0273-2289/04/117/0075/$25.00

Influence of Carbon and Nitrogen Sources and Temperature on Hyperproduction of a Thermotolerant β-Glucosidase From Synthetic Medium by *Kluyveromyces marxianus*

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Abstract

The effect of carbon source and its concentration, inoculum size, yeast extract concentration, nitrogen source, pH of the fermentation medium, and fermentation temperature on β-glucosidase production by *Kluyveromyces marxianus* in shake-flask culture was investigated. These were the independent variables that directly regulated the specific growth and β-glucosidase production rate. The highest product yield, specific product yield, and productivity of β-glucosidase occurred in the medium (pH 5.5) inoculated with 10% (v/v) inoculum of the culture. Cellobiose (20 g/L) significantly improved β-glucosidase production measured as product yield ($Y_{P/S}$) and volumetric productivity (Q_p) followed by sucrose, lactose, and xylose. The highest levels of productivity (144 IU/[L·h]) of β-glucosidase occurred on cellobiose in the presence of CSL at 35°C and are significantly higher than the values reported by other researchers on almost all other organisms. The thermodynamics and kinetics of β-glucosidase production and its deactivation are also reported. The enzyme was substantially stable at 60°C and may find application in some industrial processes.

Index Entries: β-Glucosidase; cellobiose; corn steep liquor; *Kluyveromyces marxianus*; shake flask.

Introduction

β-Glucosidase (EC 3.2.1.21) is one of the component enzymes of the cellulase complex and is widely distributed in nature. It catalyzes the

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hydrolysis of alkyl and aryl glycosides as well as that of cellobiose to glucose, which is inhibitory of other enzymes, namely endoglucanases (EC 3.2.1.4), and exoglucanases (EC 3.2.1.91), during cellulose hydrolysis *(1)*. The enzyme has great economic significance. In plants, the function of β-glucosidase is to provide defense against pathogens by releasing coumarins, thiocyanates, terpenes, and cyanide and activation of phytohormones (*see* ref. *2*). In humans, its deficiency is the cause of Gaucher's disease *(2)*. In industry, it is employed for hydrolysis of bitter compounds from grapefruit during juice extraction and liberation of aroma from grapes during wine making *(2)*. β-Glucosidases act in synergism with other cellulases to convert cellulose in abundantly available lignocellulosic biomass into glucose, and they should be present in the right proportion to cause effective saccharification *(1)*. Glucose could be converted either into fructose (sweetener) by glucose isomerase or into ethanol, butanol, and so on, which can be used as fuels *(3)*. β-Glucosidase provides inducer for cellulase production and also increases saccharification of lignocellulosic biomass *(3,4)*. It has been observed that a deficiency of β-glucosidase may reduce the saccharification rate of cellulose to produce glucose *(5)*.

Supplemental β-glucosidase from *Aspergillus* cultures improves the process of hydrolysis by commercial enzyme preparations of *Trichoderma reesei (3,5)*. In view of their biotechnological potential, we have screened several local strains of fungi *(5)*, bacteria *(2,6)*, and yeasts and have found that *Kluyveromyces marxianus* is a good β-glucosidase and ethanol producer. Some strains of the thermotolerant yeast *K. marxianus* can grow up to 52°C and may produce thermostable glycosidases.

K. marxianus has been employed for production of biomass, enzymes, and ethanol *(7–11)*. Studies on production and secretion of β-glucosidase are important to develop enzyme systems that could be directly used for industrial purposes such as safe alternative energy sources. The production of enzyme may be influenced by several factors, such as growth of the organism on substrate, induction of the enzyme, and catabolite repression *(12)*. Inductive production of β-glucosidase in *K. marxianus* has been reported *(10)*, but no information is available on the effect of other carbon and nitrogen sources.

We report here the overproduction of β-glucosidase by an indigenous strain of *K. marxianus* by exploring the influence of process variables as reported in cultures of *Candida peltata (13)*. The achievement of high β-glucosidase/cellobiase-containing preparation has been proposed to eliminate the inhibition of FPase and endoglucanase by converting cellobiose to glucose, the former being a strong inhibitor of these activities. Activation enthalpy and entropy of this process were determined to clarify the phenomena involved in both β-glucosidase production and its thermal inactivation as reported for glucose isomerase, phytase, alcohol, and xylitol production *(14–16)*. Three different approaches are available in the literature for their estimation: thermodynamic approach, kinetic approach, and Arrhenius plots *(17)*. The last one was selected because it proved to

be the best tool for the present system as observed for other fermentation systems *(16,18)*.

Materials and Methods

Chemicals

4-Nitrophenyl-β-D-glucopyranoside (4-NPG), 4-nitrophenol, arabinose, xylose, glucose, galactose, sucrose, cellobiose, and lactose were from Sigma (St. Louis, MO). All other chemicals were of analytical grade.

Maintenance of Stock Culture

K. marxianus NIBGE Y-1 was isolated from a sugarcane molasses storage tank in Pakistan during the summer months (daytime temperature of 45–49°C) after enrichment in molasses yeast medium (17% sugar) and 1.4 g/L of K_2HPO_4, 28 g/L of KH_2PO_4, 6 g/L of $(NH_4)_2SO_4$, 0.4 g/L of $MgSO_4 \cdot 7H_2O$, and 3 g/L of yeast extract after Barron et al. *(10)* at 50°C. It was identified as *K. marxianus var. marxianus* using standard identification procedures as described by Banat et al. *(11)* by comparing its microscopic, morphologic, and biochemical characteristics. It was maintained on malt extract agar plates at 4°C after growth at 50°C.

Preparation of Inoculum

Inoculum was prepared in glucose-salts medium at 35°C in a rotary shaker at 150 rpm. These cultures were used to inoculate the basal medium. For induction studies, the cultures were centrifuged (10,000*g*, 30 min), washed twice with saline, suspended in saline water, and used at 10% (unless otherwise stated) for enhanced production of β-glucosidase.

Enzyme Production

The affinity of the organism to utilize arabinose, galactose, glucose, cellobiose, sucrose, lactose, and xylose as sole carbon sources was examined in the aforementioned basal salts medium. Carbon sources were added individually to batches of basal medium to give a carbohydrate level of 20 g/L. All carbon sources were added to autoclaved medium after filter sterilization. All media were adjusted to pH 5.5 with 1 *M* NaOH or 1 *M* HCl, dispensed in 180-mL aliquots into 1-L Erlenmeyer flasks in triplicate, and inoculated with seed culture containing 0.25 mg of dry cells/mL. Shake-flask batch production in these media was carried out at 35°C (unless otherwise stated) on a gyratory shaker incubator at 150 rpm.

Effect of Inoculum

A set of Erlenmeyer flasks containing a known volume of basal medium (pH 5.5) was inoculated with different volumes of inoculum

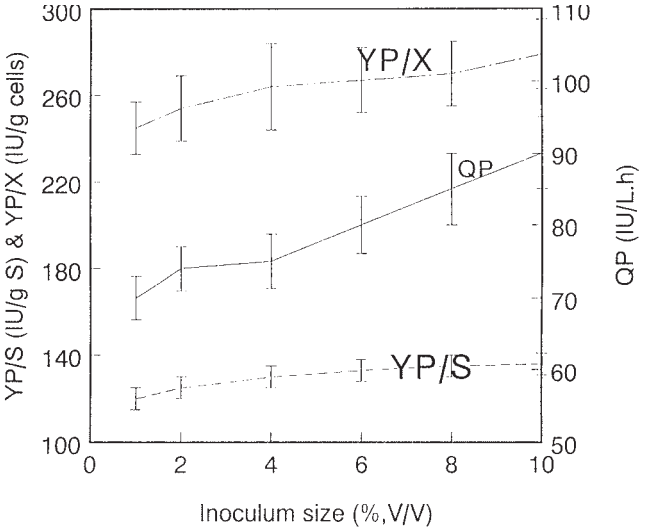

Fig. 1. Effect of initial inoculum size on volumetric productivity (Q_P), product yield ($Y_{P/S}$), and specific product yield ($Y_{P/X}$) of β-glucosidase following fermentation of cellobiose (control pH of 5.5, 0.3% yeast extract, temperature of 35°C, and time up to 48 h in time course).

(Fig. 1), and each set of flasks was grown separately. The flasks were incubated as described earlier.

Growth (g/L) was measured gravimetrically as dry cell mass. The enzyme activity present in the cell-free supernatant or cell extract was assayed as the induction or repression indicator. Clear supernatant from 150 mL of original culture broth was obtained by centrifugation (10,000*g*, 30 min). The cell pellet was used to extract cellular fractions by suspending in Na acetate buffer and disrupting the cells by probe sonication in three 1-min bursts with a Braun sonicator 2000 on ice. The remaining 50-mL portion was also centrifuged (10,000*g*, 30 min). The cell-free supernatant was preserved for enzyme assays, and solid material was washed twice with saline, suspended in 10 mL of distilled water, and dried at 70°C to constant mass.

Effect of Nitrogen Sources

The effect of different nitrogen sources (Table 1) to give equimolar nitrogen content (1.27 g/L) as contained in $(NH_4)_2SO_4$ was explored while maintaining other variables constant.

Effect of Varying pH and Temperature on Enzyme Production

To demonstrate the influence of fermentation temperature on fermentation ability of the culture, the data of batch fermentations were gained by performing experiments (three runs) on cellobiose-based media by main-

Table 1
Comparative Fermentation Kinetic Parameters of *K. marxianus* for β-Glucosidase Formation Parameters Following Growth on Cellobiose Yeast Medium (pH 5.5) in Presence of Different Nitrogen Sources in Batch Culture Fermentation at 35°C[a]

	β-Glucosidase production parameters[b]		Substrate consumption parameters[b]	
N source	Q_P (IU/[L·h])	$Y_{P/S}$ (IU/g)	Cell mass (g/L)	Protein production (mg/[L·h])
Ammonium nitrate	23.0[e]	62.0[d]	8.7[e]	4.2[f]
Ammonium sulfate	90.0[d]	136.0[c]	10.0[d]	5.8[e]
CSL	144.0[a]	230.6[a]	12.7[a]	7.9[a]
Sodium glutamate	129.4[b]	164.0[b]	11.4[c]	6.7[d]
Sodium nitrate	20.0[e]	67.0[d]	7.8[f]	3.9[g]
Urea	115.0[c]	143.0[c]	11.2[c]	6.8[c]
Peptone	110.0[c]	162.0[b]	12.1[b]	6.9[b]

[a]The organism was grown on cellobiose yeast medium containing nitrogen sources to contain 1.24 g/L of nitrogen to replace ammonium sulfate in the medium in a time course study. Sample flasks in triplicate were removed and processed to test substrate, cell mass, and β-glucosidase in the fermentation medium under uncontrolled pH conditions. Each value is an average of three replicates. Values followed by different superscript letters differ significantly according to Duncan multiple range test.

[b]All treatments had a highly significant effect on all kinetic parameters.

taining other variables constant and varying temperature on an orbital shaker (150 rpm) at a temperature range of 22–45°C.

Determination of Protein

The proteins were determined by Lowry's method using bovine serum albumin as the standard.

pH Tolerance

Relative pH tolerance by β-glucosidase produced by *K. marxianus* was studied after incubating the enzyme in different buffers containing 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF) for 30 min, and the remaining activity was determined at 40°C. The different buffers were citrate phosphate buffer (pH 4.0 to 5.0), phosphate buffer (pH 6.0–8.0), Tris-glycine-NaOH buffer (pH 10.0), and phosphate buffer (pH 11.0).

Temperature Tolerance

The enzyme containing 0.1 m*M* PMSF was incubated at different temperatures for 30 min, and the remaining β-glucosidase activity was determined at 40°C. An Arrhenius relationship was used to get inference about temperature tolerance.

Enzyme Assays

β-Glucosidase activity was determined using 1 m*M* 4-NPG as substrate in 1 mL of 50 m*M* Na acetate buffer, pH 5.0 (unless otherwise stated). One milliliter of the enzyme sample (preequilibrated at 40°C) was incubated with this mixture at 40°C for 10 min. The reaction was stopped by adding 2 mL of 1 *M* sodium carbonate. The liberated *p*-nitrophenol was measured at 400 nm with a spectrophotometer. One IU of β-glucosidase was defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol/min.

Determination of Saccharides

Saccharides were analyzed by high-performance liquid chromatography (HPLC) (Perkin-Elmer, Norwalk, CT). An HPX-87H column (300×78 mm; Bio-Rad, Richmond, CA) was maintained at 45°C in a column oven. Sulfuric acid (0.002 *N*) in HPLC-grade water served as a mobile phase at 0.6 mL/min. The samples were detected using a refractive index detector and quantified using Turbochron4 software (Perkin-Elmer).

Determination of Kinetic Parameters

Kinetic parameters for the batch fermentation process were determined according to Lawford and Rousseau *(19)* and Pirt *(20)*. The volumetric rate of β-glucosidase production (Q_P) was determined from a plot between β-glucosidase (IU/mL) and time of fermentation. The process product yield ($Y_{P/S}$) was determined from dP/dS, specific product yield ($Y_{P/X}$, IU/g of cells) was determined from dP/dX, and volumetric rate of substrate consumption was determined from a plot between substrate (g/L) present in the fermentation medium and time of fermentation. Cell mass productivity, expressed as g of dry cells/(L·h), intracellular (P_i) or extracellular protein (P_e) productivity (mg of protein/[L·h]), was determined from a plot of g of dry cells/L, intracellular or extracellular protein (mg/L) vs time. Specific growth rate was determined from $\mu t = \text{Ln } X$, and specific productivity was a multiple of μ and $Y_{p/x}$. Specific enzyme activity was obtained by dividing Q_P by P_I or P_E.

Statistical Analysis

Treatment effects were compared by the protected least significant difference method and were presented as a two-factor factorial design in the form of probability (p) using MstatC software.

Results and Discussion

Production of β-Glucosidase From Different Substrates

β-Glucosidase is generally considered to be synthesized in the presence of an inducer. Initial studies on the induction of β-glucosidase indicated that cellobiose medium was essential to induce high β-glucosidase

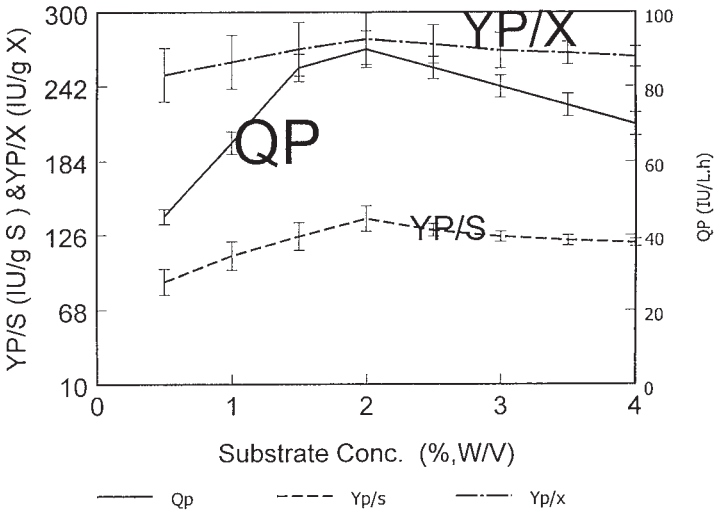

Fig. 2. Effect of initial substrate concentration in fermentation medium on volumetric productivity (Q_p), product yield ($Y_{P/S}$), and specific product yield ($Y_{P/X}$) of β-glucosidase production following liquid culture fermentation of cellobiose (control pH of 5.5, inoculum of 10 mL/100, 0.3% yeast extract, temperature of 35°C, and time up to 48 h in time course).

activities. These studies were conducted in time course at 35°C in shake flasks with salts media containing cellobiose (0.25–1.5%). The results (Fig. 2) indicated that 2.0% carbohydrate was optimum for biosynthesis of β-glucosidase and that 36–40 h was the optimum time for production of enzymes. The decreased concentration encountered with the highest concentration treatment was probably owing to osmotic effects. It has been reported that above a critical substrate concentration, the decreased water activity and the onset of plasmolysis combine to cause a decrease in the rates of fermentation and product formation *(20)*. All further studies on the time course production of β-glucosidase were performed with 2% (w/v) cellobiose. The use of commercial cellobiose as feedstock is not economical for large-scale production of β-glucosidase; therefore, several other substrates (Table 2) were included in these studies. *K. marxianus* was grown in shake-flask batch culture in mineral culture media containing either lactose, sucrose, cellobiose, arabinose, galactose, glucose, or xylose as the sole carbon source as described in Materials and Methods.

K. marxianus produced very low levels of cell-bound β-glucosidase on glucose. These low levels of activity represent the basal level of this enzyme necessary for cellular metabolism. Cellobiose, the primary product of cellulose hydrolysis, is believed to be a strong inducer of the cellulase complex, and β-glucosidase as glucose is liberated slowly and cannot cause catabolite repression *(4)*.

Growth promoters in yeast extract can affect enzyme synthesis markedly. The effect of yeast extract concentrations on biosynthesis of β-glucosi-

Table 2
Comparative Substrate Consumption Kinetic Parameters of *K. marxianus* Following Growth on Different Substrates in Yeast Fermentation Medium (pH 5.5)[a]

	Substrate consumption parameters[b]				
C source	μ (1/h)	$Y_{X/S}$ (g/g)	Q_S (g/[L·h])	q_S (g/[g·h])	Q_{IP} (mg/[L·h])
Arabinose	0.54^{ab}	0.49^{a}	0.76^{b}	0.95^{b}	6.6^{a}
Galactose	0.49^{c}	0.48^{a}	0.74^{b}	0.85^{c}	6.2^{b}
Glucose	0.59^{a}	0.50^{a}	0.91^{a}	1.06^{a}	6.4^{a}
Xylose	0.45^{d}	0.47^{a}	0.65^{cd}	0.73^{d}	6.2^{b}
Cellobiose	0.49^{c}	0.50^{a}	0.68^{c}	0.75^{d}	5.4^{c}
Lactose	0.26^{e}	0.48^{a}	0.32^{e}	0.57^{e}	4.5^{e}
Sucrose	0.27^{f}	0.52^{a}	0.60^{d}	0.44^{f}	5.0^{d}

[a]Each value is an average of three replicates. Values followed by different superscript letters differ significantly at $p \leq 0.05$ according to Duncan multiple range test.

[b]μ = specific growth rate calculated as slope of Ln X (X is cell mass, g/L) vs time of fermentation; Q_X = g of cells synthesized/(L·h); $Y_{X/S}$ = g of cells/g of substrate consumed; Q_S = g of substrate consumed/(L·h); and q_S = g of substrate consumed/(g of cells·h).

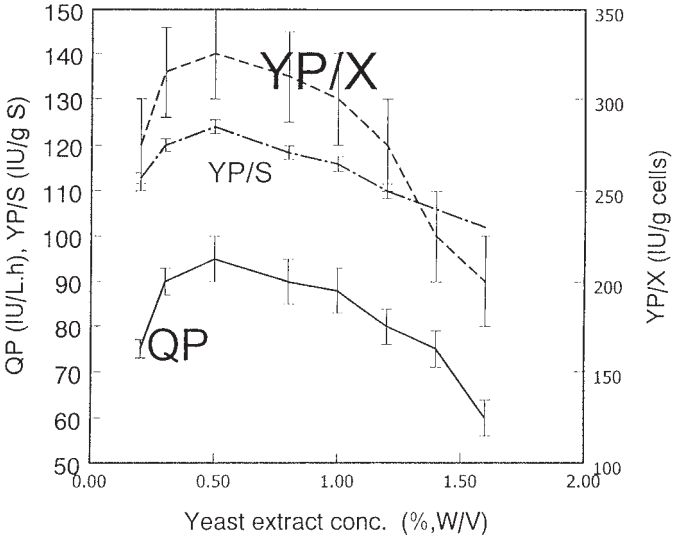

Fig. 3. Effect of initial yeast extract concentration on volumetric productivity (Q_P), product yield ($Y_{P/S}$), and specific product yield ($Y_{P/X}$) of β-glucosidase following fermentation of cellobiose (control pH of 5.5, inoculum of 10 mL/100, temperature of 35°C, and time up to 48 h in time course).

dase in time course was also studied. The cells were grown in 2.0% cellobiose in yeast salt broth at 35°C, pH 5.5. The enzyme was prepared as described in Materials and Methods and β-glucosidase activities were measured (Fig. 3). Yeast extract concentrations influenced β-glucosidase production up to 3 g/L, but higher concentrations had no pronounced

effect on enzyme overproduction. All further time courses were performed with 3 g/L of yeast extract in the fermentation medium.

The effect of inoculum on β-glucosidase activity is shown in Fig. 1. The maximum activity was obtained when the basal medium was inoculated with 10% (v/v) of the culture. In media inoculated with 2, 4, 6, 8, and 10% (v/v) inoculum, the β-glucosidase productivity was 75, 81, 85, 88, and 90 IU/(L·h), respectively. The activity of β-glucosidase was low (75 IU/[L·h]) when the substrate was inoculated with 2% inoculum. Barron et al. *(10)* and Furlan et al. *(21)* reported 2% inoculum for production of β-glucosidase and β-galactosidase, respectively, using *K. marxianus* as biocatalyst. Based on the foregoing observations, all the media were inoculated with 10% (v/v) inoculum for all subsequent studies.

The potential of production of β-glucosidase in shake-flask batch culture studies was tested after the growth of *K. marxianus* in yeast culture medium containing arabinose, cellobiose, galactose, glucose, lactose, sucrose, and xylose (2% [w/v]), with each carbohydrate as the sole carbon source. Representative kinetics of enzyme production is presented in Fig. 4A–D, following growth on galactose, lactose, cellobiose, and xylose, respectively. Figure 4A–D reveals that production of β-glucosidase was maximum on cellobiose and least on xylose. The curves also indicate that bulk production of β-glucosidase was apparently growth associated.

The kinetic parameters for substrate consumption from different substrates are presented in Table 2. Overall, values of these parameters were reasonably high and substantiated the work of Inchaurrondo et al. *(22)*. This may have occurred owing to the substantial amount of enzymes of the metabolic pathways provided by these substrates as well as to greater protein for cell mass production. Enhanced substrate consumption and cell mass formation rates by *K. marxianus* could lead to increased productivities in commercial β-glucosidase production from industrial substrates (cheese whey and molasses), as reported by Furlan et al. *(21)*.

Growth rate, protein productivities, and substrate uptake rates were considered as the factors that might contribute to differences in enzyme synthesis from different growth substrates. The β-glucosidase production profiles presented in Table 3 establish the relationship between substrates and enzyme production by *K. marxianus*. There was significant ($p \leq 0.05$) variation in specific growth rate (μ, per h) and specific substrate uptake rates during growth on cellobiose, and other carbon sources and permitted larger variation in synthesis of β-glucosidase although values of $Y_{X/S}$ from both easily and slowly metabolizable carbon sources were comparable. The q_S values from cellobiose (0.75 g/[g of cells·h]), sucrose (0.57 g/[g of cells·h]), and lactose (0.44 g/[g of cells·h]) were different and displayed a direct relationship to β-glucosidase synthesis, whereas in easily metabolizable substrates—arabinose, galactose, glucose, and xylose—which exhibited 0.95, 0.85, 1.06, and 0.73 g/(g of cells·h), respectively, q_S values (Table 2) were significantly ($p \leq 0.05$) different and showed an inverse relationship to β-glucosidase synthesis. Thus, induction of β-glucosidase may be influ-

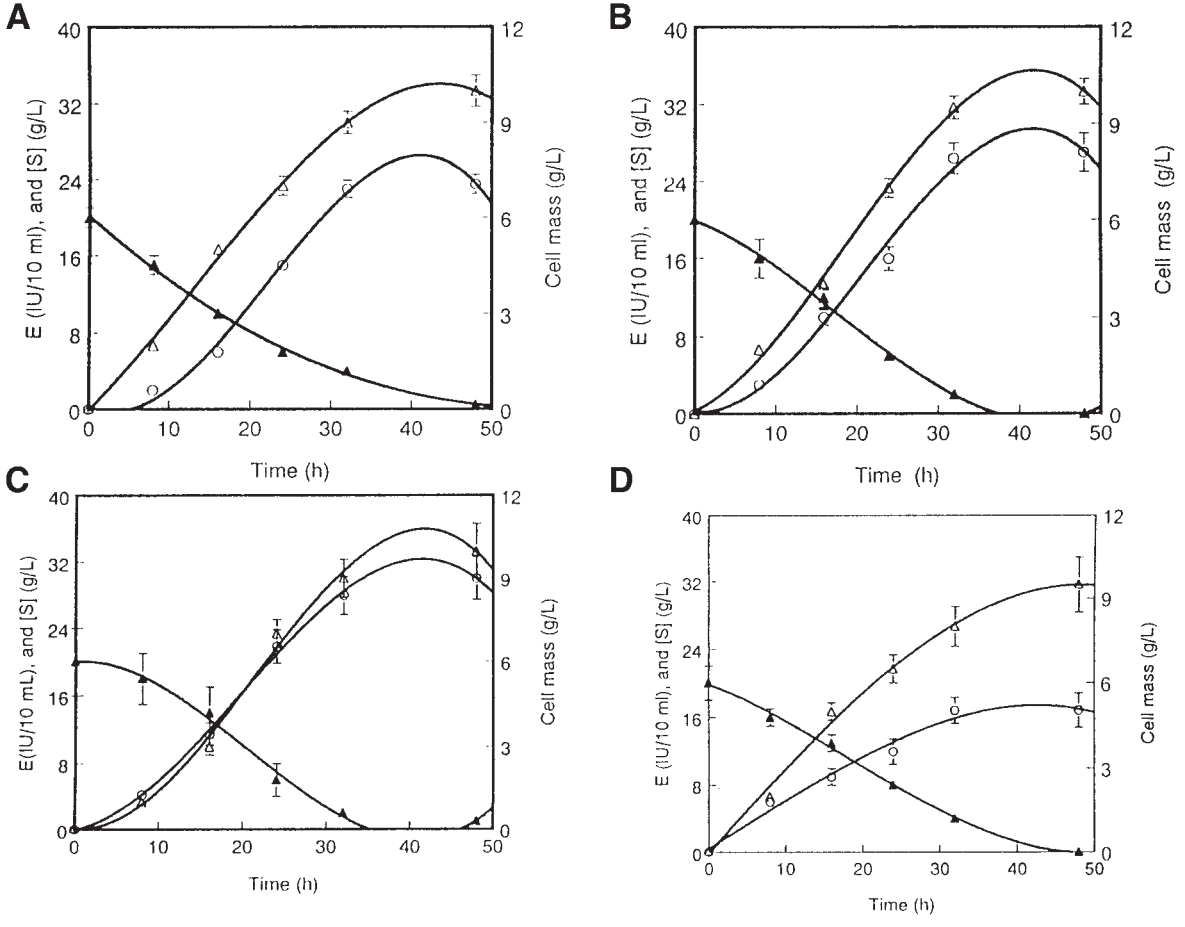

Fig. 4. Kinetics of β-glucosidase production in shake-flask fermentation of four representative substrates: **(A)** galactose; **(B)** lactose; **(C)** cellobiose; and **(D)** xylose. The initial pH of the medium was 5.5, inoculum size was 10% on 2% (w/v) substrates, and temperature was 35°C.

Table 3
Comparative Fermentation Kinetic Parameters of *K. marxianus* for β-Glucosidase Formation Parameters Following Growth on Different Substrates in Yeast Growth Medium (pH 5.5) in Shake-Flask Culture at 35°C[a]

	Product formation parameters[b]			
C source	Q_P (IU/[L·h])	$Y_{P/S}$ (IU/g)	q_P (IU/[g·h])	Specific activity (IU/mg protein)
Arabinose	9.0[e]	33.0[e]	31.0[f]	1.4[e]
Galactose	13.0[d]	117.0[c]	36.0[e]	2.1[d]
Glucose	0.24[f]	1.00[f]	01.1[g]	0.1[f]
Xylose	35.0[c]	84.0[d]	48.0[d]	5.6[c]
Cellobiose	90.0[a]	136.0[a]	64.0[a]	16.7[a]
Lactose	55.8[b]	132.0[b]	58.0[c]	12.3[b]
Sucrose	55.5[b]	118.0[c]	60.0[b]	11.1[b]

[a]Each value is an average of three replicates. Values followed by different superscript letters differ significantly at $p \leq 0.05$ according to Duncan multiple range test.

[b]Q_P = volumetric productivity (IU/[L·h]); $Y_{P/S}$ = product yield (IU/g of substrate utilized); q_P = specific productivity (IU/[g of cells·h]) calculated as multiple of μ and $Y_{P/X}$.

enced owing to the presence of different metabolites supported by each carbon source in the biosynthetic pathways. Volumetric substrate uptake rates (Q_s) from lactose (0.32 g of substrate/[L·h]) and sucrose (0.36 g of substrate/[L·h]) were marginally different but differed significantly with that on cellobiose and supported significantly ($p \leq 0.05$) different parameters of β-glucosidase production. There was also variation in intracellular protein synthesis, and a positive correlation was established between growth and protein productivity (Q_{IP}, mg/[L·h]).

Among monomeric saccharides, galactose proved to be a significantly ($p \leq 0.05$) greater stimulator of β-glucosidase product yield ($Y_{P/S}$). About 88% of activity accumulated intracellulary; twelve percent was found in the medium (and has not been included in the results). The maximum specific activity in Table 3 (12.4 IU/mg of protein) is 6- to 36-fold improved over that produced by *Escherichia coli* recombinant harboring *bgl* gene from *Caldocellum saccharolyticum* and crude enzyme from almonds sold by Sigma. These levels are higher than those reported on *K. marxianus (10)* following growth on cellobiose. One novel aspect of the study is that regulation of β-glucosidase synthesis has been observed using almost all the carbon sources that this organism can consume *(23)*. Moreover, this is the first ever report describing the potential kinetic parameters for substrate consumption and product formation using *K. marxianus*.

Effect of Nitrogen Sources on Enzyme Production

Replacement of one nitrogen source for another in the medium causes a change in protein synthesis as well as product formation. To explore the

influence of nitrogen sources on β-glucosidase synthesis, easily metabolizable nitrogen sources such as ammonium sulfate, ammonium nitrate, and sodium nitrate were comparatively evaluated with reference to sodium glutamate, peptone, urea, and corn steep liquor (CSL) (which release NH^{+4} slowly) when added to cellobiose medium. The results in Table 1 show that these nitrogen compounds influenced the production of β-glucosidase and cell growth to different degrees. All nitrogen sources except ammonium sulfate, ammonium nitrate, and sodium nitrate were significantly good nitrogen sources. These results clearly show that CSL is an appropriate nitrogen source for the production of β-glucosidase by *K. marxianus.* The replacement of ammonium sulfate with other nitrogen sources resulted in a lower increase in β-glucosidase profiles (Table 1), because there was considerable variation in cell mass of the organism. In the cultures grown in medium containing CSL, cell mass was 11.2 g/L, while it increased slightly in the presence of urea, and glutamate. On the other hand, in cultures grown in ammonium nitrate and sodium nitrate, the biomass concentration remained rather low (7.5–8.7 g/L) owing to a change in terminal pH. Generally, the results confirmed that CSL, a low-cost byproduct of the starch industry, gave the maximum profiles of β-glucosidase compared to other nitrogen compounds. The organism produced lower enzyme titers from sodium nitrate and ammonium nitrate, which was attributed to low nitrate reductase activity in the organism. The highest volumetric productivity (Q_p, 144 IU/[L·h]) of β-glucosidase is 6- to 100-fold higher than the values reported on *Cellulomonas* spp. and other organisms (see refs. *6* and *24*), *Saccharomyces cerevisiae* recombinants *(25,26)*, and fungi *(27–29)* and 1.4-fold higher than those displayed by the best *E. coli* recombinant harboring heterologous *bgl* gene from *Cellulomas biazotea (30)*.

Effect of Fermentation Temperature on β*-Glucosidase Production*

The inoculated fermentation medium was incubated at 22, 24, 30, 32, 35, 38, 40, 43, and 45°C (Fig. 5). Maximum enzyme productivities were supported at 35°C. At lower and higher temperatures, the organism supported lower volumetric or specific productivity. At lower temperature, the transport of nutrients in the cells is hindered, while at high temperature, the maintenance energy requirement increases owing to inactivation of proteins of the metabolic pathway *(20)* and less product formation occurs.

Thermodynamics of β*-Glucosidase Production*

It has been demonstrated *(14–16)* that both volumetric and specific productivities can be used to determine the thermodynamic parameters of all microbial processes provided that a sufficient number of data at different temperatures are available. In the present studies, specific productivities at different temperatures used to calculate thermodynamic parameters for β-glucosidase production and its deactivation. Maximum β-glucosidase-specific or volumetric productivity actually increased with an increase in temperature up to 35°C (Fig. 5). An empirical approach of Arrhenius *(17)*

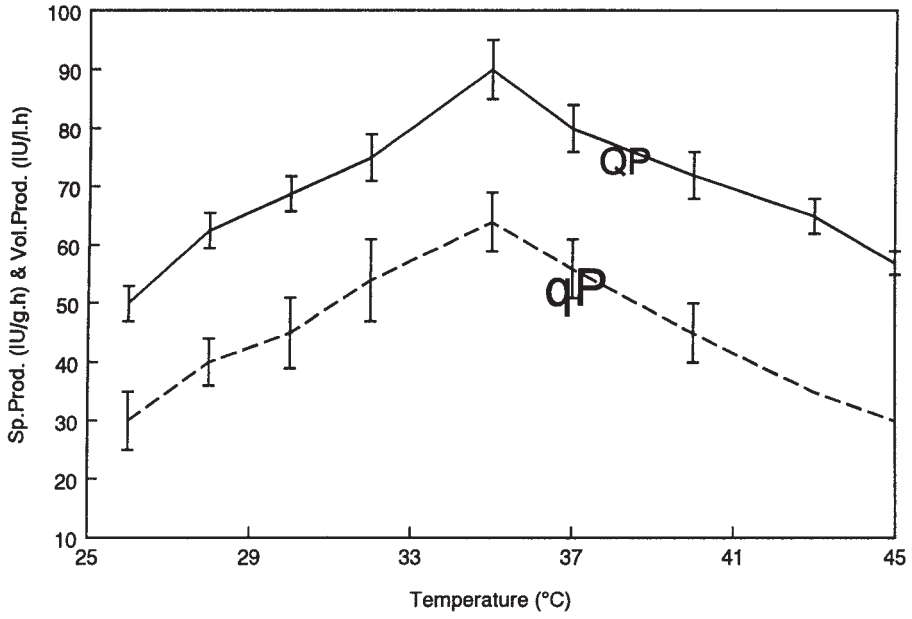

Fig. 5. Effect of fermentation temperature on volumetric (Q_p) and specific (q_p) productivity of β-glucosidase following fermentation of cellobiose (control pH of 5.5, 0.3% yeast extract, and time up to 48 h in time course).

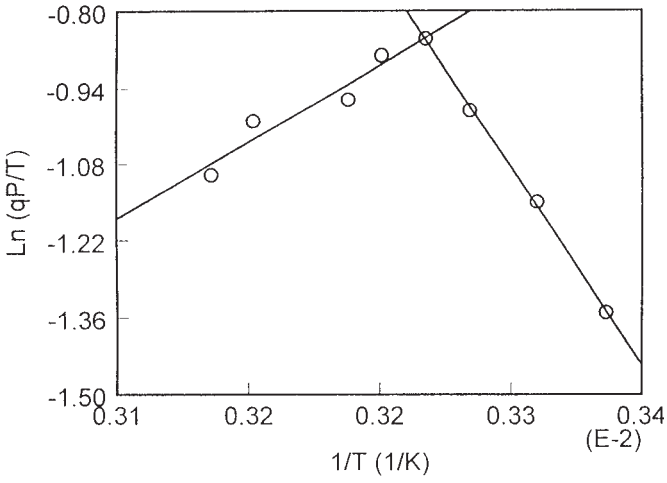

Fig. 6. Arrhenius plot to calculate enthalpy (ΔH^*) and entropy (ΔS^*) of β-glucosidase formation and its deactivation applying relationship $\mathrm{Ln}(q_p/T) = \mathrm{Ln}(k_B/h) + \Delta S^*/R - \Delta H^*/R \cdot 1/T$ (for details *see* Table 4).

was used to describe the relationship of temperature-dependent reversible and irreversible inactivation of β-glucosidase. The activation enthalpy of β-glucosidase production was graphically calculated from Fig. 6 by the application of an Arrhenius approach extended to microbial

Table 4
Thermodynamic Parameters Estimated by Arrhenius Approach for Batch Formation of β-Glucosidase and Its Deactivation[a]

	Enzyme formation	Thermal inactivation
Activation enthalpy (kJ/mol)	45 ± 3[a]	19.0 ± 2[b]
Activation entropy (kJ/[mol·K])	–0.06 ± 0.01[b]	–0.266 ± 0.012[a]

[a]Enthalpy and entropy values of product formation and its deactivation were calculated by applying the following equation:

$$\mathrm{Ln}(q_p/T) = \mathrm{Ln}(k_B/h) + \Delta S^*/R - \Delta H^*/R \cdot 1/T$$

in which q_P, T, k_B, h, ΔS^*, ΔH^*, and R are specific rate of product formation, absolute temperature, Boltzmann constant, Planck's constant, entropy of activation, enthalpy of activation, and gas constant, respectively. The values of ΔH^* were calculated as slopes of the straight line between $\mathrm{Ln}(q_P/T)$ and $1/T$ and $\mathrm{Ln}(k_B/h) + \Delta S^*/R$ = intercept on y-axis. The values of k_B, h, and R are 1.38×10^{-23} J/K, 6.63×10^{-34} J/S, and 8.314 J K^{-1} mol^{-1}, respectively.

processes *(18)* with some modification. For this purpose, K_r (specific rate of product formation, IU/[g of cells·h]) *(17)* was replaced with q_p (specific productivity or specific rate of product formation, IU/[g of cells·h]) and the following relationship emerged:

$$q_P = T \cdot k_B/h e^{\Delta S^*/R} e^{\Delta H^*/R \cdot T} \quad (1)$$

$$\mathrm{Ln}(q_P/T) = \mathrm{Ln}(k_B/h) + \Delta S^*/R - \Delta H^*/R \cdot 1/T \quad (2)$$

in which q_P, T, k_B, h, ΔS^*, ΔH^*, and R are specific rate of product formation, absolute temperature, Boltzmann constant, Planck's constant, entropy of activation, enthalpy of activation, and gas constant, respectively. The values of the thermodynamic parameters estimated with this model are summarized in Table 4. The activation enthalpy of β-glucosidase formation (ΔH^* = 45 kJ/mol) is lower than that for phytase production (70–80 kJ/mol) reported by Al-Asheh and Duvniak *(15)* but compares favorably with those estimated for many different whole-cell bioprocesses, such as cell growth (34–74 kJ/mol) *(14,15)*.

The phenomena responsible for thermal inactivation of enzyme is characterized by an activation enthalpy (ΔH_D^* = 19 kJ/mol) and is remarkably lower than that for β-glucosidase production. This means that its rate does not decrease faster with temperature than product formation rate. Therefore, the overall productivities do not fall sharply above a threshold value (Fig. 5). The value of ΔH_D^* is significantly lower than the values reported for a glucose isomerase system (160–235 kJ/mol) *(16)*. The activation entropy of β-glucosidase formation (–0.060 kJ/[mol·K]) is very low and compares favorably with other fermentation processes *(15,31)*.

The activation entropy value of thermal inactivation (–0.266 kJ/[mol·K]) is also very low (and has a negative symbol), which reflects that this inactivation phenomenon implies a slight increase in randomness during the

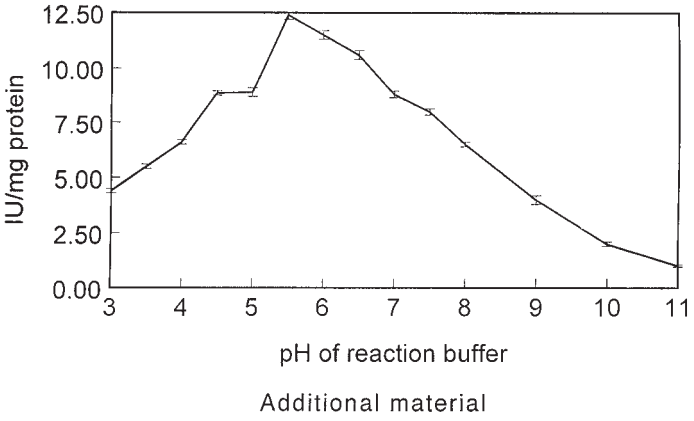

Fig. 7. Relative tolerance of pH by β-glucosidase produced by *K. marxianus* (control temperature of 55°C and time of 30 min, as described in Materials and Methods).

activated-state formation. Practically this value is lower than those estimated for some other enzymatic systems (0.89 kJ/[mol·K]) (*see* ref. *16*). This suggests a sort of protection exerted by the cell system against thermal inactivation. In the present studies, q_p values were used to calculate activation enthalpy and entropy for product formation and its deactivation according to Aiba et al. *(17)*, whereas other researchers used Q_p values to calculate these parameters *(14)*. It was found that the values calculated by this model were comparable with those obtained with the latter method and validated the application of approaches suggested by Arni et al. *(18)* that both volumetric and specific productivities can be used to calculate the activation enthalpy and entropy for product formation and its thermal deactivation

Enzyme Properties

The enzyme showed maximum activity at 55°C. Thermal inactivation followed first-order kinetics in the presence of PMSF (1 m*M*). The half-life of the enzyme was 192, 102, and 12 h at 50°C, 55°C, and 60°C, respectively, while the enzyme system was stable at 60°C for 48 h in the presence of substrate. This can be compared with a half-life of 24.85 h at 65°C exhibited by thermostable β-glucosidase produced by *E. coli* recombinant (purchased from Sigma) harboring heterologous *bgl* genes from *C. saccharolyticum* and 23.5 h by β-glucosidase by thermophilic cultures of *Thermoascus aurantiacus (28)*. Similarly, enzyme was stable at pH 5.0–7.0 (Fig. 7) and corroborated the work of Brady et al. *(32)*. That the enzyme system was thermodynamically resistant to denaturation at elevated temperatures, as was visualized by the application of an Arrhenius approach ($\text{Ln } V_{\max} = -E_A/RT$) on crude enzyme preparation in the presence of PMSF (Fig. 8), was encouraging. The values of activation energy were 50.3 and 36.7 kJ/mol for hydrolysis of 1 *M p*-nitrophenyl-β-D-glucopyranoside and deactivation of enzyme, respectively. These characteristics revealed that the enzyme was fairly ther-

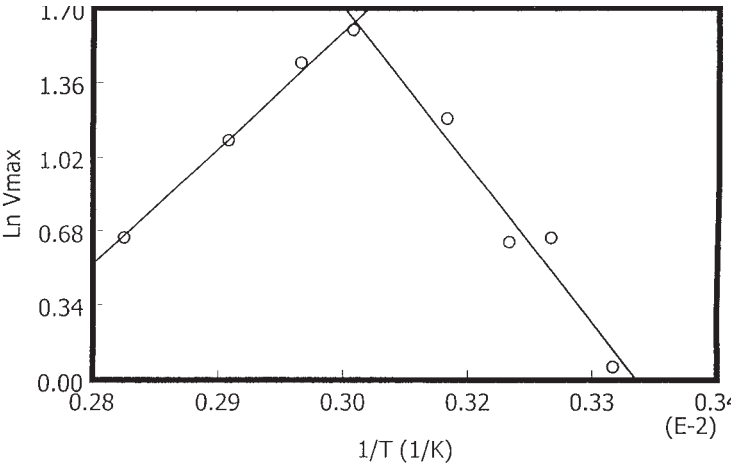

Fig. 8. Arrhenius plot to calculate the activation energy (E_A) for hydrolysis of enzyme substrate.

mostable and that the organism can be exploited for simultaneous saccharification and fermentation application to produce ethanol using *K. marxianus* as an ethanologenic organism *(9)*.

Conclusion

Improved β-glucosidase production was obtained by using other carbon sources to select a cheaper carbon source, such as sucrose in molasses or lactose in cheese whey, for its production. CSL contains potential nitrogen for improved production of β-glucosidase. The greatest enzyme production occurred at 35°C and pH 5.5 when grown in a medium containing cellobiose and CSL as carbon and nitrogen sources, respectively. The thermodynamics and kinetics of β-glucosidase production indicated that the enzyme system exerted a defense against thermal inactivation. The enzyme was substantially stable at 60°C and pH 7.0–8.0 and may be selected for commercial applications.

The volumetric and specific productivities of β-glucosidase were significantly higher than the deduced values reported by other researchers. We intend to improve the production of β-glucosidase by isolating a mutant derivative altered in translation along with optimizing other nitrogen and carbon sources to support production of elevated profiles of this important enzyme in cellulolysis for production of value-added commodity products from renewable biomass.

Nomenclature

h = Planck's constant
k_B = Boltzmann constant
q_P = specific rate of enzyme production (IU/[g of cells·h])

q_S = specific rate of substrate consumption (g of substrate consumed/[g of cells·h])
Q_{IP} = rate of intracellular protein formation (mg/[L·h])
Q_P = rate of β-glucosidase formation (IU/[L·h])
Q_S = rate of substrate consumption (g of substrate consumed/[L·h])
Q_X = rate of cell mass formation (g of cells/[L·h])
R = gas constant
T = absolute temperature
$Y_{P/S}$ = β-glucosidase yield (IU/g of substrate utilized)
$Y_{P/X}$ = specific yield of enzyme production (IU/g of cells)
$Y_{X/S}$ = cell yield coefficient (g of cells formed/g of substrate utilized)
ΔH^* = enthalpy of activation
ΔS^* = entropy of activation
μ = specific growth rate (h^{-1})

Acknowledgments

We are grateful to the director general of NIBGE/NIAB for providing research facilities. This work was supported by Pakistan Atomic Energy Commission, Islamabad. Some chemicals were purchased from a United States Agency for International Development (USAID) grant under PCST Proposal 6.163.

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