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KINETICS OF INVERTASE PRODUCTION BY *SACCHAROMYCES CEREVISIAE* IN BATCH CULTURE

IKRAM-UL-HAQ AND SIKANDER ALI

Institute of Industrial Biotechnology, GC University, Lahore, Pakistan

Abstract

The present study is concerned with the improved invertase production by *Saccharomyces cerevisiae* on kinetic basis. The wild-culture (IIB-1) and putative mutant (NA6) of *S. cerevisiae* were compared on time course basis when grown in batch culture. The values for $Y_{x/s}$, $Y_{p/s}$ and $Y_{p/x}$ with urea were significantly improved ($p \leq 0.05$) over the control (peptone+yeast extract) as well as peptone supplementation. The maximum growth in terms of volumetric rate for cell mass formation (Q_x), 48 h after the incubation was only marginally different from control. The values of both K_m and V_{max} of the invertase from mutant culture were significantly improved, as were the specificity constant and k_{cat} for the enzyme. The activation energy and substrate binding for sucrose fermenting were found to be highly significant for yeast culturing.

Introduction

Invertase catalyses α-1, 4 glycosidic linkage between α-D-glucose and β-D-fructose molecules of sucrose by hydrolysis releasing monosaccharides such as glucose and fructose (Barlikova *et al*., 1991). It also hydrolyses β-fructans such as raffinose into simple sugars. Sucrose is the best sole carbon source for its bioproduction as the availability of glucose for yeast is dependent on sucrose hydrolysis (Nakano *et al*., 2004). The common yeast *Saccharomyces cerevisiae* is the organism of choice for invertase production (Bokosa *et al*., 1992). Invertase has wide range of commercial applications including the production of confectionary with liquid or soft centres and fermentation of cane molasses into ethanol. Invertase is also used in pharmaceutical industry, as in digestive aid tablets, powder milk for infants and other infant foods. Invert syrup production by microbial invertase is not widespread because of ease in chemical hydrolysis and high price of the enzyme. But golden syrup, generated by acid hydrolysis of sucrose, is not preferable over invert syrup produced by invertase (Sanchez *et al*., 2001). Fermentation efficiency of *S. cerevisiae* at higher temperatures (>35°C) is low because of increased fluidity in membranes, which changes the fatty acid composition. The increase in growth temperature results in the biosynthesis of heat-shock proteins that are implicated in conferring thermal cross-tolerance in various organisms (Vrabel *et al*., 2003). In the present study the putative mutant (NA6) of *S. cerevisiae* was compared with wild-culture (IIB-1) for enhanced substrate uptake rate and concomitant production of invertase. Activation enthalpy and entropy of invertase were determined to get an insight of kinetics of the system and to clarify the phenomenon involved in improved enzyme production.

Materials and Methods

The wild (IIB-1) and mutant (NA6) cultures of *S. cerevisiae* were obtained from Institute of Industrial Biotechnology, GCU Lahore, Pakistan. The mutant culture was developed from the wild after treatment with 50 mM HNO_2 (-*NA*) for 20 min., in a sterilized Mackonkey bottle. Both the cultures were maintained on the medium containing (g/l): sucrose 20.0, agar 20.0, peptone 5.0, yeast extract 3.0 at pH 6.0 and stored at 4°C in a lab cool (Sanyo - Japan). Twenty five millilitre of this medium (-agar) was transferred to a 250 ml Erlenmeyer flask. The flask was cotton plugged, autoclaved at 15 lbs/in^2 pressure (121°C) for 15 min., and cooled to 20°C. One millilitre of cell suspension (1.2×10^7 cells/ml) was transferred to the flask, aseptically prior to incubation in a rotary shaking incubator (Gallenkamp - UK) at 30°C for 24 h (160 rpm). Production of invertase was carried out by shake flask technique using 250 ml Erlenmeyer flasks. The medium having the same composition (pH 6.0) was used for batch fermentation. The medium (25 ml) was transferred to the individual conical flasks and cotton-plugged. The flasks were autoclaved and cooled at room temperature. One millilitre of inoculum was aseptically transferred to each flask. Flasks were incubated in a rotary shaking incubator (200 rpm) at 30°C for 48 h.

Yeast dry cell mass was determined by centrifugation of the fermented broth at 5,661×g in weighed centrifuge tubes at 20°C. The tubes were dried at 105°C for 2 h in an oven (Model: 1442A, Memmert, Germany). Sugar was estimated by DNS method (Miller, 1959) using a double beam UV/Vis scanning spectrophotometer at 546 nm. Enzyme activity was determined after Chen *et al*., (1996). One invertase unit is defined as the amount of enzyme, which releases 1.0 mg of inverted sugar in 5 min at 20°C, pH 4.5. For invertase activity, 2.5 ml acetate buffer (50 mM, pH 5.5) and 0.1 ml sucrose (300 mM) was added into the individual test tubes. The tubes were pre-incubated at 35°C for 5 min. After the addition of 0.1 ml of appropriately diluted enzyme solution, incubation was continued for another 5 min. The reaction mixture was placed in a boiling water bath for 5 min., to stop the reaction and allowed to cool at room temperature. A blank was also run parallel replacing the enzyme solution with distilled water. To 1.0 ml of each reaction mixture 1.0 ml of DNS was added and the tubes placed in boiling water for 5 min. After cooling to 20ºC, volume was raised up to 10 ml. Transmittance was measured at 546 nm using spectrophotometer.

The empirical approach of Arrhenius equations was used to describe the temperature-dependent irreversible enzyme inactivation (Aiba *et al*., 1973). The plot of $\ln(q_p/T)$ against $1/T$ gave a straight line whose slope was $-\Delta H/R$ and intercept was $\Delta S/R + \ln(K_B/h)$, where h (Planck's constant) = $6{\cdot}63\times10^{-34}$ Js, K_B (Boltzman constant $[R/N]$ = $1{\cdot}38\times10^{-23}$ J K^{-1} and N (Avogadro's No.) = $6{\cdot}02\times10^{23}$ mol^{-1}. Specific rate of product formation (q_p, Enzyme units/g cells/h) was used to calculate different variables using the following equations:

$$q_p = T \cdot k_B/h e^{\Delta S^*/R} e^{-\Delta H^*/RT}$$

$$\ln(q_p/T) = \ln(k_B/h) + \Delta S^*/R - \Delta H^*/RT$$

Kinetic parameters were based on the methods described earlier (Pirt, 1975). Treatment effects were compared after Snedecor & Cochran (1980). Significance was presented as Duncan's Multiple Range (DMR) in the form of probability (<p>).

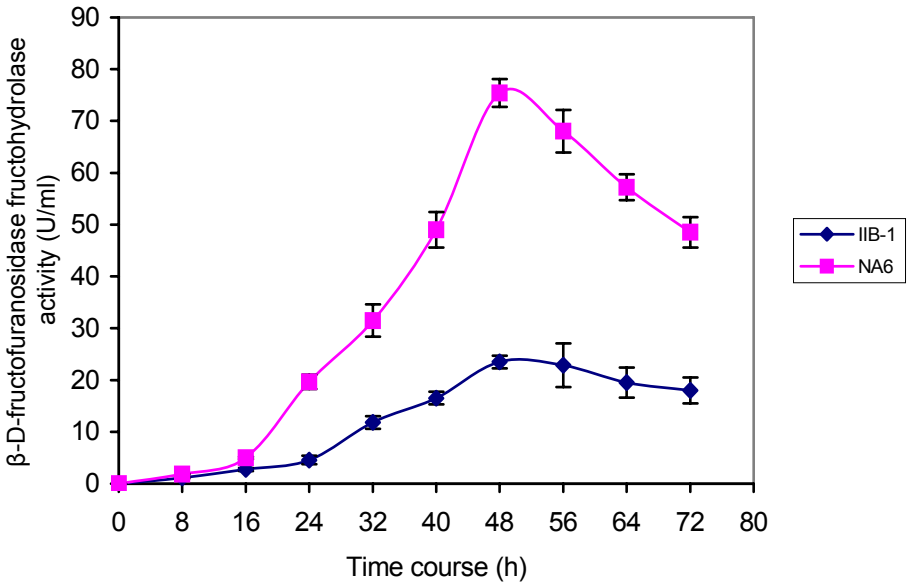

Fig. 1. Comparison of kinetics of invertase production by wild and mutant *S. cerevisiae*.

Sucrose concentration 30.0 g/l, temperature 28°C, initial pH 6.5, agitation rate 200 rpm.Y-error bars indicate standard deviation among the three parallel replicates.

Results and Discussion

Cultivation conditions directly affect the microbial growth and thereby monitor the metabolic behaviour to secrete primary or secondary metabolites (Vitolo *et al*., 1995). In the present study, wild-culture (IIB-1) and a mutant (NA6) of *S. cerevisiae* were compared on time course basis when grown in batch culture (Fig. 1). Maximum invertase production (78.96±2.0 U/ml) was observed from the mutant 48 h after inoculation. Further increase in incubation period did not enhance invertase production. In batch culture, the enzyme production starts after a lag phase of about 8 h and reaches maximal at the onset of stationery phase. Afterwards, enzyme activity declines due to the decreased nutrients availability in the medium or carbon catabolite repression (Herwig *et al*., 2001). The mutant NA6 has lower rate of biomass formation while conversely has a higher rate of sucrose fermentability compared to the wild-culture.

The influence of environmental factors on product formation in batch culture is determined by biomass concentration, the specific production rate, product yield from the substrate, the duration of synthetic activity and rate of decomposition of the product (Chen *et al*., 1996). In the present study, the effect of sole nitrogen sources i.e., urea and peptone (5.0 g/l each) added into the fermentation medium were compared with the control (Table 1). Among the kinetic parameters, the values for $Y_{x/s}$, $Y_{p/s}$ and $Y_{p/x}$ at different concentrations of urea were significantly improved ($p \leq 0.05$) over the control (peptone+yeast extract) as well as peptone supplementation. The maximum growth in terms of volumetric rate for cell mass formation (Q_x), 48 h after the incubation was only marginally different from control. Similar, findings have previously been reported by Pirt *et al*., (1975). However, present report is several folds (≈3.8-4.9) improved over to those

Table 1. Kinetic parameters for invertase production by wild and mutant *S. cerevisiae*.

Kinetic parameters	Control		Urea (5.0 g/l)		Peptone (5.0 g/l)	
	IIB-1	NA6	IIB-1	NA6	IIB-1	NA6
$Y_{x/s}$ (g cells/g)	0.16±0.02	0.09±0.01	0.13±0.02	0.22±0.04	0.12±0.02	0.13±0.02
Q_s (g/l/h)	0.34±0.01	0.41±0.03	0.43±0.01	0.48±0.02	0.31±0.02	0.40±0.01
q_s (g/g cells/h)	0.15±0.02	0.17±0.04	0.11±0.01	0.16±0.02	0.13±0.02	0.18±0.01
Q_x (g cells/l/h)	0.04±0.02	0.08±0.01	0.07±0.01	0.06±0.01	0.07±0.02	0.09±0.01
Q_p (U/ml/h)	0.28±0.01	0.72±0.01	0.60±0.01	1.36±0.5	0.38±0.01	1.66±0.04
$Y_{p/s}$ (U/g)	0.53±0.02	1.51±0.08	1.39±0.6	3.32±1.2	0.93±0.02	3.90±1.0
$Y_{p/x}$ (U/g cells)	5.41±0.1	18.6±2.4	7.80±2.8	26.70±4.1	6.28±1.8	33.96±2.2
q_p (U/g cells/h)	0.13±0.04	0.43±0.2	0.16±0.01	0.55±0.04	0.15±0.02	0.80±0.02
LSD	0.09	0.18	0.02	0.03	0.01	0.03
Significance level	-	-	-	S	-	-

Kinetic parameters: Q_p = U of invertase produced/ml/h, $Y_{p/s}$ = U of invertase produced/g substrate consumed, $Y_{p/x}$ = U of invertase produced/g cells formed, q_p = U of invertase produced/g cells/h, $Y_{x/s}$ = g cells/g substrate utilized, Q_s = g substrate consumed/l/h, q_s = g substrate consumed/g cells/h, Q_x = g cells formed/litre/h.
± Indicates standard deviation among replicates. LSD for least significant difference, HS denotes highly significant and S for significant values.

Table 2. Kinetic properties of invertase produced by wild and mutant *S. cerevisiae*.

Kinetic parameters	IIB-1	NA6
k_{cat} (min^{-1})[a]	122	216
K_m (%, w/v)	3.1	2.7
*k*cat/*K*m	19.51	49.62
E_a (kJ mol^{-1})[b]	38	26
Optimal pH	7.8	7.0
pK_{a1} (acidic limb)[c]	5.4	6.0
pK_{a2} (basic limb)[d]	8.6	9.1
ΔH_1 (kJ mol^{-1})[e]	11	14
ΔH_2 (kJ mol^{-1})[e]	18	17.5
$\Delta G^{\#}_{E\text{-}T}$ (kJ mol^{-1})[f]	-14.9	-20.8
$\Delta S^{\#}_{E\text{-}S}$ (kJ mol^{-1})[g]	2.3	1.5

[a]Turnover number (k_{cat}) = V_{max}/[e].
[b]Activation energies (Ea).
[c]Nucleophilic carboxyl group.
[d]Proton donating carboxyl group.
[e]Heat of ionization.
[f]$\Delta G^{\#}_{E\text{-}T}$ (free energy of transition state binding = -RT in *k*cat/*K*m.
[g]$\Delta S^{\#}_{E\text{-}S}$ (free energy of substrate binding) = -RT ln Ka, where $K_a = 1/K_m$.

from some other yeast cultures or filamentous fungi reported by Gomez *et al*., (2000). In addition, when the culture was monitored for Q_p and q_s, there was a significant enhancement ($p \leq 0.05$) in these variables with urea.

The increase in the hydrophobicity around active site may be explained as a result of local conformational change due to substrate (i.e., sucrose) binding and is a common phenomenon (Egorov *et al*., 2000). The values of both K_m and V_{max} of the mutant culture were significantly improved as were the specificity constant and k_{cat} for the invertase (Table 2). This could be due to the ionized state of the mutant-derived invertase whose charges have been reserved and that the sucrose binds more strongly to the active site than transition state of the substrate substantiating with the work of Myers *et al*., (1997). The mutant-derived enzyme required less free energy (ΔG) for substrate binding. In

addition, the mutant-derived invertase released a higher amount of transition state formation ($\Delta G_{E\text{-}T}$), signifying a higher catalytic enzyme efficiency due to the transition state stabilization. The requirement of lower energy of activation (E_a) for growth and product formation may be considered as potential indices for thermostability of cultures during production processes (Fig. 2), as these parameters may be considered for thermostable enzymes (Declerck *et al*., 2003).

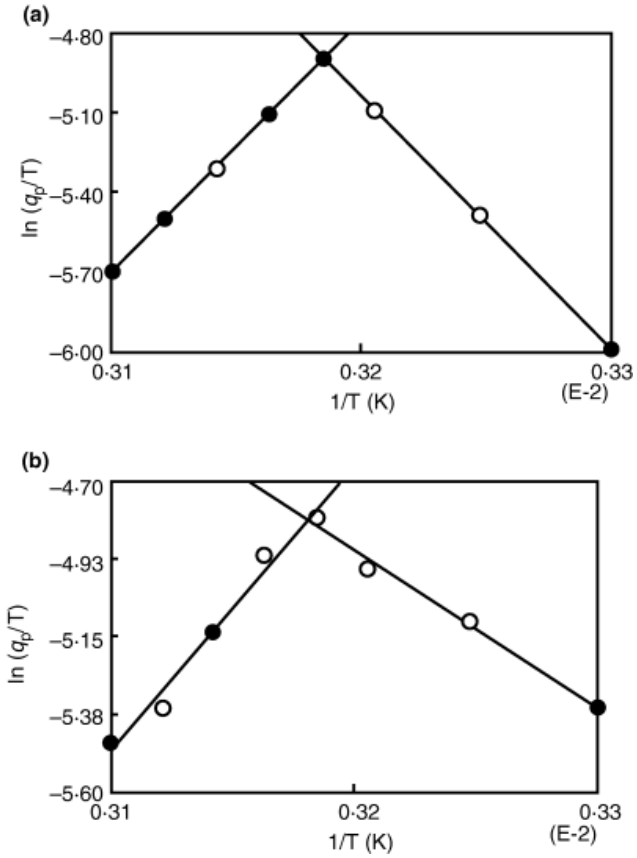

Fig. 2. Arrhenius plots for invertase production by wild and mutant *S. cerevisiae*.

Growth of a) wild-culture (IIB-1), b) mutant strain (NA6). Temperature was varied while all other variables were kept constant.

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