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Optimization of β-galactosidase production by response surface methodology using locally isolated *Kluyveromyces marxianus*

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Abstract

Five parameters including initial sugar concentration, agitation speed, initial pH, incubation time and temperature were studied for the optimization of β-galactosidase production in synthetic medium containing lactose as carbon source by *Kluyveromyces marxianus* DIYS11 and using Response Surface Methodology (RSM) as statistical analysis. The optimum conditions for the highest enzyme activity were sugar concentration = 10%, agitation speed = 250 rpm, pH =3, incubation time = 64 hours and temperature=20˚C which generated 4997 U/ml/min and made the yeast *Kluyveromyces marxianus* DIYS11 a promise organism for industrial β-galactosidase production, and the RSM a good tool for the optimization of enzyme production.

Introduction

β-galactosidase (EC,3,2,1,23) known as lactase is the enzyme responsible for catalyses the hydrolysis of the disaccharide lactose to its two mono carbohydrates glucose and galactose by breaking down B-1,4 de-galactose in lactose (Quyen *et al.*, 2011). This enzyme is very important in research, bioremediation, diagnosis, and food industries such as, bakeries-baking and soft drinks (Panesar *et al*., 2010).

Problems with lactose fall within three main areas, health (lactose intolerant people), food technology (lactose crystallization in ice-cream and condensed milk, low sweetness and solubility) and environment (the high biochemical and chemical oxygen demand (BOD/COD) of whey, the main source of lactose. Because of its high transgalactosylation activity, β-galactosidase is used in the synthesis of prebiotic galactooligosaccharides (Iqbal *et al*., 2010). Moreover, the β-galactosidase activity contributes to the glycoprotein degradation (Terra *et al.*, 2010).

Production of β-gal by GRAS (Generally Recognized As Safe) microorganisms is very important (Saad, 2004). 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 in 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 are influenced by the type of strain, cultivation conditions (temperature, pH, aeration, agitation and incubation time) and the growth medium composition (particularly carbon and nitrogen sources) (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*.

Kluyveromyces marxianus offers great advantages such as, good growth yield, which has an important economic impact in food industry; acceptability as a safe microorganism, an important technical aspect when considering that the fermented products have food or pharmaceutical applications; and a higher β-galactosidase activity than other yeasts (Manera *et al*., 2008). The optimization studies conducted by varying one parameter while keeping the others at constant level do not reflect the interaction effects among the employed variables, and this kind of optimization studies do not depict the net effect of the various factors on the enzyme activity (Gumgumjee and Danial, 2011; Gupte and Nair, 2010).

Optimization through factorial design and

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response surface methodology has been used in biotechnical processes, and several research works for the production of enzymes have applied this technique for the optimization of culture conditions, namely for the production of β-galactosidase from *Streptococcus thermophiles*, *lactobacillus* sp. (Tari *et al*., 2008), and *Pichia pastoris* (Li *et al*., 2013), in addition to screen the nutritional factors affecting β-galactosidase production by *Lactobacillus fermentum* CM33 (Sriphannam *et al.*, 2012). Many authors studied the role of factors such as, pH, agitation speed, substrate concentration, temperature and fermentation time in β-galactosidase enzyme production (Furlan *et al*., 2001; Hsu *et al.*, 2005). Manera *et al*. (2008) tested the effect of different concentrations of nutrients such as, yeast extract and $(NH_4)_2SO_4$ on the production of β-galactosidase from *Kluyveromyces marxianus* CCT 7082. Berini *et al.* (2013) studied β-galactosidase production from whey considering lactose concentration, temperature and different corn steep concentration using central composite rotatable design (CCRD). Dagbagli and Goksungur (2008) conducted the optimization of β-galactosidase production by studying four different factors (pH, agitation, substrate concentration and incubation time) by *Kluyveromyces lactis* NRRL Y-8279 using response surface methodology. All the results presented that the RSM statistical analysis was proved to be a useful and powerful tool in developing optimum fermentation conditions. The aim of the present work was to optimize five factors affecting the production of β -galactosidase by *Kluyveromyces marxianus* in submerged fermentation in agitated flasks using response surface methodology (RSM).

Materials and Methods

Microorganism

Kluyveromyces marxianus isolated from Syrian dairy products (labneh), identified by PCR-sequencer to the ITS1-5.8S- ITS2 fragment, and was kept in 4˚C cultured on YPD (Yeast Peptone Dextrose Agar) petri dishes after 24 hours of incubation at 30˚C monthly refreshed.

Inoculum preparation

One loop from YPD was transferred to 50 ml of YPL (yeast peptone lactose broth) medium containing 2% lactose, 2% peptone, 1% yeast extract and 0.01% chloramphenicol (Lins and Leão, 2002), and incubated at 30˚C, 200 rpm, for 24 h. OD_{600} was determined prior to inoculation.

Fermentation

Fermentations were carried out in 250 ml flasks using 50 ml of fermentation medium (1% peptone, 0.5% yeast extract and 0.01% chloramphenicol), sterilized at 121ºC for 15 min. Lactose was added in different concentration according to the RSM experimental design of five parameters. The flasks were inoculated with the pre-inoculum to give an initial cell count 1.5×10^7. The cultures were incubated in a rotary shaker incubator. The levels of initial sugar concentration, incubation time, agitation speed, initial pH and temperature used in the optimization studies by RSM are given in Table 1.

Measurement of beta- galactosidase activity

According to Moeini *et al*. (2004) with some modifications, the OD_{600} nm was recorded and then 1ml of yeast culture was spun out. The yeast cells were washed twice with cold Z buffer (0.06 M Na_2HPo_4, 0.04 M NaH_2Po_4, 0.01 M KCl and 0.001 M $MgSo_4$). For permeabilization of cells, 50 µl 0.1% SDS and 100 µl chloroform were added to 100 µl of washed yeast and incubated at 30C° for 10 min. 200 µl ONPG (ortho nitrophenol-β-D-galactopyranoside) solution (4 mg of ONPG in 1ml Z buffer) was added in tubes and the time was recorded. The reaction was allowed to run until the solution turned yellow. The reaction was stopped with the addition of 400 µl of 1M Na_2CO_3 and the time was recorded. The cells were spun out, OD_{420} nm of supernatant was read and Miller units were calculated (Units= $1000\times OD_{420}$/ Volume (ml) × t (min.) $\times OD_{600}$).

Experimental design

The statistical analysis of the data was performed using Minitab Statistical Software (13.2). The levels of factors used in the experimental design are listed in Table 1. The data of the factors were selected to cover a wide range of values which have not been studied before. Response surface model was fitted to the response variable, namely specific β-galactosidase activity (U/ml/min). The second order response function for the five quantitative factors is given by Equation [1]:

$$Y = \beta0 + \beta1X1 + \beta2X2 + \beta3X3 + \beta4X4 + \beta5X5 + \beta11X1^2 + \beta22X2^2 + \beta33X3^2 + \beta44X4^2 + \beta55X5^2 + \beta15X1X5 + \beta23X2X3 + \beta25X2X5 + \beta34X3X4 + \beta45X4X5$$

where Y is the predicted response and X1, X2, X3, X4 and X5 represent the levels of the factors according to Table 1 and β0, β1,....., β35 represent coefficient estimates with β0 having the role of a scaling constant.

Table 1. Levels of factors used in the experimental design

Symbol	Parameter	Level				
		-2	-1	0	+1	+2
X1	Lactose con. %	2	4	6	8	10
X2	Incubation time / h.	16	28	40	52	64
X3	Agitation/rpm	50	100	150	200	250
X4	pH	3	4	5	6	7
X5	Temperature/C°	20	25	30	35	40

Table 2. Experimental design

Assay run	Temp. C	Time /h	pH	Agitation rpm	Sugar Con.%	Enzyme activity U/ml/min.
1	30	40	5	150	6	4756
2	25	28	6	200	8	3386
3	25	52	4	100	4	1657
4	25	28	4	200	4	2957
5	25	52	6	200	4	2827
6	30	40	5	150	10	3266
7	35	52	4	100	8	74
8	25	52	4	200	8	4892
9	20	40	5	150	6	3491
10	30	40	5	150	6	4675
11	30	40	7	150	6	4124
12	35	52	6	100	4	2997
13	35	52	4	200	4	3113
14	30	40	5	250	6	4505
15	30	40	3	150	6	2990
16	30	40	5	50	6	1883
17	35	28	4	100	4	1490
18	30	40	5	150	6	5075
19	30	64	5	150	6	4438
20	30	40	5	150	2	2821
21	40	40	5	150	6	1282
22	30	40	5	150	6	4710
23	35	28	6	200	4	3090
24	35	28	4	200	8	2280
25	35	28	6	100	8	3346
26	30	16	5	150	6	3663
27	35	52	6	200	8	695
28	25	28	4	100	8	1420
29	30	40	5	150	6	4521
30	25	28	6	100	4	2084
31	30	40	5	150	6	4837
32	25	52	6	100	8	3400
33	20	64	3	250	10	4997

Results and Discussions

In our study, the level of five factors (pH, agitation, substrate concentration, incubation time and temperature) were applied in the optimization of β-galactosidase production by locally isolated Kluyveromyces marxianus using RSM were determined in a wide range of values (Table 1). The effect of the five previously mentioned variables, each at five levels, and their interactions on β-galactosidase enzyme synthesis were determined by carrying out thirty two experiments given by the model (Table 2).

A central composite design was used to determine the optimum levels of these parameters leading to a maximum β-galactosidase enzyme synthesis, and the value of alpha equal to 2 (where cube points =16, center points in cube =6, axial points =10, center points in axial =0). In order to determine the maximum enzyme activity corresponding to the optimum levels of pH, agitation, initial sugar

Table 3. Analysis of variance for enzyme activity

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Regression	20	52827875	52827875	2641394	16.82	<0.000
Linear	5	11809401	24699903	4939981	31.45	<0.000
Square	5	25239445	25239445	5047889	32.14	<0.000
Interaction	10	15779029	15779029	1577903	10.05	<0.000
Residual Error	11	1727569	1727569	157052		
Lack-of-fit	6	1555586	1555586	259264		
Pure Error	5	171983	171983	34397		
Total	31	54555444				

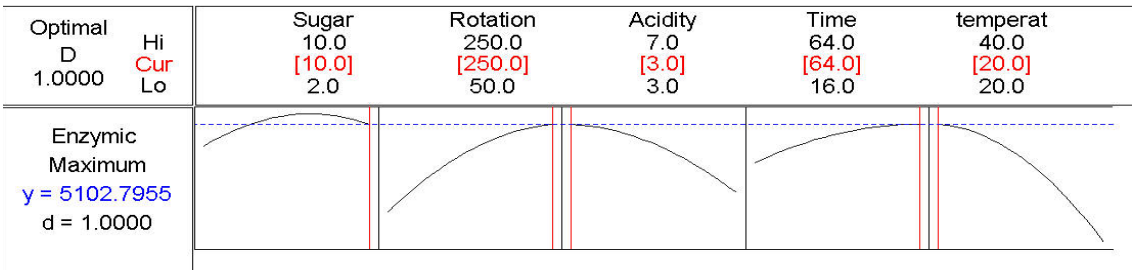

Figure 1. Response optimization plots for the optimal values of the tested variables

concentration, incubation time and temperature, a second order polynomial model was used to calculate the values of these variables (Equation [2]):

$$Y= -72206.7+ 3232.9\ x_1+ 165.1\ x_2 + 5702.4\ x_3+ 466.7\ x_4 + 2075.1\ x_5 -125\ x_1^2 -0.2\ x_2^2 -371.4\ x_3^2 -1.7\ x_4^2 -26.6\ x_5^2 -49.2\ x_1 x_5 -13\ x_2 x_3 -1.1\ x_2 x_5 -18.6\ x_3 x_4 – 6.5\ x_4 x_5$$

Analysis of variance (ANOVA) for the enzyme activity is presented As shown in Table 3, the determination coefficient (R^2) was 91.1% which suggested that design was an efficient tool to determine the effects of medium constituents on β-galactosidase by *K.marxianus* DIYS11, indicating that the model as fitted explained 91.1% 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.

Estimated regression coefficients for enzymatic activity (Table 4) of the experimental data showed that pH, agitation speed, initial sugar concentration, incubation time and temperature demonstrated significant positive linear effects on enzyme synthesis *(P< 0.05)*. Among the five factors tested, these findings are agreed with the result of Dagbagli and Goksungur (2008) . On the other hand, the tested factors showed significant negative quadratic effects on enzyme production indicating that the specific enzyme activity increased as the level of these factors increased and decreased as the level of these parameters increased above certain values. Interactions between these parameters were also significant. The interactions between initial sugar concentration-temperature, agitation-pH, agitation-temperature, pH-time, incubation time-temperature were significant *(P < 0.05)* as shown in Table 4. However, the other interactions were found to be insignificant *(P > 0.05)*, and hence the insignificant terms were excluded from the polynomial Equation [2] used for this model.

pH, incubation temperature and time, sugar concentration and agitation of the fermentation medium are important factors and have insightful influence on metabolic activities of microorganisms. Manera *et al*. (2008) reported that elevated pH degrees improved enzyme activity, and found the best β-galactosidase production when pH= 5 and lactose concentration =28.2 g/l. Gupte and Nair (2010) found that the optimal condition for Kluyveromyces marxianus NCIM3551were pH=5, incubation temperature =25°C, 20 hours of incubation time. Fulan *et al*. (2001) indicated an optimum temperature

Table 4. Estimated regression coefficients for enzymatic activity

Term	Coefficient	SE coefficient	T	P
Constant	**-72206.7**	**6566.93**	**-10.996**	**<0.000**
Sugar	3232.9	498.86	6.481	<0.000
Agitation	165.1	19.95	8.273	<0.000
pH	5702.4	1086.77	5.247	<0.000
Time	466.7	84.16	5.545	<0.000
Temperature	2075.1	228.79	9.070	<0.000
Sugar*Sugar	-125.0	18.29	-6.831	<0.000
Agitation*Agitation	-0.2	0.03	-6.316	<0.000
pH*pH	-371.4	73.17	-5.076	<0.000
Time*Time	-1.7	0.51	-3.390	<0.006
temperature*temperature	-26.6	2.93	-9.075	<0.000
Sugar*Agitation	-0.5	0.99	-0.471	0.647
Sugar*pH	11.9	49.54	0.240	0.815
Sugar*Time	-6.1	4.13	-1.479	0.167
Sugar*temperature	-49.2	9.91	-4.963	<0.000
Agitation*pH	-13.0	1.98	-6.580	<0.000
Agitation*Time	0.0	0.17	0.016	0.987
Agitation*temperature	-1.1	0.40A	-2.668	<0.022
pH*Time	-18.6	8.26	-2.256	<0.045
pH*temperature	30.0	19.81	1.514	0.158
Time*temperature	-6.5	1.65	-3.947	<0.002

$Y = -72206.7 + 3232.9\ x_1 + 165.1\ x_2 + 5702.4\ x_3 + 466.7\ x_4 + 2075.1\ x_5 - 125\ x_1^2 - 0.2\ x_2^2 - 371.4\ x_3^2 - 1.7\ x_4^2 - 26.6\ x_5^2 - 49.2\ x_1 x_5 - 13\ x_2 x_3 - 1.1\ x_2 x_5 - 18.6\ x_3 x_4 - 6.5\ x_4 x_5$

S = 396.3 R-Sq = 96.8% R-Sq(adj) = 91.1%

Global Solution

Sugar % = 10.000

Agitation rpm = 250.000

Acidity = 3.000

Time h = 64.000

Temperature C° = 20.000

Predicted Responses

Enzymatic activity = 5103.00; desirability = 1.00000

of 35°C for the production of β-galactosidase by *Kluyveromyces marxianus*. Dagbagli and Goksungur (2008) proved that 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 by *Kluyveromyces lactis* NRRL Y-8279 using response surface methodology. In our study in order to determine the maximum specific enzyme activity the response optimization choice from Minitab program showed the optimum values of the tested variables (Figure 1). The fitting of the experimental data to Equation [2] allowed to determine the level of pH (X4 = 3), indicated that high pH degrees were unfavorable for the tested organism, and the concluded low pH value could be due to the origin of the yeast which isolated from very acidic material (Labneh) and was adapted to the environment. The optimal agitation speed was (X3 = 250 rpm), and this high value allowed a good contact between substrate (lactose) and the yeast cells. The optimal initial sugar concentration was (X1 = 10%), incubation time (X2 = 64 hrs) and temperature (X5=20°C). Differences between published results are due to the different medium components used, different strains of yeast employed and also to differing cultivation condition. A final fermentation experiment was performed at the optimal values to optimize β-galactosidase enzyme production from synthetic medium containing yeast extract 5g/ l^{-1}, peptone 10 g/l^{-1}, chloramphenicol 0.01% by a new

yeast isolated from Labneh identified as *K. marxianus* in shake flask culture, and the maximum specific enzyme activity was (4997 U/ml), which was very closed to the value given by the model (5103 U/ml).

Conclusion

RSM was used to determine the effects of five important factors (sugar concentration, agitation speed, pH, incubation time and temperature) on β-galactosidase enzyme production from locally *Kluyveromyces marxianus* in patch shake flasks. 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 initial sugar concentration (10%) agitation speed (250 rpm), pH (3), incubation time (64 hrs) and temperature (20C˚) were determined. Using the optimum levels of fermentation parameters, a maximum specific enzyme activity of 4997U /ml was obtained.

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