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UDC 577.152.34:663.1:547.455
ISSN 1330-9862

original scientific paper

(FTB-1237)

Kinetics and Regulation Studies of the Production of β-Galactosidase from *Kluyveromyces marxianus* Grown on Different Substrates

Muhammad Ibrahim Rajoka, Samia Khan and Riaz Shahid*

National Institute for Biotechnology and Genetic Engineering,
P.O. Box 577, Jhang Road, Faisalabad, Pakistan

Received: May 16, 2003
Accepted: November 10, 2003

Summary

Lactose-intolerance is manifested in 50 % of the world's population. This can be remediated by removing lactose from the diet or converting it into glucose and galactose with β-galactosidase (EC 3.2.1.23). In this work, batch production of this enzyme in the presence of lactose, galactose, cellobiose, xylose, arabinose, sucrose and glucose was investigated using *Kluyveromyces marxianus* in shake flask culture studies. Substrate type and temperature were the independent variables that directly regulated the specific growth and β-galactosidase production rates. Lactose (2 %) supported the maximum specific product yield ($Y_{P/X}$), followed by galactose, sucrose, cellobiose, xylose, arabinose and glucose. Its synthesis was regulated by an induction and a growth-dependent repression mechanism. The optimum temperature for the production was found to be 35–37 °C. The highest volumetric productivity of enzyme (80.0 IU/L/h) occurred on lactose-corn steep liquor medium. This was significantly higher than the calculated values reported in the literature. Thermodynamic studies revealed that the cells provided a defence mechanism against thermal inactivation. The enzyme was stable at 60 °C and pH=5.0–7.0, and it may find application in commercial lactose hydrolysis.

Key words: β-galactosidase; *Kluyveromyces marxianus*; lactose, galactose, cellobiose, xylose and glucose as substrates

Introduction

Maldigestion of lactose is manifested in 50 % of the world's population (*1*). This trait is determined genetically and is due to reduction in enzyme protein. Remediation of this genetic disorder consists in removing lactose from the diet or converting it into glucose and galactose by application of β-galactosidase (EC 3.2.1.23) or lactase (*2*,*3*). Moreover, conversion to galactose and glucose is useful because it has 70 % of the sweetness of sucrose (*4*). Lactose hydrolysis is also of great interest for physiological, nutritional and technological studies (*3*). The enzymatic hydrolysis of lactose to glucose and galactose also constitutes the basis of the most biotechnological processes currently developed to exploit the sugar content of milk whey, a by-product of cheese industry whose disposal now contributes to a considerable pollution problem.

Kluyveromyces marxianus has been employed for the production of biomass, enzymes (including β-galactosidase) and ethanol (*3*,*5*). Some strains of *K. marxianus* can grow at a temperature of up to 52 °C and are expected

* Corresponding author; Fax ++91 41 651 475; E-mail: mirajoka@nibge.org

to produce thermostable β-galactosidase. Galactose and lactose are common inducers of this enzyme but as for other enzymes (*6*), other carbon sources may serve as inducers of β-galactosidase as reported for β-glucosidase production in *Candida peltata* (*7*).

Activation enthalpy and entropy of β-galactosidase production and its inactivation were determined to get insight into the kinetics of the whole system and clarify the phenomena involved in both the production of β-galactosidase and its thermal inactivation, as reported for glucose isomerase, phytase (*8*), alcohol and xylitol production (*9*) reactions. Three different approaches for their estimation are available in literature: (*i*) a thermodynamic approach used by Roels (*10*) to elucidate the thermal inactivation of enzyme systems, (*ii*) a kinetic approach (*11*) only useful to describe the time dependent phenomena of irreversible inactivation and thermal death of cells, and (*iii*) Arrhenius plots used to study growth and product formation by different organisms (*11*). The last one was selected for the present system, as it was applied by Converti and Dominguez (*9*). This work reports regulation of β-galactosidase production following its growth on different carbon sources and fermentation temperatures under optimized conditions (initial medium, pH=5.5, yeast extract (0.2 %), nitrogen source (corn steep liquor) and inoculum size (volume fraction of 10 %) using kinetics and thermodynamics of its production and its deactivation.

Materials and Methods

Chemicals

2-Nitrophenyl-β-D-galactopyranoside (2-NPGa), 2-nitrophenol, arabinose, xylose, glucose, galactose, sucrose, cellobiose and lactose were from Sigma Chemical Co. USA. All other chemicals were of analytical grade.

Source and maintenance of *Kluyveromyces marxianus*

The strain was collected from NIBGE Culture Collection, National Institute for Biotechnology and Genetic Engeneering (NIBGE Y-1). It was isolated from molasses during summer and it acquired the ability to grow at higher substrate concentrations. Stock culture was maintained on malt extract agar plates at 4 °C after growth at 50 °C.

Preparation of inoculum

The inoculum was prepared in glucose medium (*5*) at 35 °C on a rotary shaker (150 rpm). The cultures were centrifuged (10 000 g, 30 min), washed twice with saline, and used (10 %) containing 0.25 mg(dry cells)/mL (*7*).

Enzyme production

The ability of the strain to utilize arabinose, galactose, glucose, cellobiose, sucrose, lactose or xylose as a sole carbon source was examined in basal salts medium (*5*). Filter-sterilized carbon sources (20 g/L) were added to basal medium (pH=5.5) at the time of inoculation.

Shake-flask batch production was carried out at 35 °C (unless otherwise stated) on a gyratory shaking incubator at a shaking speed of 150 rpm as described earlier (*6*). The growth (g/L) was measured gravimetrically as dry cell mass. Clear supernatant from 100 mL of original culture broth was obtained by centrifugation (10 000 g, 30 min). The cell pellet was used to extract cellular fractions (*5*).

Effect of temperature on enzyme production

The data of batch fermentations were gained by performing 3 separate experiments on lactose-based media by maintaining other variables constant and varying temperature in an orbital shaker (150 rpm) at a temperature range of 22–45 °C.

Determination of kinetic parameters

Erlenmeyer flasks containing 20–30 % medium volume and shaken at 150–200 rpm are able to ensure product yields comparable with tests carried out in stirred tank reactors, at K_La values ranging from 4.8–35.4 h^{-1} and volumetric air flow/system volume ratios up to 0.29, respectively (*9*). Kinetic parameters for batch fermentation process in shake-flasks were determined according to Lawford and Rousseau (*12*).

Enzyme assays

β-Galactosidase activity was determined using 1 mM 2-NPGa as substrate in 50 mM sodium acetate buffer, pH=5.0. One millilitre of the enzyme sample (preheated at 40 °C) was incubated with the above mixture (equilibrated for 5 min) at 40 °C for 10 min. The reaction was terminated by the addition of 2 mL of 1 M sodium carbonate. The liberated *o*-nitrophenol was measured at 400 nm. One IU of β-galactosidase is defined as the amount of enzyme that releases 1 μmol of nitrophenol per min under the assay conditions.

Protein determination

Proteins were determined by Bradford method (*13*) using bovine serum albumin as standard.

The determination of saccharides

Saccharides were analyzed by HPLC (Perkin Elmer, USA) using column HPX-87H (300x78 mm, Bio-Richmond, CA, USA) maintained at 45 °C in a column oven. Sulphuric acid (0.002 N) served as a mobile phase at 0.6 mL/min. The samples were detected using refractive index detector and quantified using Turbochron4 software by Perkin Elmer, USA.

Empirical approach of the Arrhenius equations (*11*) was used to describe the relationship of temperature dependent reversible and irreversible inactivations of β-galactosidase production in the temperature range of 22–45 °C. For this purpose, K_r (specific rate of product formation, IU/g (cells)/h) (*11*) was replaced by q_p (specific productivity, IU/g (cells)/h) from which the following relationship emerged:

$$q_P = T\, K_B/h\, e^{\Delta S^*/R}\, e^{-\Delta H^*/RT} \qquad /1/$$

$$\ln(q_P/T) = \ln(K_B/h) + \Delta S^*/R - \Delta H^*/RT \qquad /2/$$

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 constant) is $6.63 \cdot 10^{-34}$ J s and K_B

(Boltzman constant, (R/N)) is $1.38 \cdot 10^{-23}$ J/K (where N (Avogadro's No.) is $6.02 \cdot 10^{23}$ mol^{-1}).

Statistical analysis

The effects of treatment were compared by the protected least significant difference method and are presented in the form of probability (*p*) using Duncan multiple range test with MstatC software.

Results and Discussion

Initial studies on the induction of β-galactosidase indicated that galactose medium (pH=5.5) was essential to induce high activities of this enzyme. The use of commercial galactose as feedstock is uneconomical for large-scale production; therefore, several other substrates and lactose were included in these studies. All further studies were performed to produce the enzyme using 2 % of substrates, which we found to be optimum to calculate kinetic parameters.

Time course production of β-galactosidase from different substrates

The kinetics of *K. marxianus* growth and production of β-galactosidase in shake-flask batch culture were examined by growing cells in mineral culture media as described in Materials and Methods. Representative batch culture kinetics of enzyme production from galactose (a), xylose (b), cellobiose (c) and lactose (d), respectively, are presented in Fig. 1. These results suggested that bulk β-galactosidase production reached a maximum activity after 30–40 h. The curves indicate that the production of β-galactosidase was apparently growth-associated. Application of Luedeking and Piret model (*14*), however, indicated that the enzyme production was both growth-associated as well as non-growth-associated and confirmed the work of Inchaurrondo *et al*. (*4*).

Complete consumption of all carbon sources (except xylose) took place within 60 h of fermentation. Mean protein formation rates and the values of volumetric rate of substrate consumption (Q_S=g/L/h of sugar consumed) of the organism were significantly higher ($p \leq 0.05$) on arabinose and glucose than the values from all other carbon sources (Table 1). These values were significantly higher than those reported by other researchers (*4*). Volumetric substrate uptake rates from lactose, galactose, sucrose and cellobiose were significantly different and supported significantly higher ($p \leq 0.05$) kinetic parameters of β-galactosidase production (Table 1).

Enhanced substrate consumption rates by *K. marxianus* could increase its application in commercial β-galactosidase production from industrial substrates (cheese whey and molasses), as reported by Furlan *et al*. (*15*). Growth rates, intracellular protein production rates and substrate uptake rates were considered the factors that might contribute to differences in enzyme synthesis from different growth substrates. The values of Q_S from cellobiose (0.68 g/L/h), sucrose (0.60 g/L/h), lactose (0.52 g/L/h) and galactose (0.74 g/L/h) were different and all except galactose displayed inverse relationship on intracellular β-galactosidase synthesis. Similarly, intracellular protein production from the above carbon sources was significantly different and all of them except galactose provided inverse relationship on the regulation of enzyme. Among easily metabolizable substrates, values of growth parameters on arabinose and glucose (non-inducers) were higher than those of galactose and xylose (both induced enzyme production) and exhibited inverse relationship on β-galactosidase formation.

Specific enzyme yield ($Y_{P/X}$) was 480.0 IU/g of cells on lactose, and only 5.2 IU/g of cells as basal enzyme activity in the presence of glucose (non inducer). The induction ratio, defined as the ratio of inducer activity and basal activity, was 15.0–92.4. It appeared that enzyme synthesis was controlled by two regulatory mech-

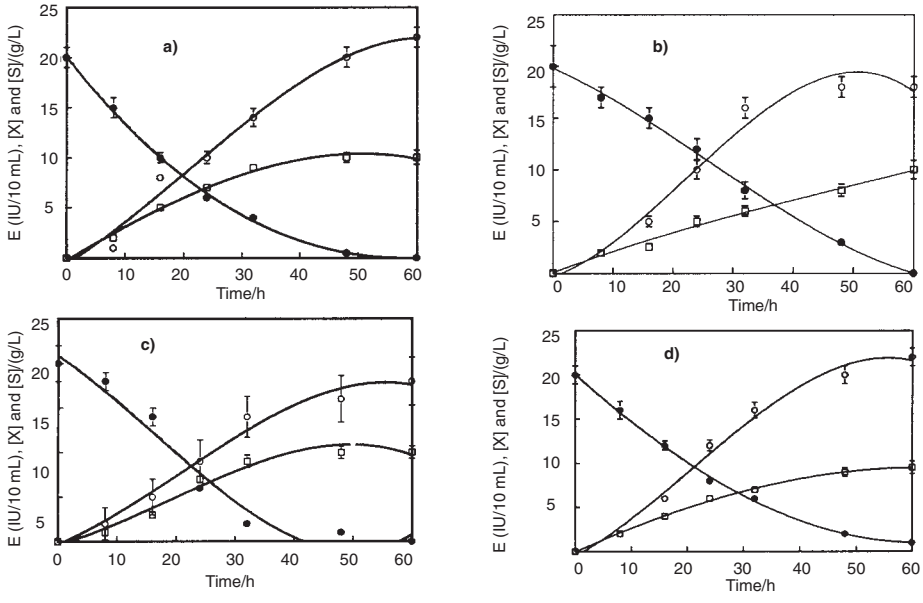

Fig. 1. Kinetics of β-galactosidase production in shake flask fermentation of four representative substrates (2 %), namely (a) galactose, (b) lactose, (c) cellobiose, and (d) xylose. The initial pH of the medium, inoculum size, and temperature were 5.5, 10 % and 35 °C, respectively. ○ = E, β-galactosidase, □= X, cell mass and ● = S, substrate

Table 1. Comparative growth kinetics of *Kluyveromyces marxianus* grown on different substrates (2 %)

C-source	Substrate consumption parameters*			Product formation parameters**		
	μ	Q_S	Q_{IP}	Q_P	Spec. act.	$Y_{P/X}$
	(1/h)	(g/L/h)	(mg/L/h)	(IU/L/h)	(IU/mg)	(IU/g of cells)
Arabinose	0.54^{ab}	0.76^{b}	6.6^{a}	12^{e}	4^{f}	78^{f}
Galactose	0.49^{c}	0.74^{b}	6.2^{b}	71^{b}	11^{b}	434^{b}
Glucose	0.59^{a}	0.91^{a}	6.4^{a}	1^{f}	1^{g}	5^{g}
Xylose	0.45^{d}	0.65^{cd}	6.2^{b}	5^{d}	7^{e}	129^{e}
Cellobiose	0.49^{c}	0.68^{c}	5.4^{c}	65^{c}	12^{b}	377^{d}
Lactose	0.36^{e}	0.52^{e}	4.5^{e}	80^{a}	16^{a}	480^{a}
Sucrose	0.37^{f}	0.60^{d}	5.0^{d}	66^{c}	12^{b}	391^{c}

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

*μ = specific growth rate calculated as slope of ln*X* (*X* is cell mass, g/L) *vs.* time of fermentation

Q_x = g(cells) synthesized per litre per h (g/L/h)

$Y_{x/s}$ = g (cells)/g (substrate) consumed

Q_s = g(substrate) consumed per L per h

Q_{IP} = intracellular protein productivity

**Q_p = Product formation rate, specific activity (IU/mg of protein) and $Y_{P/X}$ (specific product yield, IU/g of cells), respectively

anisms: (*i*) an induction mechanism that enhanced the specific enzyme yield ($Y_{P/X}$) of β-galactosidase up to 92.4-fold, and (*ii*) a growth-dependent repression mechanism that changed the rate of synthesis up to 14.9- and 24.8-fold in arabinose- and xylose-induced over non-induced (glucose-grown) cultures, respectively (Table 1).

Following growth on all substrates, about 88 % of total activity was located within the cell; 12 % was secreted in the culture medium (and has not been included in the results). Maximum specific enzyme activity and volumetric yield were 16.3 IU/mg of protein (Table 1) and 4.9 IU/mL (Fig. 1) from lactose and are significantly ($p \leq 0.05$) higher than those reported for *K. lactis*, *K. fragilis* and *K. marxianus* (*4,5*). The highest volumetric productivity (Q_P=80 IU/L/h) of β-galactosidase was 6- to 100-fold higher than the values reported on *Cellulomonas* spp. and other organisms (*6*), filamentous fungi (*16*), other yeasts (*4*), and *Saccharomyces cerevisiae* recombinants (*17,18*), and is comparable with that reported by Furlan *et al.* (*15*). In these studies, substrate regulation of β-galactosidase synthesis has been observed using almost all carbon sources which this organism can consume or ferment (*19*). In almost all other cases, β-galactosidase synthesis has been mainly evaluated using lactose or galactose as the carbon source (*5,16*). Only Furlan *et al.* (*15*) reported its production from molasses-based media.

Effect of temperature

The inoculated fermentation medium was incubated at 22–45 °C (Fig. 2a). Maximum enzyme volumetric (Q_P) and specific (q_p) productivity values were supported at 35 °C. Optimum temperature coincides with the optimum reported in the literature for this organism in synthetic medium (*4,5,15*).

The enzyme showed 90 % of its maximum activity at 60 °C in buffer solution (pH=5.0–7.0).

Thermodynamic parameters of β-galactosidase production

Maximum β-galactosidase-specific or volumetric productivity actually increased with the increase in temperature up to 35 °C (Fig. 2a). Activation enthalpy of β-galactosidase production was graphically calculated from Fig. 2b. The values of the thermodynamic parameters (Table 2) indicated that the activation enthalpy of β-galactosidase formation (ΔH^*=36.6±3 kJ/mol) is lower than that for phytase production (70–80 kJ/mol) (*9*) but compares favourably with those estimated for many different whole-cell bioprocesses, such as cell growth (34–74 kJ/mol) (*9,11*).

The phenomena responsible for thermal inactivation of enzyme are characterized by an activation enthalpy (ΔH_D^*) of 31.6±2 kJ/mol; this is remarkably lower than that for its production. It means that its rate of deactivation does not decrease faster with temperature than

Table 2. Thermodynamic parameters§ estimated by Arrhenius approach for batch formation and deactivation of β-galactosidase

	Enzyme formation	Thermal inactivation
Activation enthalpy/(kJ/mol)	36.6 ± 3^{a}	31.6 ± 2^{b}
Actvation entropy/(J/mol/K)	$(-)64.9\pm4^{a}$	$(-)291.6\pm20^{b}$

§ Thermodynamic parameters were determined using the following equation:

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

where q_P, T, K_B, h, ΔS^*, ΔH^* and R are specific productivity, absolute temperature, Boltzmann constant, Planck's constant, entropy of activation, enthalpy of activation and gas constant, respectively. The values of K_B, h and R are $1.38 \cdot 10^{-23}$ J/K, $6.63 \cdot 10^{-34}$ J s and 8.314 J/K/mol, respectively. From the above straight line equation, ΔH^* was calculated as slope and $\ln(K_B/h) + \Delta S^*/R$ as intercept on Y-axis

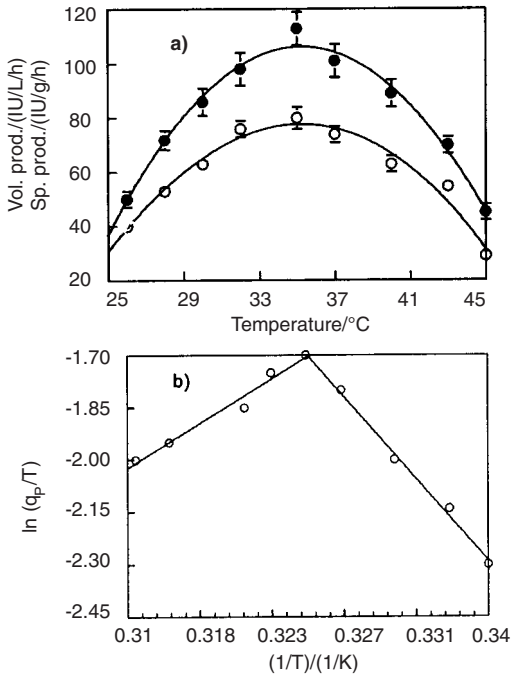

Fig. 2a. Effect of fermentation temperature on β-galactosidase volumetric productivity (Vol. prod. ○) and specific productivity (Sp. prod. ●), medium pH=5.5

Fig. 2b. Arrhenius plot on enthalpy and entropy of β-galactosidase formation and its deactivation using specific productivity values at different temperatures

product formation rate. The value of ΔH_D^* is significantly lower than the values reported for glucose isomerase system (160–235 kJ/mol) (*8*). The activation entropy of β-galactosidase formation (–0.065 kJ/mol/K) is very low and compares favourably with that of xylitol formation reactions (*8*) as well as other fermentation processes (*10,11*). The activation entropy value of thermal inactivation (–0.292 kJ/mol/K) is also very low (and has negative symbol), which reflects that this inactivation phenomenon implies a little disorderliness during growth on the substrate at higher temperature. Practically this value is lower than those estimated for some other enzymatic systems (0.89 kJ/mol/K) (*11*). This suggests a sort of protection exerted by cell system (possibly due to chaperone activity) against thermal inactivation.

Conclusion

Lactose was the best carbon source, followed by galactose and sucrose, but galactose is an expensive substrate, therefore, sucrose present in molasses could be used for commercial β-galactosidase production. The highest enzyme production level occurred at 35 °C, and pH=5.5. The volumetric productivities of β-galactosidase were significantly higher than the deduced values reported by some other authors. The enzyme was remarkably stable at 60 °C and pH=7.0 and may find application in commercial lactose hydrolysis. Thermodynamic studies suggested that the cell system exerted defence (probably due to chaperone activity) against thermal inactivation.

Acknowledgements

This work was supported by Pakistan Atomic Energy Commission, Islamabad. Some chemicals were purchased from a USAID grant under PCST Proposal 6.163. This work formed a part of M. Ph. thesis of S. K. Research facilities provided by Director General NIBGE/NIAB are gratefully appreciated.

References

1. V. Gekas, M. Lopez-Leiv, *Process Biochem. 20* (1985) 2–12.
2. D. B. G. Martins, C. G. De Souza, D. A. Simoes, M. A. De Morais, *Curr. Microbiol. 44* (2002) 379–382.
3. M. A. F. Belem, B. H. Lee, *Crit. Rev. Food Sci. Nutr. 38* (1998) 565–598.
4. V. A. Inchaurrondo, O. M. Yantorno, C. E. Voget, *Process Biochem. 29* (1994) 47–54.
5. D. Brady, R. Merchant, A. P. McHale, *Biotechnol. Lett. 16* (1994) 737–740.
6. M. I. Rajoka, A. Bahir, K. A. Malik, *Biores. Technol. 62* (1997) 99–108
7. B. C. Saha, R. J. Bothast, *Appl. Environ. Microbiol. 62* (1996) 3165–3170.
8. A. Converti, M. Del Borghi, *Microb. Technol. 21* (1997) 511–517.
9. A. Converti, J. M. Dominguez, *Biotechnol. Bioeng. 75* (2001) 39–45.
10 J. A. Roels, *Elsevier Biomedical Press*, Amsterdam (1983).
11. S. Aiba, A. E. Humphrey, N. F. Millis, *Biochemical Engineering (2nd ed.)*, Academic Press, New York (1973) 92–127.
12. H. G. Lawford, J. D. Rousseau, *Biotechnol. Lett. 15* (1993) 615–620.
13. M. M. Bradford, *Anal. Biochem. 72* (1976) 248–254.
14. R. Luedeking, E. L. Piret, *J. Biochem. Microbiol. Technol. Eng. 1* (1957) 393.
15. A. S. Furlan, A. S. L. Schneider, R. Merckle, M. F. Carvalho-Johans, R. Jonas, *Biotechnol. Lett. 22* (2000) 1195–1198.
16. R. Rech, C. F. Cassini, A. Sacci, M. A. Z. Ayub, *Appl. Microbiol. 23* (1999) 91–96.
17. L. Domingues, J. A. Teixiera, M. Pentitila, N. Lima, *Appl. Microbiol. Biotechnol. 58* (2002) 645–650.
18. M. Beccerra, S. D. Prado, E. Rodriguez-Balmonte, M. E. Cerdan, M. I. G. Siso, *Biotechnol. Lett. 24* (2002) 1391–1396.
19. D. Singh, P. Nigam, I. M. Banat, R. Marchant, A. P. McHale, *World J. Microbiol. Biotechnol. 14* (1998) 823–834.

Proučavanje kinetike i regulacije proizvodnje β-galaktozidaze od bakterije *Kluyveromyces marxianus* uzgojene na raznim supstratima

Sažetak

Laktozu ne podnosi približno 50 % populacije u svijetu. To se može spriječiti uklanjanjem laktoze iz hrane ili njezinom razgradnjom na glukozu i galaktozu djelovanjem β-galaktozidaze (EC 3.2.1.23). Proučavana je šaržna proizvodnja toga enzima u prisutnosti laktoze, galaktoze, celobioze, ksiloze, arabinoze, saharoze i glukoze, koristeći *Kluyveromyces marxianus* u tikvicama na tresilici. Vrsta supstrata i temperatura nezavisne su varijable koje izravno reguliraju specifični rast i brzinu proizvodnje β-galaktozidaze. Dodatak laktoze (2 %) omogućava najveće specifično iskorištenje proizvoda ($Y_{P/X}$), a sve manje iskorištenje postiže se s galaktozom, saharozom, celobiozom, ksilozom, arabinozom i glukozom. Sinteza β-galaktozidaze regulirana je indukcijom i mehanizmom represije ovisnom o rastu. Optimalna temperatura proizvodnje bila je 35–37 °C. Najviša volumetrijska proizvodnja enzima (80 IU/L/h) postignuta je na podlozi laktoza-kukuruzni ekstrakt, što je puno više od izračunatih vrijednosti opisanih u literaturi. Na osnovi termodinamičkih studija vidi se da stanice imaju obrambeni mehanizam prema toplinskoj inaktivaciji. Enzim je bio stabilan pri 60 °C i pH=5,5–7,0 pa se može primijeniti u komercijalnoj hidrolizi laktoze.