,* 5: 1131-1134.

Djegui, K.Y., E.W. Gachomo, D.J. Hounhouigan, A.P. Kayode and S.O. Kotchoni. 2014. Biochemical characterization and growth patterns of new yeast isolates. *Mol. Biol. Rep.,* 41: 5199-206.

Egorov, S.N., I.N. Semenova and V.N. Maksimov. 2000. Mutual effect of invertase and acid phosphatase from the yeast *Saccharomyces cerevisiae* on their secretion into culture media. *Mikrobiol.,* 69: 34-37.

Elorza, M., R. Villanuena and R. Sentandreu. 1977. The mechanism of catabolite inhibition of β-D-fructofuranosidase fructohydrolase by glucose in *Saccharomyces cerevisiae*. *Biochimie Biophy. Acta,* 475: 103-112.

Fernandez, R.C., C.A. Ottoni, E.S. da Silva, R.M. Matsubara, J.M. Carter, L.R. Magossi, Wada, M.A., M.F. de Andrade Rodrigues, B.G. Maresma and A.E. Maiorano. 2007. Screening of beta-fructofuranosidase-producing microorganisms and effect of pH and temperature on enzymatic rate. *Appl. Microbiol. Biotechnol.,* 75: 87-93.

Gomez, S.J.R., C. Augur and G. Viniegra-Gozalez. 2000. β-D-fructofuranosidase fructohydrolase production by *Aspergillus niger* in submerged and solid-state fermentation. *Biotechnol. Lett.,* 22: 1255-1258.

Haq, I. and S. Ali. 2007. Kinetics of improved invertase production by *Saccharomyces cerevisiae* in batch culture. *Pak. J. Bot*., 39: 907-912.

Haq, I., K. Shafiq and S. Ali. 2003. Substrate induced repression of invertase synthesis by *Saccharomyces cerevisiae* in submerged culture. *Pak. J. Bot*., 35: 527-531.

Iraqi, D., V.Q. Le, M.S. Lamhamedi and F.M. Tremblay. 2005. Sucrose utilization during somatic embryo development in black spruce: involvement of apoplastic invertase in the tissue and of extracellular invertase in the medium. *J. Plant Physiol.,* 162: 115-124.

Lazar, Z., T. Rossignol, J. Verbeke, A.M.C. Crutz-Le, J.M. Nicaud and M. Robak. 2013. Optimized invertase expression and secretion cassette for improving *Yarrowia lipolytica* growth on sucrose for industrial applications. *J. Ind. Microbiol. Biotechnol.,* 40: 1273-1283.

Madhan, S.S., R. Sathyavani and B. Niket. 2010. Production and partial purification of invertase using *Cympopogan caecius* leaf powder as substrate. *Ind. J. Microbiol.,* 50: 318-324.

Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem*., 31: 426-428.

Myers, D.K., D.T. Lawlor and P.V. Attfield. 1997. Influence of invertase activity and glycerol synthesis and retention on fermentation of media with a high sugar concentration by *Saccharomyces cerevisiae. Appl. Environ. Microbiol.,* 63: 145-150.

Nakano, H., H. Murakami, M. Shizuma, T. Kiso, T.L. Dearaujo and S. Kitahata. 2004. Transfructosylation of thiol group by beta-fructofuranosidases. *Biosci. Biotechnol.,* 64: 1472-1476.

Persike, D., S.T.M. Bonfim, M.H. Santos, S.M. Lyng, M.D. Chiarello and J.D. Fontana. 2002. Invertase and urease activities in the carotenogenic yeast *Xanthophyllomyces dendrorhous*. *Bioresour. Technol.,* 82: 79-85.

Pirt, S.J. 1975. Principles of Microbes and Cell Cultivation. Blackwell's Scientific Corporation, London, UK. pp. 86-118.

Sanchez, M.P., J.F. Huidobro, I. Mato, S. Munigategui and M.T. Sancho. 2001. Evolution of invertase activity in honey. *J. Agr. Food Chem.,* 49: 416-422.

Schnierda, T., F.F. Bauer, B. Divol, E. van Rensburg and J.F. Gorgens JF. 2014. Optimization of carbon and nitrogen medium components for biomass production using non-Saccharomyces wine yeasts. *Lett. Appl. Microbiol.,* 58: 478-485.

Silveira, M.C., E.M. Oliveira, E. Carvajal and E.P. Bon. 2000. Nitrogen regulation of *Saccharomyces cerevisiae* invertase. Role of the URE2 gene. *Appl. Biochem. Biotechnol.,* 84: 247-254.

Snedecor, G.W. and W.G. Cochran. 1980. Statistical Methods, 7th Edition, Iowa State University, USA. pp. 32-43.

Stowers, C.C., E.M. Ferguson and R.D. Tanner. 2008. Development of activity-based cost functions for cellulase, invertase, and other enzymes. *Appl. Biochem. Biotechnol.,* 147: 107-117.

Vitolo, M., M.A. Duranti and M.B. Pellegrim. 1995. Effect of pH, aeration and sucrose feeding on β-D-fructofuranosidase fructohydrolase activity of intact *Saccharomyces cerevisiae* cells grown in sugarcane black strap molasses. *J. Ind. Microbiol.,* 15: 75-79.

Weber, H. and T. Roitsch. 2003. Invertases and life beyond sucrose cleavage. *Trends Pl. Sci.,* 5: 47-48.

(Received for publication 8 January 2015)