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Optimization of the Production of Extracellular α-Amylase by *Kluyveromyces marxianus* IF0 0288 by Response Surface Methodology

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ABSTRACT

The aim of this work was to study the production of extracellular α-amylase by Kluyveromyces marxianus *IF0 0288 using optimized nutritional and cultural conditions in a complex yeast medium under aerobic batch fermentation. By applying the conventional "one-variable-at-a-time" approach and the response surface methodology, the effect of four fermentation parameters (type of carbon source, initial culture pH, temperature, and incubation time) on the growth and α-amylase production was evaluated. The production of α-amylase during 60 h of fermentation increased 13-fold under optimized conditions (1% starch, pH 6.0, 30°C) in comparison to the conventional optimization method. The initial pH value of 6.13 and temperature of 30.3°C were optimal conditions by the response surface methodology, leading to further improvement (up to 13-fold) in the production of extracellular α-amylase. These results constituted first evidence that* K. marxianus *could be potentially used as an effective source of extracellular α-amylase.*

Key words: Extracellular enzymes, α-amylase, *Kluyveromyces marxianus*, fermentation, RSM

INTRODUCTION

The enzyme α-amylase (α-1,4-glucanohydrolase, EC 3.2.1.1, endoamylase and dextrogenic) is of widespread occurrence in nature. It hydrolyzes α-1,4-glucosidic linkages in amylose, amylopectin and glycogen in an endo-acting mechanism (Hiromi 1988). Amylases are obtained from various sources such as plant, animal, bacterial and fungal and have many applications in food, textile, paper and pulp, pharmaceuticals, baking and beverages, detergent and leather industries (Pandey et al. 2000; Spier 2005; Kar et al. 2010). The varied applications of these enzymes support a continuing search for new amylase producers, which could expand the assortment of specificities and feasible operating conditions. Some commonly used sources of amylolytic enzymes are *Aspergillus oryzae*, *A. niger*, and *Rhizopus oryzae* (Bigelis 1992; Spier et al. 2006).

The production of amylases is greatly influenced by the nutritional and physicochemical factors such as pH, incubation temperature and carbon and nitrogen sources, which differ significantly with the biochemical nature of the microbial strain (De Mot et al. 1984; Kar et al. 2010; Santos and Martins 2003; Aiyer 2004). Hence, optimization of the components of the fermentation medium and physiological growth conditions is essential in optimizing the product synthesis pattern. The

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classical optimization method (single variable optimization) is not only time-consuming and tedious but also does not depict the complete effects of the parameters in the process and ignores the combined interactions between the physicochemical parameters. In contrast, application of the statistical experimental design technique in a fermentation process development can improve the product yield, reduce process variability and time, and can achieve more cost effective process (Elibol 2004). The present study deals with the optimized extracellular amylase production from a complex medium by *K. marxianus* (Hansen) van der Walt IFO 0288.

MATERIALS AND METHODS

Strain and reagents

The strain *K. marxianus* (Hansen) van der Walt IFO 0288, its working stocks as well as the starting cultures, were handled and prepared as previously described (Stergiou et al. 2012). All the chemicals used in this work were of analytical grade and were purchased from Sigma (U.K.), while the culture media were purchased from Lab M (U.K.).

Cell growth and α-amylase production

All fermentations were performed in a stirred tank bioreactor using a standard medium supplemented with a fixed concentration of carbon source as described previously (Stergiou et al. 2012). The experimental scales of the operating variables (pH and temperature) are depicted in Tables 1 and 2. Cell growth and α-amylase activity assays were carried out at regular intervals in aliquots of 5.0 mL which were withdrawn from the culture medium (Stergiou et al. 2012).

α-amylase activity assays

α-amylase activity was determined spectrophotometrically by monitoring the color variation of the starch-iodine complex (Ramesh and Lonsane 1989). Accordingly, 945 μL of 0.1M phosphate buffer of the appropriate pH value were mixed with 25 μL of sample (cell-free crude supernatant) and 15 μL of 0.6 % starch solution was added. The mixer was incubated in a water bath at 30^{o}C for 30 min and the reaction was stopped by the addition of 250 μL cold HCl solution. Then, 5.0 μL of iodine solution containing 3.75 ‰ I_2 and 37.5 ‰ KI was added and the color of the starch-iodine complex developed was measured at 650 nm. The blank solution was prepared similarly by replacing the quantity of sample by buffer solution. One unit (IU) of α-amylase activity was defined as the amount of enzyme required to hydrolyze 1.0 mg of starch at 30 min under the assay conditions.

Statistical modeling and process optimization

The "one-variable-at-a-time" approach was used in order to optimize the various nutrient and physicochemical parameters for α-amylase production. Each subsequent factor was examined after taking into account the previously optimized factor(s). The effect of the employed carbon sources such as starch, glucose, maltose, and maltotriose (10g/L), was studied by supplementing them in the basal medium consisting of 0.4% yeast extract and 0.3% peptone. Studies were also carried out on the influence of initial pH (5.0-7.0), temperature (20-40^{o}C) and time length (0-96 h) of the fermentation process on the yeast growth and α-amylase production.

Subsequently, the response surface methodology (RSM) was applied using a full 3^2 factorial design to study the interactive effects of two variables, i.e., pH (A) and incubation temperature (B). All the experiments were carried out in triplicates and the results presented as the average of three independent trials. Each experimental design consisted of nine runs and the independent variables were kept at three different levels. Average values (amylase activity and/or cell growth) were taken into account as the dependent variables or responses (y). The minimum and maximum ranges of variables were investigated, and all full experimental designs with respect to their values in actual and coded form, as well as the observed and predicted values of α-amylase production were obtained and listed in Tables 1 and 2.

Table 1 - Actual and coded values of the variables (pH-value and temperature) used in the full 3^2 factorial design.

Variables		Actual	Coded	Actual	Coded	Actual	Coded
α-amylase production	pH-Value	5.00	-1	6.00	0	7.00	1
	Temp. (°C)	20	-1	30	0	40	1
Cell growth	pH-Value	5.00	-1	6.00	0	7.00	1
	Temp. (°C)	20	-1	30	0	40	1

Table 2 - Observed and predicted values of the full 3^2 factorial designs of α-amylase production and cell growth.

			Response	Activity	Response	Cell growth
Standard Order			Actual Value	Predicted Value	Actual Value	Predicted Value
1	5.0	20	0.014	0.013	1.84	1.815
2	6.0	20	0.05	0.049	3.1	3.055
3	7.0	20	0.021	0.022	1.3	1.245
4	5.0	30	0.026	0.025	3.56	3.53
5	6.0	30	0.128	0.127	3.83	3.88
6	7.0	30	0.064	0.0625	2.93	2.91
7	5.0	40	0.009	0.008	2.72	2.75
8	6.0	40	0.054	0.0555	3.52	3.55
9	7.0	40	0.028	0.0285	1.31	1.38
10	5.0	20	0.012	0.013	1.79	1.815
11	6.0	20	0.048	0.049	3.01	3.055
12	7.0	20	0.023	0.022	1.19	1.245
13	5.0	30	0.024	0.025	3.5	3.53
14	6.0	30	0.126	0.127	3.93	3.88
15	7.0	30	0.061	0.0625	2.89	2.91
16	5.0	40	0.007	0.008	2.78	2.75
17	6.0	40	0.057	0.0555	3.58	3.55
18	7.0	40	0.029	0.0285	1.45	1.38

Likewise, the corresponding mathematical expressions related to the RSM approach were formulated, and empirical quartic polynomial equations of the general form of equation (1) were provided as previously described (Khuri and Cornell 1996; Singh et al. 2011; Stergiou et al. 2012).

Then, the obtained models were validated using suitable statistical methodologies, i.e., analysis of variance (ANOVA), Fisher's test value, and R^2 value. In all the cases, the developed mathematical models were drawn as both response surface curves and contour plots using the statistical software-package "Design Expert" (2012).

$$y = a_0 + \sum_{i=1}^{2}\left\{a_i x_i + \sum_{j=1}^{2}\left[a_{ij} x_i x_j + \sum_{k=1}^{2}\left(a_{ijk} x_i x_j x_k + \sum_{l=1}^{2} a_{ijkl} x_i x_j x_k x_l\right)\right]\right\} \quad \text{Equation 1}$$

RESULTS AND DISCUSSION

It should be mentioned that although *K. marxianus* IF0 0288 has firstly used in this work as an effective α-amylase producer, however, other yeasts as well as bacteria have been employed as good sources of amylases by either batch fermentation, or solid-state fermentation by *Aspergillus* sp (Suganthi et al. 2011; Mamatha et al. 2012) and various bacteria (Sivaramakrishnan et al. 2006; Akcan et al. 2011; Welker and Campbell 1963) with excellent results. The results on the effect of the carbon source of the fermentation medium indicated that all the supplemented compounds supported the growth but affected α-amylase production. The use of either glucose and/or of maltose resulted in the very low amylolytic activity of 0.013 and 0.028 IU/mL, respectively. The maximum α–amylase yield of 0.115 IU/mL was obtained with starch as the carbon source. The results showed that the carbon source in the form of monosaccharide, or

polysaccharide strongly influenced the production of extracellular α-amylase. Both, the maximum α-amylase activity (0.125 IU/mL) and the maximum cell growth were achieved at initial pH 6.0 of the fermentation medium; deviations from the optimum pH-value, and especially towards the alkaline region had an opposite effect on the yeast growth.

The yeast strain produced α-amylase in the temperature range from 20-40^{o}C, and exhibited maximum production of α-amylase and of cell growth at 30^{o}C, whereas at temperatures higher than 40^{o}C, the extracellular amylolytic activity was almost completely lost. Figure 1 showed that cell growth and α-amylase production were strongly associated, and the production of α-amylase reached its maximum (0.127 IU/mL) at the end of the exponential phase of the growth.

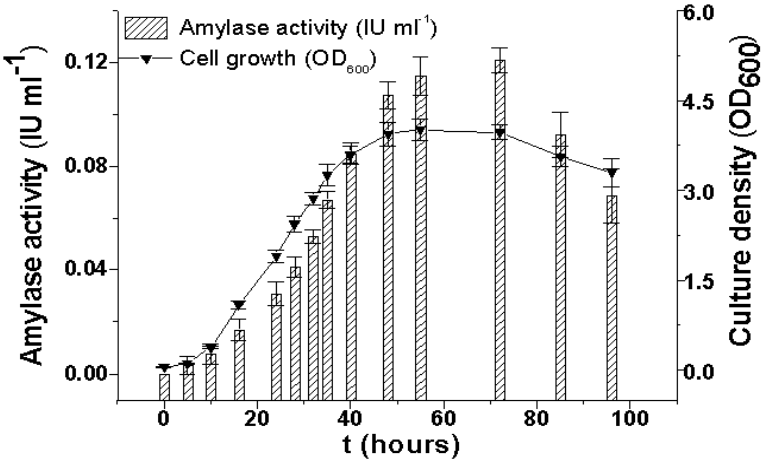

Figure 1 - The produced activity of α-amylase, as well as the growth response of *Kluyveromyce marxianus* in the optimized medium (composition is given in the text), are shown at 150 rpm during 96 h growth; the starting pH-value and temperature were 6.0, and 30°C, respectively.

Full 3^2 factorial design in association with response surface methodology were used for the examination of the effective interactions among the most appropriate optimized factors, i.e., pH and incubation temperature. The experimental data were treated by multiple regression analysis and the polynomial equations (2) and (3) were derived, where A and B corresponded to the pH-value and temperature, respectively:

α-Amylase activity =
$0.127+0.01875*A+0.00325*B+0.002875A*B-0.08325A^2-0.07475*B^2-0.00288\ A^{2*}B-0.01138A*B^2+0.048875\ A^{2*}B^2$ Equation 2

Cell growth = $3.88-0.31*A+0.2475*B-0.2\ A*B-0.66A^2-0.5775*B^2+0.02\ A^{2*}B-0.175A*B^2-0.845A^{2*}B^2$ Equation 3

By means of the ANOVA and Fisher's analyses, the above model equations were evaluated as adequate, in so far as the estimated R^2 values suggested a marked agreement between the experimentally and theoretically obtained values. The low values of Probability>F, which were $P<0.050$, revealed that the estimated model terms were significant in all the cases (Table 3). In all the cases, the employed models in the optimization procedure were considered as adequate as both significant F-values and <10 standard deviation values were estimated.

Figures 2 and 3 depicteded the combined three-dimensional convex response surface plots and two dimensional contour plots, which represented graphically the regression equations to estimate the interactions of the required variables for maximum α-amylase production and cell growth, respectively. The three-dimensional response surface plots were not enough convex but rather symmetric, and partially flat near the optimum, indicating that the RSM-optimized values for the

combined effects of pH and temperature were not largely different from the single variable optimized conditions. The obtained operating conditions from of the RSM-optimization were at pH 6.13 and 30.3°C, i.e. about 4.5% higher than those found from the single variable optimization (pH 6.0 and 30°C). The optimum operating conditions for the cell growth were at pH 5.64 and 31.0°C, with the maximum optical density of 3.91 at 600nm. Thus, the production of extracellular α-amylase from *K. marxianus* IF0 0288 was increased and instead of a yield of 0.127 IU/mL under the "one-variable-at-a-time" method, a yield of 0.133 IU/mL was obtained at 60 h incubation, showing a small, but important improvement under the optimization approach using the RSM-method. Fonseca et al. (2008) and Lane and Morrissey (2010) have described the biotechnological potential of *K. marxianus* for industrial applications. This study was the first report on extracellular production of α-amylase from *K. marxianus* in batch fermentation.

Table 3 - Analysis of variance for the response in α-amylase production, and the corresponding cell growth of strain *Kluyveromyce marxianus.*

	α-amylase Production					Cell Growth				
Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob>F**	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob>F**
Model	0.021	8	2.7E-3	1123	1.0E-4	15.23	8	1.903	530	1.0E-4
A	1.4E-3	1	1.4E-3	589	1.0E-4	0.384	1	0.384	107	1.0E-4
B	4.2E-5	1	4.2E-5	17.69	2.3E-3	0.245	1	0.245	68.2	1.0E-4
AB	6.6E-5	1	6.6E-5	27.68	5.0E-4	0.321	1	0.321	89	1.0E-4
A^2	9.2E-3	1	9.2E-3	3868	1.0E-4	0.581	1	0.581	162	1.0E-4
B^2	7.4E-3	1	7.4E-3	3119	1.0E-4	0.445	1	0.445	124	1.0E-4
A^2B	2.2E-5	1	2.2E-5	9.226	1.4E-3	0.001	1	0.001	0.30	0.59
AB^2	3.4E-4	1	3.4E-4	144	1.0E-4	0.081	1	0.081	22.7	1.0E-3
A^2B^2	2.1E-3	1	2.1E-3	889	1.0E-4	0.635	1	0.635	176	1.0E-4
Pure Error	2.1E-5	9	2.4E-6			0.032	9	0.004		
Corr. Total	0.021	17				15.26	17			

Variables A and B correspond to the pH-value and temperature, respectively.

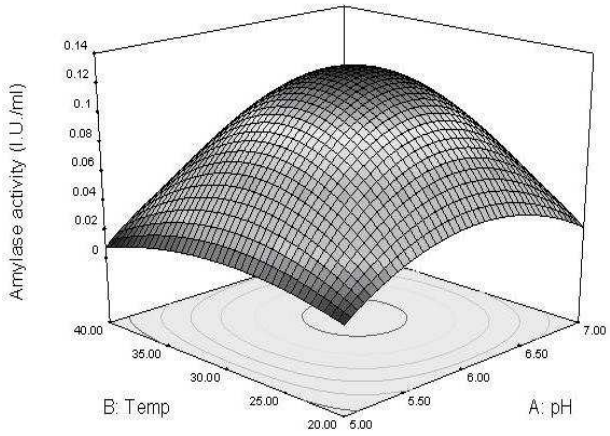

Figure 2 - Three-dimensional response surfaces and contours, which were drawn according to Equation 1 (Table 2), and correspond to the combined effects of pH-value and temperature on α-amylase production by *Kluyveromyce marxianus*.

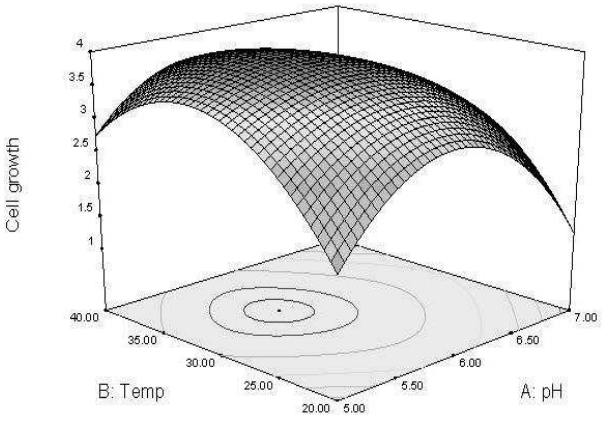

Figure 3 - Three-dimensional response surfaces and contours, which were drawn according to Equation 1 (Table 2), and correspond to the combined effects of pH-value and temperature on cell growth of *Kluyveromyce marxianus*.

CONCLUSIONS

In this work, a 12.7-fold increase in α-amylase production with optimized nutritional and cultural conditions at the end of exponential growth phase (60 h) employing the "one-variable-at-a-time" method was achieved. By applying the RSM approach, extracellular α-amylase production was enhanced to 13.5-fold by optimizing the initial pH-value to 6.13 and temperature to 30.3^{o}C. This is a first report on the production of extracellular α-amylase from *K. marxianus* using RSM.

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