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RESEARCH ARTICLE

Optimization of β-galactosidase production using *Kluyveromyces lactis* NRRL Y-8279 by response surface methodology

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Abbreviations: DNS: dinitrosalicylic acid
ONP: *o*-nitrophenol
ONPG: *o*-nitrophenol-β-D-galactopyranoside
P: probability
RSM: response surface methodology
SDS: sodium dodecyl sulfate

This paper investigates the production and optimization of β-galactosidase enzyme using synthetic medium by *Kluyveromyces lactis* NRRL Y-8279 in shake flask cultures. Among the different cell disintegration methods used, the highest specific activity was obtained when the cells were permeabilized using isoamyl alcohol. Response surface methodology was used to investigate the effects of four fermentation parameters (agitation speed, pH, initial substrate concentration and incubation time) on β-galactosidase enzyme production. Results of the statistical analysis showed that the fit of the model was good in all cases. Maximum specific enzyme activity of 4218.4 U g^{-1} was obtained at the optimum levels of process variables (pH 7.35, agitation speed 179.2 rpm, initial sugar concentration 24.9 g l^{-1} and incubation time 50.9 hrs). The response surface methodology was found to be useful in optimizing and determining the interactions among process variables in β-galactosidase enzyme production.

The enzyme β-galactosidase (lactase, EC 3.2.1.23) catalyzes the hydrolysis of lactose to glucose and galactose. This enzyme is industrially important because it can be used to avoid lactose crystallization in sweetened, condensed and frozen dairy products such as ice cream and condensed milk and solve problems associated with whey utilization and disposal. In addition, β-galactosidase is used to avoid the problems of lactose intolerance by individuals who are deficient in lactase (Artolozaga et al. 1998). New applications for β-galactosidase, such as in the production of biologically active galacto-oligosaccharides, have also been reported in the literature (Boon et al. 2000; Albayrak and Yang, 2002). Commercial β-galactosidases are produced from yeasts such as *Kluyveromyces lactis* and *Kluyveromyces marxianus* (formerly known as *Kluyveromyces fragilis* and *Saccharomyces fragilis*), and moulds such as *Aspergillus niger* and *Aspergillus oryzae* (Shaikh et al. 1997; Santos et al. 1998). 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 (Santos et al. 1998).

The activity and stability of enzymes is influenced by the type of strain, cultivation conditions (temperature, pH, aeration, agitation, incubation time) and the growth medium composition (particularly carbon and nitrogen

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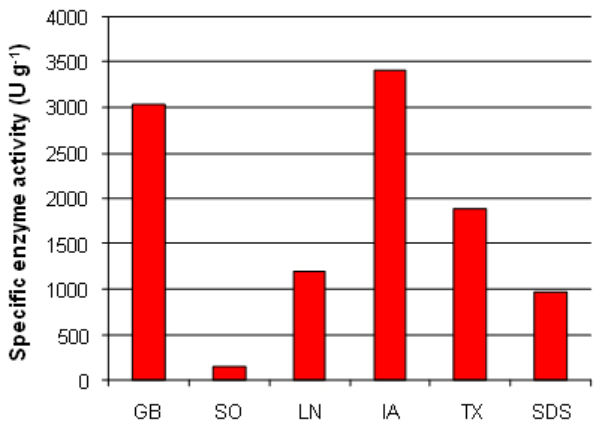

Figure 1. Effect of cell disruption methods on specific β-galactosidase enzyme activity by *K. lactis* NRRL Y-8279. (GB: Glass beads, SO: Sonication, LN: Liquid nitrogen, IA: Isoamyl alcohol, TX: Triton X-100, SDS: Sodium dodecyl sulfate) (pH = 7.0, Initial substrate concentration = 30 g/l, incubation time = 48 hrs, T = 30ºC).

sources) (Schneider et al. 2001; Jurado et al. 2004; Tari et al. 2007). Several papers have been published (Chen et al. 1992; Fiedurek and Szczodrak, 1994; Bojorge et al. 1999; Furlan et al. 2000; Furlan et al. 2001) reporting the optimization of a variety of culture conditions for the production of β-galactosidase by *Kluyveromyces marxianus*.

The optimization studies done by varying one parameter while keeping the others at constant level do not reflect the interaction effects among these variables employed and this kind of optimization studies do not depict the net effect of the various factors on the enzyme activity. In order to overcome this major problem, optimization studies are done using response surface methodology (RSM) which is a mathematical and statistical technique widely used to determine the effects of several variables and to optimize different biotechnological processes (He and Tan, 2006). RSM has been extensively applied to optimize culture medium and other process parameters for the production of lipase (He and Tan, 2006; Liu et al. 2006), tannase (Battestin and Macedo, 2007), α-amylase (Uma Maheswar Rao and Satyanarayana, 2007), β-cyclodextrin glucanotransferase (Ibrahim et al. 2005), dextran dextrinase (Naessens et al. 2004) and chitinase (Nawani and Kapadnis, 2005).

Chen et al. (1992) studied the optimization of β-galactosidase production using a statistical and mathematical approach. These authors used a central composite design to optimize the medium composition for lactase production by *Kluyveromyces marxianus* and found the optimal medium composition as 80.6 g l^{-1} lactose, 107.7 g l^{-1} corn steep liquor, 4.1 g l^{-1} glucose and 9.6 g l^{-1} glycerol. Becerra and González Siso (1996) used a full-factorial design to optimize β-galactosidase production by *Kluyveromyces lactis* in solid-state fermentations on corn grits or wheat bran moistened with deproteinized milk whey. No previous work has used RSM or other statistical techniques to optimize cultivation conditions and to determine the interactions among pH, agitation speed, incubation time and initial sugar concentration in β-galactosidase production.

The present study examined β-galactosidase production from synthetic medium containing lactose by *Kluyveromyces lactis* NRRL Y-8279. The effects of cell disruption methods on β-galactosidase activity were determined. RSM was used to optimize fermentation parameters to obtain maximum enzyme activity. Four factors (pH, agitation speed, initial sugar concentration and incubation time) considered to have significant impact on enzyme production were selected as parameters for optimization studies. This study is the first detailed work on the use of response surface methodology for the optimization of fermentation parameters in β-galactosidase production using *K. lactis* in shake flask cultures.

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 4ºC and transferred monthly to fresh slants incubated at 30ºC for 2 days. Two wire loops of culture were taken from fresh slants and transferred to 250 ml Erlenmayer flasks containing 50 ml of culture broth which had been sterilized at 121ºC for 15 min. Medium composition of agar slants was as follows (in g l^{-1}): 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^{-1}): lactose, 30; yeast extract, 1; K_2HPO_4, 2; $NH_4H_2PO_4$, 1; $(NH_4)_2HPO_4$, 1; $MgSO_4$ x 7 H_2O, 0.1 and pH was adjusted to 7.0 (unless otherwise

Table 1. Levels of factors used in the experimental design.

Factor	Name	Levels				
		-a	-1	0	+1	+a
X_1	pH	5	6	7	8	9
X_2	Agitation speed (rpm)	100	150	200	250	300
X_3	Sugar conc. (g l^{-1})	10	20	30	40	50
X_4	Time (h)	24	36	48	60	72

stated) (Fiedurek and Szczodrak, 1994). All chemicals used in this study were of analytical grade.

Fermentation conditions

Fermentations were carried out batchwise in 250 ml flasks using 50 ml of fermentation medium sterilized at 121ºC for 15 min. The flasks were inoculated with the pre-inoculum volume (2%) to give an initial cell concentration between 0.05-0.1 g l^{-1}. The cultures were incubated at 30ºC for 48 hrs in a rotary shaker incubator (Gerhardt Laboshake LS 2/5 RO 2/5, Bonn, Germany) operated at 200 rpm. The culture medium and growth conditions used for both inoculation and fermentation were the same as described above. The levels of pH, agitation speed, initial sugar concentration and incubation time used in the optimization studies by RSM are given in Table 1.

Cell disintegration methods

β-galactosidase enzyme from *K. lactis* is an intracellular enzyme. Different chemical and mechanical methods were used to permeabilize and/or disrupt *K. lactis* cells prior to the assay of intracellular β-galactosidase. Hence, the effect of these methods on enzyme activity was determined.

Isoamyl alcohol. A volume of resuspended cells in 0.2 M phosphate buffer (pH = 6.5) 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. The mixture was shaken for 15 min at room temperature to make the cell envelopes permeable and used for the enzyme assay (Barberis and Gentina, 1998).

Vortexing cells with glass beads. Cell suspensions were vortexed at 4ºC with glass beads (1 mm diameter) in a 30 ml glass tube. Vortexing continued for 10 min and the resulting mixture was centrifuged at 4000 x g. The supernatant was used for the enzyme assay (Song and Jacques, 1997).

Liquid nitrogen. Biomass was placed in mortar and ground with liquid nitrogen and centrifuged at 4000 x g for 20 min. The supernatant was used for the enzyme assay (Liu et al. 2001).

Triton X-100. Cell suspensions were shaken in presence of 0.04% Triton X-100 in a shaker at 200 rpm for 1 hr at 30ºC and centrifuged at 4000 x g for 20 min. The supernatant was used for the enzyme assay (Fiedurek and Szczodrak, 1994).

Sodium dodecyl sulfate (SDS). Cell suspensions were shaken in presence of 0.04% SDS in a shaker at 200 rpm for 1 hr (30ºC) and centrifuged at 4000 x g for 20 min. The supernatant was used for the enzyme assay (Fiedurek and Szczodrak, 1994).

Sonication. The cell suspensions were sonicated on ice in glass tubes using a Branson Sonic Power Sonicator (48 Bransonic Power, 40 W, 30 sec with 30 sec cooling periods) for 4 min. Then, they were centrifuged at 4000 x g for 20 min and the supernatant was used for measuring β-galactosidase activity (Song and Jacques, 1997).

Analytical techniques

The method described by Food Chemical Codex (1993) was used with some modifications for β-galactosidase activity. The substrate for the enzyme was 2.5 mg ml^{-1} of *o*-nitrophenol-β-D-galactopyranoside (ONPG, Sigma, N-1127), which was prepared in 0.2 M phosphate buffer, pH =

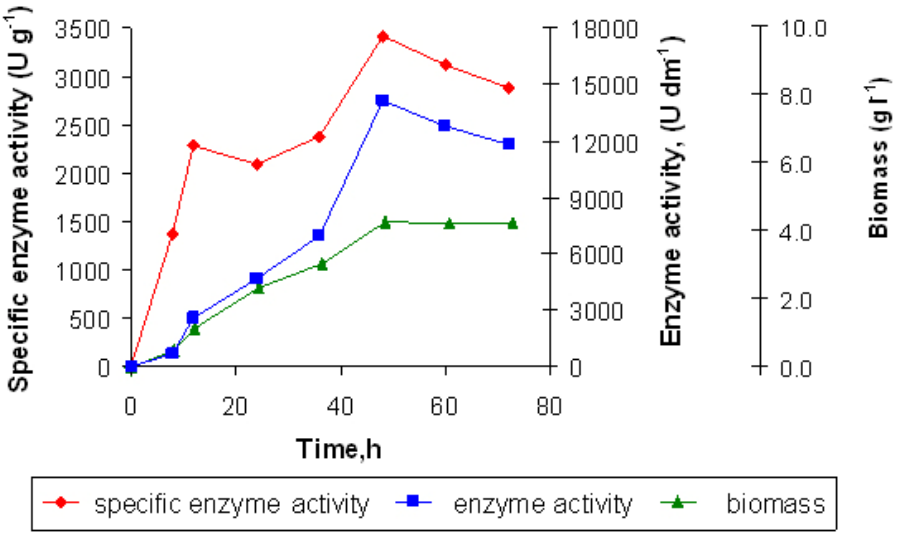

Figure 2. Kinetics of β-galactosidase production by *K. lactis* NRRL Y-8279. (pH = 7.0, Initial substrate concentration = 30 g/l, T = 30ºC).

Table 2. Experimental design.

Run	pH	Agitation speed (rpm)	Sugar conc. (g l^{-1})	Time (h)	Specific Activity (U g^{-1})
1	6	150	20	36	3025
2	7	200	30	48	3757
3	7	200	30	48	3717
4	6	150	40	60	2460
5	6	250	40	60	2042
6	8	150	40	36	2809
7	6	250	20	36	2766
8	7	200	30	48	3752
9	9	200	30	48	2598
10	7	200	30	24	3311
11	8	150	40	60	2895
12	7	200	30	48	3722
13	7	300	30	48	2690
14	7	200	30	48	3757
15	5	200	30	48	1992
16	6	250	20	60	2492
17	8	250	40	60	2542
18	7	200	30	48	3717
19	7	100	30	48	3390
20	7	200	30	48	3737
21	6	250	40	36	2664
22	8	250	20	36	2964
23	6	150	40	36	2755
24	8	150	20	36	3286
25	7	200	50	48	2210
26	8	250	40	36	2556
27	7	200	30	72	3173
28	7	200	10	48	3122
29	8	150	20	60	3679
30	6	150	20	60	3014
31	8	250	20	60	3064

6.5. 1 ml of the crude permeabilized cell suspension with isoamyl alcohol (unless otherwise stated) was incubated with 4 ml of the above substrate mixture (preheated to 37ºC) at 37ºC for 15 min. The reaction was terminated by adding 1 mL of 10% sodium carbonate. The liberated *o*-nitrophenol (ONP) was measured spectrophotometrically at 420 nm. ONP concentration was calculated using an extinction coefficient of 4.2371 ml μmol^{-1} cm^{-1}. One unit of enzyme activity was defined as the enzyme quantity that liberated 1 μmol of *o*-nitrophenol per minute under the assay conditions. Specific activity (U g^{-1}) was defined as the number of lactase units per gram of cell biomass. Specific β-galactosidase activity was used throughout the study to obtain a common basis for comparisons. Volumetric enzyme activity values were only given in determining the kinetics of enzyme production.

The biomass concentration was measured gravimetrically as dry cell mass (g l^{-1}) by centrifuging 20 ml of original culture broth at 4000 x *g* for 20 min followed by drying the cells at 80ºC overnight. Lactose was measured using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). All experiments were carried out in triplicate and the values reported are the mean of three such experiments in which 3-5% variability was observed.

Experimental design and statistical analysis

The statistical analysis of the data was performed using Minitab Statistical Software (Release 13.20). Details of response surface methodology can be found elsewhere (Myers and Montgomery, 1995). 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 central composite routable design for the study of four factors each at five levels (Table 2). The levels were $-a$, -1, 0, +1, $+a$ where a = $2^{n/4}$, n was the number of variables and 0 corresponded to the central point. The actual level of each factor was calculated using the following equation (Myers and Montgomery, 1995).

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

Response surface model was fitted to the response variable, namely specific β-galactosidase activity (U g^{-1}). The second order response function for four quantitative factors is given by Equation [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

Effect of cell disintegration methods on specific enzyme activity

Cell disruption methods can be roughly divided into those which produce cell leakage and disruption in response to the application of shear forces (mechanical methods) and those which produce digestion or permeabilization of the cell envelopes by the action of chemicals (chemical methods) (Numanoglu and Sungur, 2004). In permeabilization, the cell envelope is altered to allow small molecules, such as substrates, products or coenzymes to cross freely. The permeabilizing agent disrupts the membrane structures to allow the passive passage of low molecular weight solutes in and out of cells. Organic solvents as permeabilizing agents act on the cell membranes by decreasing the phospholipids content (Panesar et al. 2007). In this study, among all the mechanical and chemical cell disintegration methods tested, the highest specific enzyme activity was obtained when *K. lactis* cells were treated with isoamyl alcohol prior to the assay of β-galactosidase. After permeabilization of cell walls of *K. lactis* with isoamyl alcohol, ONPG diffused into the cells and was hydrolyzed by the β-galactosidase enzyme entrapped within the cell.

Cell disintegration is an important procedure applied in the production of intracellular β-galactosidase enzyme from *K. lactis*. Different mechanical and chemical disruption methods were applied to the cell mass at the end of fermentation. The results indicated that the specific activity of β-galactosidase enzyme is dependent on the cell disintegration method used. As seen in Figure 1, highest specific enzyme activity (3416.6 U g^{-1}) was obtained when the cells were permeabilized using isoamyl alcohol. Similar specific enzyme activity was obtained by disrupting the cells mechanically using glass beads (3038.9 U g^{-1}). Lower specific enzyme activities were found when Triton X-100 (1888.8 U g^{-1}), liquid nitrogen (1199.4 U g^{-1}) and SDS (964.3 U g^{-1}) were used. The lowest enzyme activity (152.6 U g^{-1}) was obtained with the sonication method.

Several studies have been reported on the permeabilization of microbial cells by organic solvents (Flores et al. 1994; Kondo et al. 2000; Numanoglu and Sungur, 2004; Panesar et al. 2007; Park et al. 2007). Flores et al. (1994) studied the permeabilization of *K. lactis* cells by chloroform, toluene and ethanol in relation to β-galactosidase activity. They found that the performance of those solvents was dependent on the incubation time, the incubation temperature and the concentration of both cells and solvents. Panesar et al. (2007) used ethanol to permeabilize *K. marxianus* cells to overcome the problem of enzyme extraction and poor permeability of cell membrane in the production of lactose-hydrolyzed milk. Park et al. (2007) investigated the use of five different solvents (dimethyl sulfoxide, petroleum ether, acetone, chloroform and hexane) to disrupt the cell wall of freeze-dried *Rhodotorula glutinis* cells to extract

Table 3. ANOVA for specific enzyme activity (R^2 = 0.998).

Source	D F	Seq SS	Adj SS	Adj MS	F	P
Regression	14	8586783	8586783	613342	998.41	<0.001
Linear	4	2550677	2787665	696916	1135.0	<0.001
Square	4	5642044	5642044	1410511	2297.3	<0.001
Interaction	6	394062	394062	65677	106.91	<0.001
Residual error	16	9829	9829	614		
Lack of fit	10	7779	7779	778	2.28	0.163
Pure error	6	2050	2050	342		
Total	30	8596612				

DF: degrees of freedom; Seq SS: sequential sum of squares; Adj SS: adjusted sum of squares; Adj M: adjusted mean square.

intracellular carotenoid pigments. Kondo et al. (2000) permeabilized flocculent yeast *Saccharomyces cerevisiae* with isopropyl alcohol and ethanol under various conditions. Other chemicals used to increase the membrane permeability of yeast cells were toluene-ethanol mixture, cethyltrimethyl-ammonium bromide, ethyl ether, digitonin, Triton X-100 and hexamethylenediamine (Kondo et al. 2000).

Mechanical methods such as sonication, high-pressure homogenizer or bead mills have been traditionally used for the disruption of microbial cells (Geciova et al. 2002). However such techniques suffer from drawbacks, including the production of fine cell debris, high capital investment cost and long processing time (Park et al. 2007). The method used should be robust enough to disrupt cell envelopes efficiently but gentle enough to preserve enzyme activity (Numanoglu and Sungur, 2004). Several papers have been published on the use of mechanical methods for the release of intracellular β-galactosidase enzyme. Numanoglu and Sungur (2004) compared chemical (toluene, ethanol-chloroform) and physical (glass bead mill) methods to permeabilize β-galactosidase producing *K. lactis* cells and found that the physical method was better because of the probable risk of chemical toxicity accompanied with the chemical methods. Fiedurek and Szczodrak (1994) used three types of extraction methods to release the β-galactosidase enzyme from *K. fragilis* cells and found that the highest yield was obtained by mechanical disintegration of cells by mortar and pestle.

In this study, among the mechanical methods tested for cell disruption, the highest specific enzyme activity was obtained by mechanical disruption of *K. lactis* cells with glass beads. In our study, sonication was found to be unsuitable for cell disruption. This might be because of the incomplete disruption of the *K. lactis* cell wall since lactase is a large enzyme and complete disruption of the cell and its cell wall is necessary to liberate the enzyme from the cell debris. Another reason for obtaining low enzyme activity units in sonication might be the significant heat emerging from the absorption of sonication energy into suspensions, probably leading to enzyme inactivation. Therefore, good temperature control is necessary throughout the disruption process.

Parallel to the findings of this study, Bury et al. (2001) studied the disruption of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 cells to compare sonication, high-pressure homogenization and bead milling for the release of β-galactosidase enzyme. They found that bead milling and high-pressure homogenizer were comparable methods while laboratory scale sonication was the least effective method on the release of β-galactosidase enzyme. Contrarily, Berger et al. (1995) studied two physical disruption methods to optimize the recovery of intracellular enzymes from *Thermus* species and found that the sonication method was superior to the glass-bead method in the recovery of the total thermophilic β-galactosidase activity. Agrawal and Pandit (2003) studied cell disruption of *S. cerevisiae* with ultrasonication for the release of intracellular α-glucosidase enzyme and optimized the pH of the disruption medium for both unit activity and specific activity of the enzyme. Iida et al. (2008) investigated the release of intracellular protein from yeast cells by the ultrasonic action and studied the effect of factors such as the concentration of yeast cells, temperature, ultrasound power, types of sonicator and the superposition with the mechanical mixing.

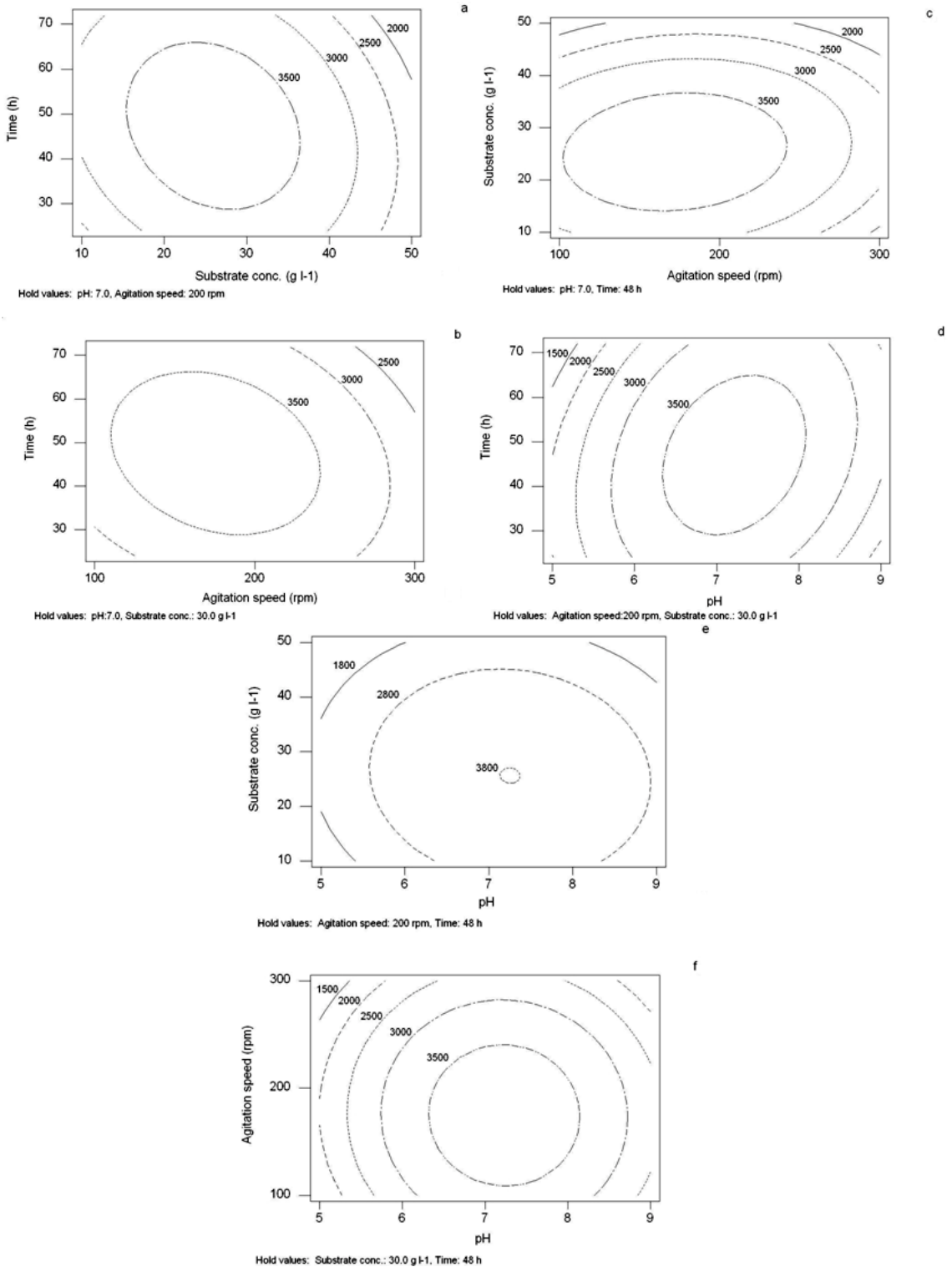

Figure 3. Contour plots for specific β-galactosidase enzyme activity at varying concentrations of:
(a) time and substrate conc.
(b) time and agitation speed.
(c) substrate conc. and agitation speed.
(d) time and pH.
(e) substrate concentration and pH.
(f) agitation speed and pH at a constant middle level of the other two factors.

In our sonication system, optimizing the operational parameters such as time and power might have helped to achieve less enzyme inactivation and higher enzyme activity. The completeness of cell disruption by sonication could have been determined by using a microscope and/or by measuring the release of nucleic acids from the disrupted cells.

Kinetics of β-galactosidase production from *K. lactis* NRRL Y-8279

The kinetics of *K. lactis* growth and production of β-galactosidase enzyme in the shake flask batch culture were examined by growing the cells in the synthetic medium containing 30 g l^{-1} of initial lactose. As seen in Figure 2, specific enzyme activity curve exhibited two distinct sharp points at the beginning and at the end of the growth phase. The specific activity remained low at the beginning of the cultivation and then increased giving a peak at the early exponential phase. During the exponential growth phase, specific activity increased reaching a maximum at the early stationary phase and decreased continuously throughout the stationary growth phase. The maximum specific activity (3416.6 U g^{-1}) was attained at the end of 48 hrs. The reason for obtaining two 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 β-galactosidase in an effort to utilize lactose in the medium leading to a second peak of enzyme activity.

It was observed from the volumetric enzyme activity curve (Figure 2) that the enzyme production was associated with the biomass production and the maximum volumetric activity (14106 U l^{-1}) was obtained at the 48^{th} hrs of fermentation. However, volumetric activity decreased drastically at 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. The decrease of β-galactosidase activity after 48 hrs indicates that the operation should be stopped after the stationary phase has been reached. Hence, the fermentation time seems to be an important parameter that should be considered in optimization studies using RSM to achieve maximum enzyme activity.

Maximum specific (189.83 U g^{-1} h) and volumetric (293.88 U l^{-1} h^{-1}) productivity values were obtained at the end of 8 and 48 hrs, 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 1.1 g l^{-1}. In all culture systems, the pH decreased slightly during fermentation and decreased from an initial value of 7.0 to 5.0-5.3 at the end of 48 hrs.

Similar activity profile curves were reported in the literature (Rech et al. 1999; Martins et al. 2002; Rajoka et al. 2003). Rech et al. (1999) studied β-galactosidase production from sweet cheese whey by using two different strains of *K. marxianus* and found that the specific activity curves exhibited two 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. (2002) studied the production of β-galactosidase enzyme from *K. marxianus* and observed that the specific enzyme activity increased and attained a maximum after 4 hrs of incubation. They found a second phase of high specific activity after 16 hrs of incubation where the culture entered the stationary phase of growth. Rajoka et al.(2003) studied the production of β-galactosidase enzyme from *K. marxianus* in shake flask cultures and reported that the bulk β-galactosidase production reached a maximum activity after 30-40 hrs and the enzyme production was apparently growth associated.

Optimization of β-galactosidase enzyme production

Many authors stated the importance of factors such as pH, aeration, substrate concentration and fermentation time in β-galactosidase enzyme production (Chen et al. 1992; Fiedurek and Szczodrak, 1994; Shaikh et al. 1997; Rech et al. 1999; Furlan et al. 2000; Furlan et al. 2001; Fekete et al. 2002; Martins et al. 2002; Rajoka et al. 2003; Hsu et al. 2005; Konsoula and Liakopoulou-Kyriakides, 2007). Our preliminary experiments also showed that the agitation speed, pH, initial substrate concentration and incubation time influenced the production of β-galactosidase enzyme by *K. lactis* NRRL Y-8279. Thus, a central composite design was used to determine the optimum levels of these parameters leading to a maximum β-galactosidase enzyme synthesis. The levels of these factors (Table 1) used in the optimization studies by RSM were determined by preliminary experiments. The effect of the four previously mentioned variables, each at five levels, and their interactions on β-galactosidase enzyme synthesis have been determined by carrying out thirty one experiments given by the model (Table 2).

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 0.998 indicating that the model as fitted explained 99.8% of the variability in specific enzyme 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 (0.163) was not significant at the 5% level ($P > 0.05$) indicating that the experimental data obtained fitted well with the model.

Table 4. Estimated regression coefficients for specific enzyme activity.

Term	Coefficient	SE Coefficient	T	P
Constant	-20297.9	415,275	-48,878	<0.001
pH	4944.30	76,226	64,864	<0.001
Agitation	28.3190	1,303	21,741	<0.001
Substrate	180.239	6,252	28,828	<0.001
Time	57.7840	5,427	10,647	<0.001
pH x pH	-359.041	4,635	-77,464	<0.001
Agitation x Agitation	-0.0691163	0,002	-37,280	<0.001
Substrate x Substrate	-2.66287	0,046	-57,452	<0.001
Time x Time	-0.849373	0,032	-26,389	<0.001
pH x Agitation	-0.191813	0,124	-1,548	0.141
pH x Substrate	-4.46805	0,620	-7,211	<0.001
pH x Time	8.68165	0,516	16,813	<0.001
Agitation x Substrate	0.0878612	0,012	7,090	<0.001
Agitation x Time	-0.112674	0,010	-10,910	<0.001
Substrate x Time	-0.600165	0,052	-11,623	<0.001

These results show that the model chosen can satisfactorily explain the effects of pH, agitation speed, initial substrate concentration and incubation time on β-galactosidase production by *K. lactis* NRRL Y-8279in shake flask cultures.

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*.

$$Y = - 20297.9 + 4944.3\ X_1 + 28.319\ X_2 + 180.239\ X_3 + 57.784\ X_4 - 359.041\ X_1^2 - 0.06912\ X_2^2 - 2.66287\ X_3^2 - 0.84937\ X_4^2 - 4.46805\ X_1X_3 + 8.68165\ X_1X_4 + 0.087861\ X_2X_3 - 0.11267\ X_2X_4 - 0.60017\ X_3X_4 \quad [2]$$

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 pH, agitation speed, initial substrate 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 four factors tested, pH had the highest impact on specific enzyme activity as given by the highest linear coefficient (4944.3), followed by initial substrate concentration (180.239), incubation time (57.7840) and agitation speed (28.3190). 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 pH-initial substrate concentration, pH-incubation time, agitation-initial substrate concentration, agitation-incubation time, initial substrate concentration-incubation time were significant as shown by low P values ($P < 0.05$) for interactive terms. But the interaction between pH-agitation speed was found to be insignificant as given by P value above 0.05. Hence this term was excluded from the polynomial Equation [2] used for this model.

Figure 3 shows the contour plots of specific enzyme activity for each pair of factors by keeping the other two 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 two factors. Further increase in these factors above the middle level (pH: 7.3, agitation speed: 180 rpm, initial substrate concentration: 25 g l^{-1}, incubation time: 50 hrs) showed decrease in specific enzyme activity. In order to determine the maximum specific enzyme activity corresponding to the optimum levels of pH, agitation speed, initial substrate 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 Equation [2] allowed the determination of the levels of pH (X_1 = 7.35), agitation speed (X_2 = 179.2 rpm), initial sugar concentration (X_3 = 24.9 g l^{-1}) and incubation time (X_4 = 50.9 hrs) giving a maximum specific enzyme activity of 4113.1 U g^{-1}. The above data optimizes β-galactosidase enzyme production from synthetic medium containing lactose by *K. lactis* NRRL Y-8279 in shake flask culture.

The final fermentation was performed using synthetic medium containing 5 g l^{-1} yeast extract with the optimized levels of pH (7.35), agitation speed (179.2 rpm), initial sugar concentration (24.9 g l^{-1}) and incubation time (50.9 hrs) given by the model. Maximum specific enzyme activity (4218.4 U g^{-1}) which was slightly higher than the value given by the model was obtained on the 50.9th hour of fermentation. The maximum enzyme productivity obtained was 339.8 U l^{-1} h^{-1}.

CONCLUDING REMARKS

In this study, β-galactosidase enzyme was produced by *K. lactis* NRRL Y-8279 using a synthetic medium containing lactose. Different chemical and mechanical methods were used for the disintegration of *K. lactis* cells and the highest specific enzyme activity (3416.6 U g^{-1}) was obtained when the cells were permeabilized using isoamyl alcohol. The maximum specific enzyme activity of 3737.2 U g^{-1} was obtained at the end of 48 hrs.

RSM was used to determine the effects of four important factors (agitation speed, pH, initial substrate concentration and incubation time) on β-galactosidase enzyme production. Linear, quadratic and interaction effects of these variables on specific enzyme activity were determined. The model generated in this study by RSM satisfied all the necessary arguments for its use in the optimization. By fitting the experimental data to a second order polynomial equation, the optimum levels of pH (7.35), agitation speed (179.2 rpm), initial sugar concentration (24.9 g l^{-1}) and incubation time (50.9 hrs) were determined. Using the optimum levels of fermentation parameters, a maximum specific enzyme activity of 4218.4 U g^{-1} was obtained. 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 from *K. lactis*. The parameters studied in this article also influence the enzyme synthesis in a bioreactor system and further studies will be focused on the scale-up of enzyme production and the optimization of fermentation parameters in a stirred tank bioreactor.

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