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Optimization of β-Galactosidase Production in Stirred Tank Bioreactor Using *Kluyveromyces lactis* NRRL Y-8279

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Abstract This paper investigates the production and optimization of β-galactosidase enzyme using synthetic medium by *Kluyveromyces lactis* NRRL Y-8279 in stirred tank bioreactor. Response surface methodology was used to investigate the effects of fermentation parameters on β-galactosidase enzyme production. Maximum specific enzyme activity of 4,622.7 U/g was obtained at the optimum levels of process variables (aeration rate 2.21 vvm, agitation speed 173.4 rpm, initial sugar concentration 33.8 g/L, incubation time 24.0 hr). The optimum temperature and pH of the β-galactosidase enzyme produced under optimized conditions were 37°C and pH 7.0, respectively. The enzyme was stable over a pH range of 6.0-7.5 and a temperature range of 25-37°C. The K_m and V_{max} values for *O*-nitrophenol-β-D-galactopyranoside (ONPG) were 1.20 mM and 1,000 μmol/min·mg protein, respectively. The response surface methodology was found to be useful in optimizing and determining the interactions among process variables in β-galactosidase enzyme production. Hence, this study fulfills the lack of using mathematical and statistical techniques in optimizing the β-galactosidase enzyme production in stirred tank bioreactor.

Keywords: stirred tank bioreactor, β-galactosidase, *Kluyveromyces lactis*, response surface methodology, synthetic medium

Introduction

The enzyme β-galactosidase (lactase, EC 3.2.1.23) is an industrially important enzyme. This enzyme hydrolyzes lactose into its monosaccharides, glucose and galactose and is used in the preparation of de-lactosed milk or whey, products used by lactose intolerant people and for increasing the value and possibilities of whey which is a high polluting waste (1). New applications for β-galactosidase enzyme, such as in the production of biologically active galacto-oligosaccharides, have also been reported in the literature (2,3). Commercial β-galactosidases are produced from yeasts such as *Kluyveromyces lactis* and *Kluyveromyces marxianus* and moulds such as *Aspergillus niger* and *Aspergillus oryzae* (4,5). β-Galactosidases produced by yeasts are the most employed for the treatment of milk, sweet whey, and neutral pH dairy products since their optimum pH is between 6.5-7.0 (5).

In the previous investigation of our group, β-galactosidase enzyme production was optimized in shake flask cultures by *K. lactis* NRRL Y-8279 using a synthetic medium and maximum specific enzyme activity was obtained at the optimum levels of process variables (6). However, shake flask cultivation has a number of significant disadvantages relative to cultivation in a fermentor, including lower oxygen transfer rate, less closely controlled environmental conditions, etc. Therefore, it is of higher practical value to determine the optimum fermentation conditions, such as incubation time, substrate concentration, agitation speed, and aeration rate in a mechanically stirred bioreactor (7).

Among other factors having an impact on the operating conditions in bioreactors are aeration and agitation. Aeration supplies the necessary oxygen for the growth and performance of microbial cells. Agitation is important for uniform mixing of the medium components within the fermentor (dispersion of cells and nutrients) as well as mass transfer phenomena (e.g., oxygen transfer rates). Agitation also creates shear forces that may affect microbial growth in some other ways, including morphological changes of the culture, cell damage, and formation of metabolites (8-10). The important influence of oxygen transfer and/or agitation of the medium on lactase activity was demonstrated by various researchers (1,11-15). Cortes *et al.* (1) investigated the effect of oscillating dissolved oxygen (DO) tension on the metabolism of *K. marxianus*. Schneider *et al.* (11) found that the relationship of agitation/aeration at constant volumetric oxygen transfer coefficient influenced the production of β-galactosidase. Barberis and Gentina (13) studied the influence of DO on β-galactosidase production in chemostat culture using a defined medium. Barberis and Segovia (15) optimized β-galactosidase production by controlling DO concentration. The statistical planning of experiments is a useful tool to optimize the processes where many variables are involved, enabling a better manipulation of the parameters, a more representative analysis of the results. The conventional practice which varies one variable at a time does not allow evaluation of the combined effects of all the factors involved in the process and constitutes a time consuming methodology. These restrictions can be overcome by the use of statistical experimental design combined with response surface methodology (RSM). RSM is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors, and searching optimum conditions of factors for desirable responses. It can identify and quantify the various interactions among different parameters (10). RSM has been extensively applied to optimize culture medium and other process parameters for the production of lipase (16,17), tannase

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(18), α-amylase (19), β-cyclodextrin glucanotransferase (20), dextran dextrinase (21), chitinase (22), and polygalacturonase (23). Chen *et al.* (24) used a central composite design to optimize the medium composition for lactase production in shake flask experiments by *K. marxianus*. Manera *et al.* (25) optimized β-galactosidase enzyme production in shake flasks using a central composite rotatable design. No previous work has used RSM to optimize β-galactosidase production in a mechanically stirred bioreactor and to determine the interactions among process parameters such as aeration rate, agitation speed, substrate concentration, and incubation time, etc.

The aim of this study was the optimization and determination of the interaction among fermentation parameters in β-galactosidase production in a stirred tank bioreactor by RSM. The optimized fermentation parameters were aeration rate, agitation speed, incubation time, and initial sugar concentration. The enzyme obtained under the optimized conditions was partially characterized. This work is the first article on the examination of the effect of factors such as aeration rate, agitation speed, and the optimization of β-galactosidase production in a mechanically stirred bioreactor by RSM.

Materials and Methods

Organism and culture media *Kluyveromyces lactis* NRRL Y-8279 used throughout this study was kindly supplied by the U.S. Department of Agriculture, National Center for Agricultural Utilization Research. The strain is maintained on yeast-malt agar slants stored at 4°C and transferred monthly to fresh slants incubated at 30°C for 2 days. Medium composition of agar slants was as follows (in g/L): glucose, 10; yeast extract, 3; malt extract, 3; peptone, 5; agar, 20 (pH=6.0±0.2). Medium composition for inoculum and fermentation were (in g/L): lactose, 25; yeast extract, 1; K_2HPO_4, 2; $NH_4H_2PO_4$, 1; $(NH_4)_2HPO_4$, 1; 7 $H_2O\cdot MgSO_4$, 0.1, and pH was adjusted to 7.35 (6). All chemicals used in this study were of analytical grade.

Fermentation conditions The cultures used to inoculate the production medium were prepared by transferring 2 loops of *K. lactis* NRRL Y-8279 cells to 250-mL conical flasks containing 50 mL of culture medium. The flasks were incubated at 30°C for 48 hr in a rotary shaker incubator (Laboshake LS 2/5 RO 2/5; Gerhardt, Bonn, Germany) operated at 200 rpm and used to inoculate the production medium at a level of 2%(v/v).

Fermentations were carried out batchwise in a 3.0-L Bio-Flo 110 Modular Benchtop Fermentor (New Brunswick Scientific Corporation, NJ, USA) with a working volume of 1.75 L. It consisted of a glass vessel with 4 equally spaced vertical baffles. Agitation was done by dual Rushton-style impellers. The production medium and the reaction vessel were sterilized at 121°C for 20 min. The pH was adjusted to 7.35 and not further controlled during the process. The temperature was kept at 30°C. Oxygen tension was measured as the percentage of DO in the culture medium in relation to air saturation using an oxygen electrode (InPro 6830; Mettler Toledo, Switzerland). The levels of aeration rate, agitation speed, initial sugar concentration, and incubation time used in the optimization studies by RSM are given in Table 1. Samples were collected under aseptic conditions to monitor β-galactosidase activity, biomass, and residual sugar concentration.

Analytical techniques For the permeabilization of *K. lactis* for β-galactosidase enzyme assay, a volume of resuspended cells amounting to 10-20 mg of dry cell mass was mixed with 5 mL isoamyl alcohol and diluted up to 25 mL with 0.2 M phosphate buffer, pH 6.5 containing 0.1 mM $MgCl_2\cdot 6H_2O$. The mixture was shaken for 15 min at room temperature to make the cell envelopes permeable and used for the enzyme assay (13). The method desribed by Food Chemicals Codex (FCC) (26) was used with some modifications for β-galactosidase activity. The substrate for the enzyme was 2.5 mg/mL of 2-nitrophenyl-β-D-galacto pyranoside (ONPG) (N-1127; Sigma-Aldrich, Steinheim, Germany), which was prepared in 0.2 M phosphate buffer, pH=6.5. One mL of the crude enzyme sample previously permeabilized with isoamyl alcohol was incubated with 4 mL of the above substrate mixture (preheated to 37°C) at 37°C for 15 min. The reaction was terminated by adding 1 mL of 10% sodium carbonate. The liberated *O*-nitrophenol (ONP) was measured spectrophotometrically (Analytik Jena Spekol 1300; Analytik Jena AG, Jena, Germany) at 420 nm. ONP (N1.970-2; Sigma-Aldrich) concentration was calculated using an extinction coefficient of 4.2371 mL/μmol·cm. One unit of enzyme activity was defined as the enzyme quantity that liberated 1 μmol of ONP/min under the assay conditions. Specific activity (U/g) was defined as the number of lactase units/g cell biomass. Specific β-galactosidase activity was used throughout the study to obtain a common basis for comparisons. Volumetric enzyme activity values were determined the kinetics of enzyme production.

The biomass concentration was measured gravimetrically as dry cell mass (g/L) by centrifuging 20 mL of original culture broth at 4,000×g for 20 min followed by drying the cells at 80°C for overnight. Lactose was measured using the 3,5-dinitrosalicylic acid (DNS) method (27). Protein concentration was determined according to the Bradford method using bovine serum albumin (BSA) (Sigma-Aldrich) as standard (28). All experiments were carried out in triplicate and the values reported are the mean of 3 such experiments in which 3-5% variability was observed.

Partial characterization of β-galactosidase The optimum pH was determined using the reaction mixtures as described previously except for with various buffers: pH 5.0, using 50 mM acetate buffer; pH 6.0, 6.5, 7.0, and 7.5, using 50 mM phosphate buffer; pH 8.0, 9.0, using 50 mM Tris-HCl buffer. To check the pH stability, enzyme was pre-incubated with the above respective buffer at 37°C for 30 min and enzyme activity was measured using the substrate solution described above without adjusting the pH. The relative activities were determined by using the maximal activity of the enzyme at a specific pH as 100%.

The effect of temperature on enzyme activity was tested by assaying the activity at different temperatures in the range 25-55°C and pH 6.5 using the reaction mixtures as indicated above. The relative activities were determined by using the maximal activity of the enzyme at a specific temperature as 100%. Thermal stability was determined by

pre-incubating the enzyme at various temperatures for 30 min and the samples were placed in ice immediately. The relative activities were then determined using the standard method described above, taking the activity of a sample without incubation as 100%.

Kinetic parameters were determined in the reaction mixtures indicated above containing variable amounts of the specific substrate ONPG (0.5-12.5 mM). The values of Michaelis constant (K_m, mM) and maximum velocity (V_{max}, μmol/min·mg protein) were determined from Lineweaver-Burk plots.

Experimental design and statistical analysis The statistical analysis of the data was performed using Minitab Statistical Software (Release 13.20). Details of RSM can be found elsewehere (29). The levels of factors used in the experimental design are listed in Table 1. The data of the factors were chosen after a series of preliminary experiments. Thirty-one experiments were conducted using a face central composite statistical design (α=1) for the study of 4 factors each at 3 levels (Table 2). The levels were −1, 0, +1 where 0 corresponded to the central point. The actual level of each factor was calculated using the following equation (29).

$$\text{Coded value}=\frac{\text{actual level}-(\text{high level}+\text{low level})/2}{(\text{high level}-\text{low level})/2}$$

Response surface model was fitted to the response variable, namely specific β-galactosidase activity (U/g). The second order response function for 4 quantitative factors is given by Eq. 1:

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{44}X_4^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{14}X_1X_4+\beta_{23}X_2X_3+\beta_{24}X_2X_4+\beta_{34}X_3X_4 \quad (1)$$

where X_1, X_2, X_3, and X_4 represent the levels of the factors according to Table 1 and β_0, β_1,……, β_{34} represent coefficient estimates with β_0 having the role of a scaling constant.

Results and Discussion

Optimization of β-galactosidase enzyme production in stirred tank bioreactor Mixing is very crucial for maximum enzyme production in microbial fermentations and it is achieved by means of aeration and agitation. However there should be an optimum balance between the aeration and the agitation of the bioreactor. For example, while low agitation speeds cause a reduction in enzyme production due to lower availability of DO, higher agitation speeds may cause disruption of free cells in the reactor by shear forces and formation of vortex which may result in poor mass transfer. Therefore, it is necessary to establish optimum combination of airflow and agitation for maximum product yield (30). Our preliminary experiments showed that the aeration rate, agitation speed, initial sugar concentration, and incubation time influenced the production of β-galactosidase enzyme in the stirred tank bioreactor. Thus, a face centered design was used to determine the optimum levels of the above mentioned variables leading to maximum specific enzyme activity. The levels of these factors (Table 1) used in the optimization studies by RSM were determined by preliminary experiments. The effect of the 4 previously mentioned variables, each at 3 levels, and their interactions on β-galactosidase enzyme synthesis have been determined by carrying out 31 experiments given by the model (Table 2).

Table 1. Levels of factors used in the experimental design

		Level		
Factor	Name	-1	0	+1
X_1	Aeration rate (vvm)	1	2	3
X_2	Agitation speed (rpm)	100	200	300
X_3	Initial sugar (g/L)	15	25	35
X_4	Incubation time (hr)	12	22	32

Table 2. Experimental design

Run	Aeration rate (vvm)	Agitation speed (rpm)	Initial sugar (g/L)	Incubation time (hr)	Specific activity (U/g)
1	3	100	15	12	3,103
2	1	300	15	32	2,932
3	1	300	35	12	2,992
4	2	200	25	22	4,420
5	3	300	15	12	3,111
6	1	100	15	12	3,208
7	1	100	35	32	3,320
8	2	200	25	22	4,493
9	1	300	15	12	3,494
10	3	100	35	32	3,994
11	2	200	25	22	4,460
12	3	300	15	32	2,939
13	1	100	35	12	3,303
14	2	200	25	22	4,416
15	3	100	15	32	2,996
16	1	200	25	22	3,892
17	2	100	25	22	4,213
18	1	100	15	32	2,710
19	2	200	25	32	4,134
20	2	200	25	22	4,528
21	3	300	35	32	3,515
22	3	100	35	12	3,611
23	2	200	35	22	4,374
24	3	200	25	22	4,049
25	3	300	35	12	3,172
26	2	200	25	12	4,212
27	2	200	25	22	4,446
28	2	300	25	22	4,104
29	2	200	15	22	4,320
30	1	300	35	32	3,096
31	2	200	25	22	4,460

Analysis of variance (ANOVA) for specific enzyme activity is presented in Table 3. The analysis gives the value of the model and determines the requirement of a more complex model with a better fit. As shown in Table 3, R^2 was 0.996 indicating that the model as fitted explained 99.6% of the variability in specific enzyme

Table 3. Analysis of variance for β-galactosidase production (R^2=0.996)

Source[1)]	DF	Seq SS	Adj SS	Adj MS	F	p
Regression	14	11,037,016	11,037,016	788,358	261.25	<0.001
Linear	4	582,774	582,774	145,694	48.28	<0.001
Square	4	9,554,131	9,554,131	2,388,533	791.51	<0.001
Interaction	6	900,110	900,110	150,018	49.71	<0.001
Residual error	16	48,283	48,283	3,018		
Lack of fit	10	38,839	38,839	3,884	2.47	0.141
Pure error	6	9,444	9,444	1,574		
Total	30	11,085,299				

[1)]DF, degrees of freedom; Seq SS, sequential sum of squares; Adj SS, adjusted sum of squares; Adj M, adjusted mean square.

activity. F-test for regression was significant at a level of 5% ($p<0.05$) indicating that the model is fit and can adequately explain the variation observed in enzyme synthesis with the designed levels of the factors. If the F-test for lack of fit is significant, then a more complicated model is needed to fit the data. As seen in Table 3, the lack of fit (0.141) was not significant at the 5% level ($p>0.05$) indicating that the experimental data obtained fitted well with the model. These results show that the model chosen can satisfactorily explain the effects of aeration rate, agitation speed, initial sugar concentration, and incubation time on β-galactosidase production by *K. lactis* NRRL Y-8279.

Thirty-one experiments were carried out from the design and by applying multiple regression analysis on the experimental data, the following second order polynomial equation was found to explain β-galactosidase production by *K. lactis*.

$$Y=-564.849+1{,}749.64X_1+16.2884X_2+49.4414X_3+78.8684X_4-503.587X_1^2-0.0315592X_2^2-1.27187X_3^2-3.01087X_4^2-0.588513X_1X_2+11.1039X_1X_3+8.66000X_1X_4-0.119414X_2X_3+1.36665X_3X_4 \quad (2)$$

where X_1, X_2, X_3, and X_4 are the actual levels of factors shown in Table 1.

Regression analysis (Table 4) of the experimental data showed that aeration rate, agitation speed, initial sugar concentration, and incubation time had positive linear effects on enzyme synthesis ($p<0.05$). Probability (p) values were used as a tool to check the significance of each of the coefficients. The smaller the magnitude of p value, the more significant was the correlation with the corresponding coefficient. Among the 4 factors tested, aeration rate had the highest impact on specific enzyme activity as given by the highest linear coefficient (1,749.64), followed by incubation time (78.8684), initial sugar concentration (49.4414), and agitation speed (16.2884). These factors also showed significant negative quadratic effects on enzyme production indicating that specific enzyme activity increased as the level of these factors increased and decreased as the level of these parameters increased above certain values. Interaction between these parameters was also significant. The interactions between aeration rate-agitation speed, aeration rate-initial sugar concentration, aeration rate-incubation time, agitation speed-initial sugar concentration, and initial sugar concentration-incubation time were significant as shown by low p values ($p<0.05$)

Table 4. Estimated regression coefficients for β-galactosidase production

Term	Coefficient	SE Coefficient	T	p
Constant	-564.849	16.30	274.105	<0.001
Aeration rate	1,749.64	12.95	6.619	<0.001
Agitation speed	16.2884	12.95	-4.731	<0.001
Initial sugar	49.4414	12.95	10.997	<0.001
Time	78.8684	12.95	-2.447	0.026
Aeration×aeration	-503.587	34.10	-14.768	<0.001
Agitation×agitation	-0.0315592	34.10	-9.255	<0.001
Sugar×sugar	-1.27187	34.10	-3.730	0.002
Time×time	-3.01087	34.10	-8.830	<0.001
Aeration×agitation	-0.588513	13.73	-4.285	0.001
Aeration×sugar	11.1039	13.73	8.085	<0.001
Aeration×time	8.66000	13.73	6.306	<0.001
Agitation×sugar	-0.119414	13.73	-8.695	<0.001
Agitation×time	-0.00522500	13.73	-0.380	0.709
Sugar×time	1.36665	13.73	9.951	<0.001

for interactive terms. But the interaction between agitation speed and incubation time was found to be insignificant as given by p value above 0.05. Hence this term was excluded from the polynomial Eq. 2 used for this model.

Figure 1 shows the contour plots of specific enzyme activity for each pair of factors by keeping the other 2 factors constant at its middle level. Maximum specific enzyme activity was obtained at middle level of each pair of factors at a constant middle level of the other 2 factors. Further increase in these factors above the middle level (aeration rate, 2.2 vvm; agitation speed, 170 rpm; initial substrate concentration, 35 g/L; incubation time, 24 hr) showed decrease in specific enzyme activity.

Many researchers have reported agitation speeds and aeration rates in β-galactosidase enzyme production between the ranges of 100-800 rpm and 0.08-2.7 vvm (volume of air/ volume of liquid/min), respectively (12-15, 31-33). As far as it is known, there is no work in the literature done on the optimization of airflow and agitation rates simultaneously. There should be an optimum balance between the aeration rate (oxygen tension) and agitation speed (shear) to have maximum enzyme synthesis and this could be achieved by optimizing the combinations of airflow and agitation rates. As seen in Fig. 1A, 1D, and 1E, β-galactosidase enzyme

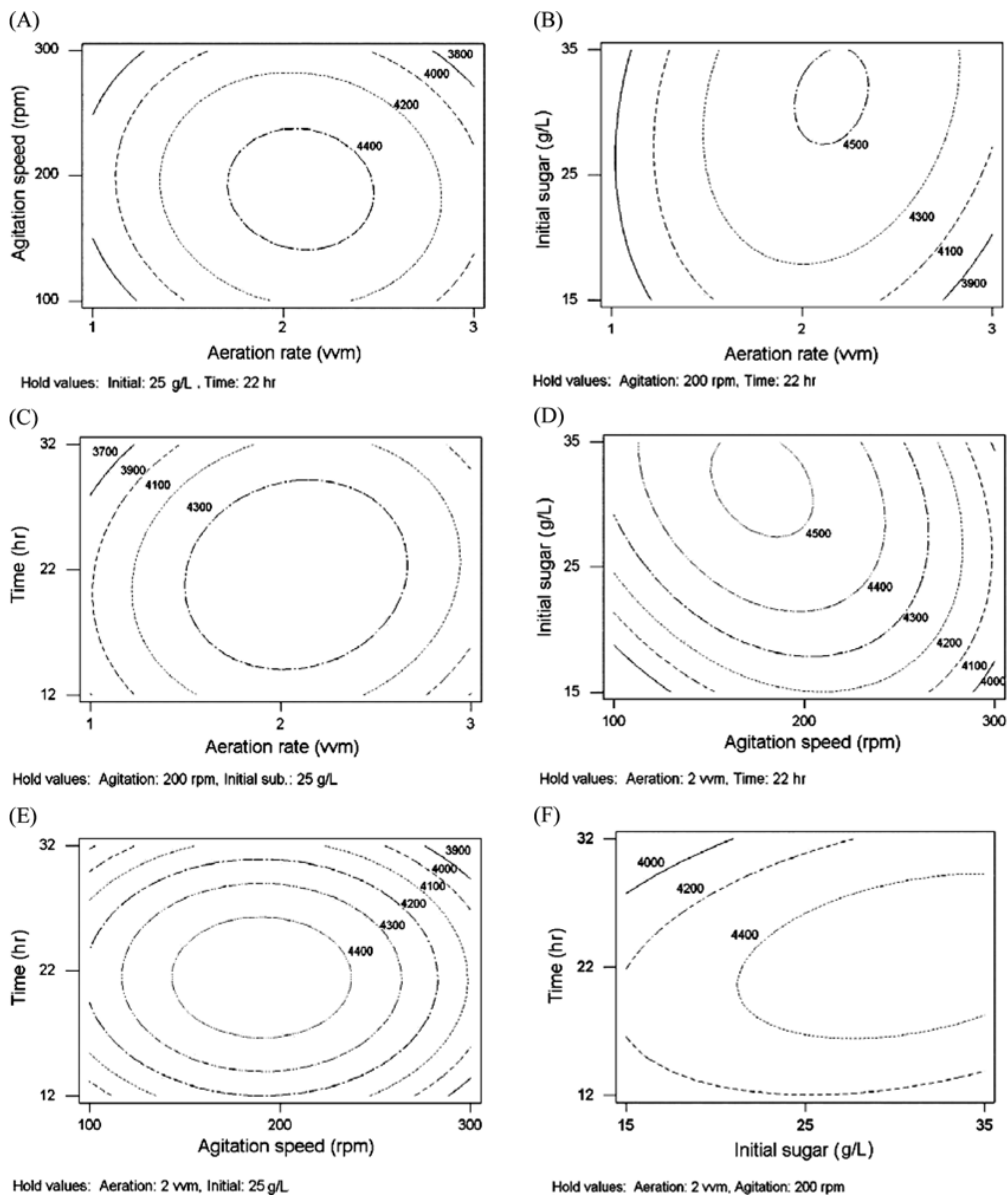

Fig. 1. Contour plots for specific β-galactosidase enzyme activity at varying conditions. (A) Agitation speed and aeration rate, (B) initial sugar concentration and aeration rate, (C) incubation time and aeration rate, (D) initial sugar concentration and agitation speed, (E) incubation time and agitation speed, and (F) incubation time and initial sugar concentration at a constant middle level of the other 2 factors.

activity increased with the increase in agitation speed at a constant middle level of the other 2 factors and decreased at higher agitation speeds. Low agitation can cause a drastic reduction in β-galactosidase production by *K. lactis* and this could be due to lower availability of DO at low mixing rates. As DO is the rate-limiting factor because of its low solubility in the aqueous solutions, it affects cell growth and yield of product in aerobic fermentations (30). However, β-galactosidase production has been found to be negatively affected by variations in agitation rates beyond 180-200 rpm. This decrease in β-galactosidase synthesis at high agitation rates can be attributed to the production of high shear stress in the broth which may cause a decrease in the growth of shear-sensitive microorganism and enzyme synthesis.

Our results also showed that the aeration rate influenced enzyme synthesis. Maximum β-galactosidase production was achieved at an aeration rate of 2.2 vvm. Aeration can help the growth and performance of microbial cells by improving the mass transfer characteristics of the fermentation medium with respect to substrate, products/by products, and oxygen. Aeration results in better mixing of the fermentation broth and thus maintains a concentration gradient between the interior and the exterior of the cells. This concentration gradient results in better diffusion of sugars and other

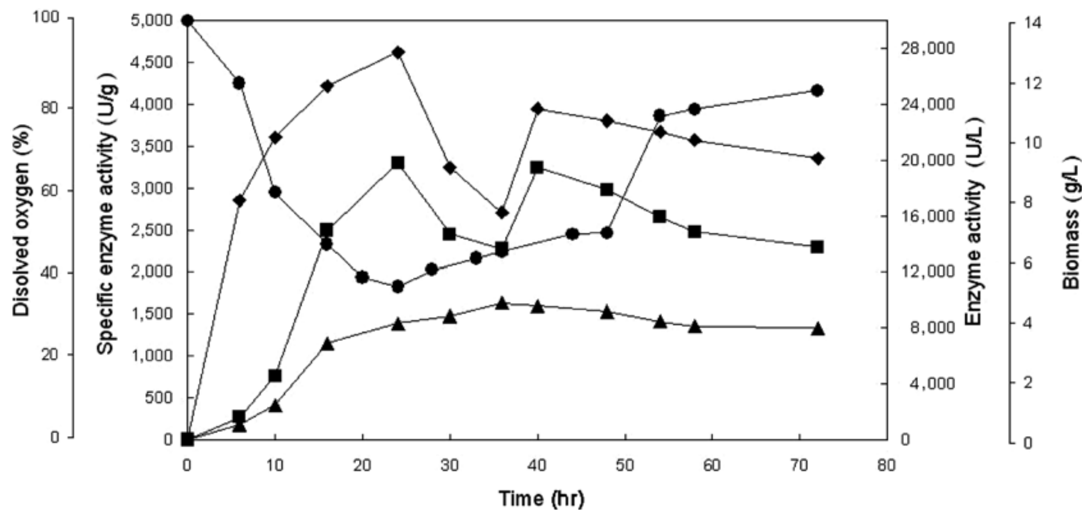

Fig. 2. Kinetics of β-galactosidase production by *K. lactis* NRRL Y-8279 in stirred tank bioreactor. ◆, Specific enzyme activity; ■, enzyme activity; ▲, biomass; ●, DO concentration. Initial pH=7.35, initial sugar concentration=33.8 g/L, T=30°C; the standard deviation of each experimental point ranged from 1.3 to 4.1.

nutrients to the cells, while it facilitates the removal of gases and other byproducts of catabolism from the microenvironment of the cells. Air supply provides oxygen to the cells and this is especially important for high biomass concentration (34). In our study, aeration rates higher than 2.2 vvm decreased β-galactosidase activity. Barberis and Gentina (13) controlled DO in stirred tank bioreactor by varying agitation speed and aeration rate. They achieved the maximum β-galactosidase production at 10% DO and stated that the metabolism of *K. fragilis* is more reductive at low aeration condition which leads to higher enzyme expression.

Our results revealed that initial sugar concentration and incubation time played a major role in enzyme production together with aeration rate and agitation speed. Figure 1B, 1D, and 1F show that the activity of β-galactosidase increased with increasing initial sugar concentrations. The highest enzyme activity was obtained at around 35 g/L initial sugar concentration and further increase in sugar concentration resulted in a decrease in enzyme synthesis. Furlan *et al.* (12) also found that the activity of lactase decreased with increasing initial sugar concentrations and the highest enzyme activities were obtained at 20 and 40 g/L of initial sugars. They stated that the oxygen uptake became insufficient with increasing initial concentrations of sugars, thus being the limiting factor in the synthesis of enzymes.

In order to determine the maximum specific enzyme activity corresponding to the optimum levels of aeration rate, agitation speed, initial sugar concentration, and incubation time, a second order polynomial model was used to calculate the values of these variables. The fitting of the experimental data to Eq. 2 allowed the determination of the levels of aeration rate (X_1=2.21 vvm), agitation speed (X_2=173.4 rpm), initial sugar concentration (X_3= 33.8 g/L), and incubation time (X_4=24.0 hr) giving a maximum specific enzyme activity of 4,566.5 U/g. The above data optimizes β-galactosidase enzyme production from synthetic medium containing lactose by *K. lactis* NRRL Y-8279 in stirred tank bioreactor.

The final fermentation was performed using synthetic medium with the optimized levels of aeration rate (2.21 vvm), agitation speed (173.4 rpm), initial sugar concentration (33.8 g/L), and incubation time (24.0 hr) given by the model. Maximum specific enzyme activity (4,622.7 U/g) which was slightly higher than the value given by the model was obtained on the 24th hr of fermentation. The maximum enzyme productivity obtained was 824.4 U/L hr.

Kinetics of β-galactosidase enzyme production from *K. lactis* NRRL Y-8279 under optimized conditions The kinetics of *K. lactis* growth and production of β-galactosidase enzyme in the stirred tank bioreactor were examined in the synthetic medium containing 33.8 g/L of initial lactose. The optimized values of 173.4 rpm agitation speed and 2.2 vvm aeration rates were used throughout the fermentation. The maximum specific activity (4,622.7 U/g) was attained at the end of 24 hr which was higher than the specific activity (3,416.6 U/g) obtained previously using the same microorganism in the shake flask cultures (6). This result is acceptable under the low oxygen transfer rate and less closely controlled environmental conditions of the shake flask cultivation.

As seen in Fig. 2, the specific enzyme activity initially increased and attained a maximum after 24 hr of fermentation. However, as the fermentation continued, specific β-galactosidase activity decreased drastically. This initial period comprised the exponential growth phase when cells were growing with a maximum specific growth rate (μ_{max}) of 0.31/hr. A second phase of high β-galactosidase activity was observed after 40 hr of fermentation which corresponds to the stationary phase of the organism. The reason for obtaining 2 distinct points on specific activity curve might be attributed to the hydrolysis of lactose to glucose by the β-galactosidase enzyme synthesized in the initial stages of fermentation. The glucose produced might have repressed the enzyme synthesis. After exhaustion of glucose, the microorganism might have synthesized more

Table 5. Some studies on batch production of β-galactosidase in stirred tank bioreactor using different *Kluyveromyces* species

Culture	Medium	Specific activity	Volumetric activity	Reference
K. marxianus NRRL Y-1109	Dehydrated whey	2,800 U/g	31,700 U/L	1
K. marxianus CDB 002	Cane molasses	760 U/g	-	11
K. fragilis NRRL Y-1109	Lactose	5,910 IU/g	-	13
K. marxianus CBS 7894	Lactose	380.9 U/g	-	14
K. fragilis NRRL Y-1109	Lactose	4,480 UI/g	-	15
K. marxianus CDB 002	Cane molasses	-	21.8 U/mL	37
K. lactis CBS 683	Lactose	1.64 ONPG/mg	-	38
K. lactis NRRL Y-8279	Lactose	4,622.7 U/g	19,785 U/L	This study

β-galactosidase in an effort to utilize lactose in the medium leading to a second peak of enzyme activity (6). Similar activity profile curves were reported in the literature (6, 33,35). Rech *et al.* (33) studied β-galactosidase production from sweet cheese whey by using 2 different strains of *K. marxianus* in a stirred tank bioreactor and found that the specific activity curves exhibited 2 distinct sharp points at the beginning and at the end of the growth phase. They also stated that volumetric β-galactosidase activity followed the pattern of the growth curve. Martins *et al.* (35) studied the production of β-galactosidase enzyme from *K. marxianus* and observed that the specific enzyme activity increased and attained a maximum after 4 hr of incubation. They found the second phase of high specific activity after 16 hr of incubation where the culture entered the stationary phase of growth. In our previous study about β-galactosidase production in shake flask culture, 2 distinct points on specific activity curve was also observed at the 12th and 48th hr of fermentation (6).

Figure 2 shows that volumetric enzyme activity increased during the exponential growth phase as a result of yeast growth. The maximum volumetric activity (19,785 U/L) was obtained at the 24th hr of fermentation. Similar to specific enzyme activity curve, volumetric activity curve exhibited 2 maximum points at the end of exponential growth phase and at the beginning of stationary phase. Volumetric activity decreased drastically throughout the stationary growth phase of the organism most probably due to the unstability of the intracellular enzyme, presence of proteases, and changes in the cell wall during the stationary phase as well as the enzymatic inhibition of the hydrolysis products (6). The decrease of β-galactosidase activity after 40 hr indicates that the operation should be stopped after the stationary phase is reached. Parallel to our findings, Ornelas *et al.* (36) produced β-galactosidase from *K. lactis* in cheese whey permeate medium and detected 2 maximum peaks of volumetric and specific β-galactosidase activity: one at the late exponential growth phase and another at the stationary growth phase when lactose has been depleted. They stated that the first decline in enzyme activity at late exponential phase could be attributed to the low concentration of the inductor (lactose) in the culture medium and the second decline in stationary growth phase could reflect the repression of enzyme synthesis in the absence of the inductor. However, by examining protein bands from the samples collected at the stationary phase, they also found that the β-galactosidase neither has changed its concentration nor has been degraded.

DO is also one of the most important environmental parameters that could affect cell growth and enzyme synthesis. As seen in Fig. 2, DO concentration in the fermentation medium decreased gradually from 100% at the beginning to 36% at the 24th hr of fermentation suggesting that no oxygen limitation occurred during cell growth. The DO concentration started to increase slightly thereafter at the end of the exponential phase.

Maximum specific (475.21 U/g·hr) and volumetric (935.67 U/L·hr) productivity values were obtained at the end of 6 and 16 hr, respectively. As expected, the concentration of residual sugars decreased during the fermentation and almost complete sugar depletion was observed in the culture medium. The residual sugar concentration in culture medium was 0.99 g/L. In all culture systems, the pH decreased slightly during fermentation and decreased from an initial value of 7.35 to 3.14 at the end of 48 hr.

Table 5 lists data from this study and some earlier reports in the literature about batch production of β-galactosidase enzyme in stirred tank bioreactors using different *Kluyveromyces* species. Table 5 shows the variations in the enzyme activity values reported in these studies. Several factors could account for the variations such as the strain of microorganism, the chemical composition, and any impurities in the medium, the conditions employed during fermentation, the configuration of the bioreactor used and even enzyme assays. However, when the results obtained in this study are compared with the literature, it can be seen that specific and volumetric enzyme activity values are among the highest values reported so far.

Partial characterization of the β-galactosidase produced by *K.lactis* NRRL Y-8279 Temperature and pH stability of the β-galactosidase produced by *K. lactis* NRRL Y-8279 were tested in order to asses its suitability for industrial applications. The enzymes for these tests were obtained from bioreactor cultivation under the optimum conditions mentioned previously. The effect of pH on the β-galactosidase activity produced by *K. lactis* NRRL Y-8279 was studied by incubating the enzyme in the buffer solution with a pH range of 5.0-9.0 (Fig. 3A). The optimum pH was found to be 7.5 and the enzyme was stable (90-99%) over a pH range of 6.0-7.5 (Fig. 3B). At pH 8.0, the enzyme retained 64% of its original activity. Fujimura *et al.* (39) studied purification and molecular characterization of β-galactosidase from *K. lactis*. Similar to our results, they found that the optimum pH of the enzyme was 7.0 and the enzyme was stable over a pH range of 7.0 to 8.5.

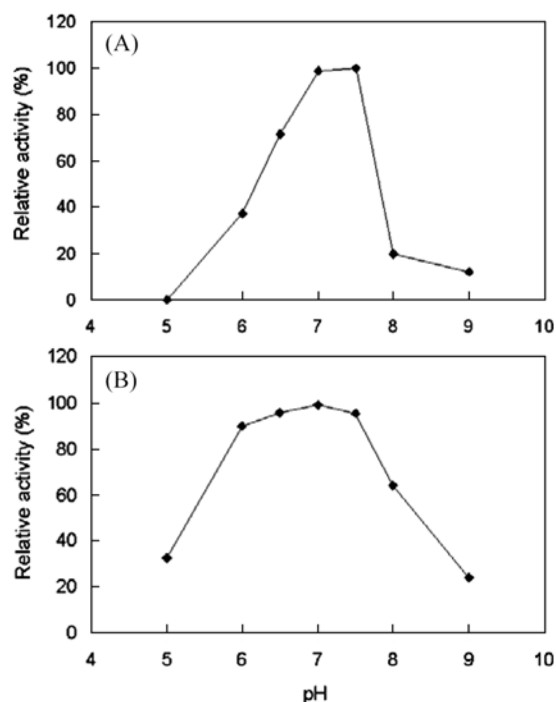

Fig. 3. Effect of pH on activity (A) and stability (B) of β-galactosidase from *K. lactis* NRRL Y-8279. The standard deviation of each experimental point ranged from 1.7 to 3.8.

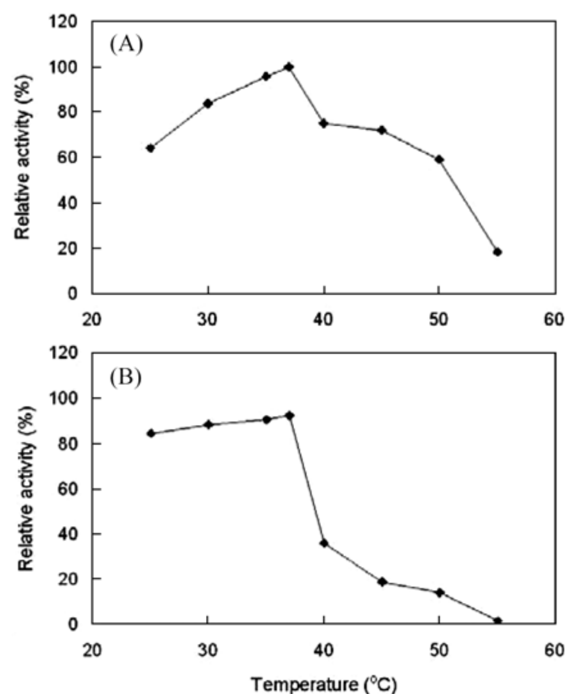

Fig. 4. Effect of temperature on activity (A) and stability (B) of β-galactosidase from *K. lactis* NRRL Y-8279. The standard deviation of each experimental point ranged from 1.2 to 3.5.

The effect of temperature on enzyme activity and stability is shown in Fig. 4. The optimum temperature for the enzyme activity was 37°C. The activity increased gradually with temperatures up to 37°C while it declined sharply at higher temperatures. The enzyme retained 75% of its original activity at 40°C. Thermal stability of the enzyme was investigated between 25-55°C. The enzyme almost maintained full activity after incubation at 37°C for 30 min and retained 84-90% of the initial activity within the range of 25-35°C after 30 min incubation. When the temperature was above 40°C, the enzymatic activity declined sharply due to enzyme inactivation. The enzyme preserved only 14% of the initial activity when exposed to 50°C for 30 min. Compared to other β-galactosidases from different sources, the optimal temperature of this enzyme was almost the same as that from *K. marxianus* CBS 6556 (33) and *K. lactis* (40) and completely different from *K. marxianus* IMB3 (41), *Treponema phagedenis* (42), *Thermotoga maritime* (43), and *Bacillus stearothermophilus* (44) β-galactosidases which have optimal temperatures of 50-55, 50, 80, and 70°C, respectively.

High temperature stability at 25-35°C and neutral pH optima of the β-galactosidase enzyme produced from *K. lactis* NRRL Y-8279 allows the treatment of milk, sweet whey, and neutral pH dairy products at low temperatures using this enzyme.

The kinetic parameters of β-galactosidase enzyme were also studied by determining the rates of hydrolysis of different concentrations of ONPG at pH 7.5 and 37°C. The Lineweaver-Burk curve was plotted and from these fits (results not shown here), Michaelis constant (K_m) and maximum reaction velocity (V_{max}) of β-galactosidase were found as 1.20 mM and 1,000 μmol/min·mg protein, respectively. Samoshina and Samoshin (45) collected literature values of K_m for different microorganisms and found *K. lactis* strains to have K_m values in 1.18-3.62. The relatively low K_m (the higher rate of reaction) obtained with *K. lactis* NRRL Y 8279 makes this enzyme more useful for practical applications, e.g., for hydrolysis of lactose in whey.

This article provides a detailed study that used statistical analysis to determine the optimum levels and interactions among the above mentioned parameters in β-galactosidase enzyme production in the stirred tank bioreactor using *K. lactis*. Further studies will be focused on the scale-up of enzyme production and purification of β-galactosidase enzyme produced.

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