Türk Biyokimya Dergisi [Turkish Journal of Biochemistry–Turk J Biochem] 2011; 36 (2) ; 83–92.

Research Article [Araştırma Makalesi]

Yayın tarihi 15 Haziran, 2011 © TurkJBiochem.com

[Published online 15 June, 2011]

Optimization of cold-adapted amylases and protease production by psychrotrophic *Streptomyces* 4 Alga using response surface methodology

[Cevap yüzey metoduyla yeni fizotropik *Streptomyces* 4 Alga'dan soğuk uyumlu amilazların ve proteazların birlikte üretiminin optimizasyonu]

Mihaela Cotârleţ,
Gabriela-Elena Bahrim

Dunarea de Jos University, Faculty of Food Science and Engineering, Bioengineering Department, Domneasca Street, No. 111, Galati, Romania

Yazışma Adresi
[Correspondence Address]

Dr. Mihaela Cotarlet

Phone: +40741537336
Fax: 0236461353
E-mail: Mihaela.Cotarlet@ugal.ro

Registered: 24 September 2010; Accepted: 24 January 2011

[Kayıt Tarihi : 24 Eylül 2010; Kabul Tarihi : 24 Ocak 2011]

ABSTRACT

Response surface methodology (RSM) employing central composite design (CCD) was used to optimize the fermentation medium constituents for co-production of cold-adapted amylases (α and β amylase) and protease from newly psyhrotrophic *Streptomyces* 4 Alga under submerged fermentation (SmF). A 2^4 full factorial central composite design was chosen to explain the combined effects of the four medium constituents; starch and glycerol as carbon sources and sodium caseinate and urea as nitrogen sources. The independent variables were chosen using "one-variable-at-a-time" conventional approach. A linear model, response surface method and the numerical optimization showed that the optimum conditions for cold-adapted α-amylase production (starch 0.75% (w/v), glycerol 0.75% (v/v), sodium caseintate 0.75% (w/v) and urea 0.26% (w/v)) results in 1.71-fold improvement in cold-adapted α-amylase production after 24 h of submerged cultivation, at 7.276xg. For beta-amylase a higher production was not obtained. Instead, in the protease case a higher production was obtained but the concentration was still too small to be industrially useful. The studied enzyme may have tremendous applications in food industry, detergents in processes that are running at lower temperature.

Key words: *Streptomyces* sp., α-amylase, β-amylase, central composite design (CCD), response surface methodology (RSM), submerged fermentation cultivation (SmF)

ÖZET

Amaç: Soğuk uyumlu amilazlar (α ve β amilaz) ve yeni fizotropik *Streptomyces4Alga*'dan elde edilmiş proteazın batık fermentasyonda birlikte üretiminde fermentasyon bileşenlerinin optimizasyonunda merkezi kompozit dizaynı ile çalışan yüzey cevap metodu kullanılmıştır.

Materyal ve metot: Dört ortam bileşeninin (karbon kaynağı olarak nişasta ve gliserol ile azot kaynağı olarak sadyum kazeinat ve üre) birlikte etkilerini açıklamak için 2^4 tam faktöryel merkezi bileşen kullanılmıştır.

Bağımsız değişkenler her seferinde bir değişken konvansiyonel yaklaşımı ile seçilmiştir.

Bulgular: Lineer model, yüzey cevap metodu ve nümerik optimizasyon, optimum koşulların soğuk uyumlu a-amilaz üretimi için optimum koşulların (% 0.75 nişasta (ağırlık/ hacım), % 0.75 gliserol (ağırlık/hacım), % 0.75 sodyum kazeinat (ağırlık/hacım) ve % 0.26 üre), 7.276xg devirdeki 24 saatlik batık kültürde soğuğa uyumlu a-amilaz verimini 1.71 kat arttırmıştır. Beta-amilaz üretiminde artış elde edilmezken proteaz üretiminde artış elde edilmiş ancak miktar endüstride yararlı olamayacak kadar az olmuştur.

Sonuç: Çalışılan enzimin düşük sıcaklıkta çalışan deterjanlarda, gıda endüstrisinde birçok uygulaması olabilir,

Anahtar kelimeler: *Streptomyces* sp., α-amilaz, β-amilaz, merkezi kompozit dizaynı (CCD), cevap yüzey metodu (RSM), batık fermentasyon kültürü (SmF)

Introduction

Amylases constitute a class of industrial enzymes having approximately 25% of the enzyme market [1]. Proteases play an important role in many industrial technologies and they are a convenient tool whenever protein removal is needed [2]. The possibility of using actinomycetes, specifically *Streptomyces*, for enzyme production has recently been investigated. Starch hydrolyzing activity was widely distributed in species of *Streptomyces* and some of them can attack and hydrolyze raw starch granules with the release of maltose as the predominant product, such enzymes are used for the industrial conversion of raw starch into sugar for fermentation [1]. *Streptomyces* species served as an important source for numerous secondary metabolites, enzymes [3] and antibiotics [4] mainly due to their shorter generation time, and the ease of genetic and environmental manipulation. Amylase has applications in different industries; *i.e.* baking, brewing, starch liquefaction and distillery [5] and also in textile, pharmaceutical, and detergents [6-9]. Cold-adapted protease has large industrial application, especially in detergents, textile, leather, also in molecular biology [10-15] and in food industry (cheese making, meet tenderization) [12,14-16].

It is well documented that extracellular enzyme production by microorganisms is greatly influenced by media components, especially carbon and nitrogen sources, minerals and physical factors such as pH, temperature, agitation, dissolved oxygen and inoculum density [17]. Studies on optimization of amylase and protease have been reported earlier with effects of different media ingredients on its production; however, using a one-factor-at-a-time approach. This has not led to an understanding of factors that can exert an interactive effect on enzyme production.

Response surface methodology (RSM) has already been successfully applied for optimization of the media and culture conditions in many cultivation processes for the production of primary and secondary metabolites including lovastatin [16], exopolymer [18], amino acid, ethanol and enzymes as α-amylase [5,19-21], xylanase [17,22], chitinase [22], protease [6], transglutaminase [23], and α-galactosidase [24]. The traditional 'one-factor at a time' technique used for optimizing a multivariable system is not only time consuming but also often easily misses the alternative effects between components. Also, this method requires carrying out a number of experiments to determine the optimum levels. These drawbacks of single factor optimizing process can be eliminated by optimizing all the affecting parameters collectively by central composite design (CCD) using RSM which is the one suitable for identifying the effect of individual variables and for seeking the optimum conditions for a multivariable system efficiently [5,19]. RSM, which includes factorial design and regression analysis, helps in evaluating the effective factors and building models to determine interaction and select optimum conditions of variables for a desirable response [21].

The present study was designed for optimize the effect of four crucial factors, starch, glycerol, sodium caseinate, and urea concentrations on cold-adapted amylase and protease produced by the filamentous *Streptomyces* 4 Alga in submerged fermentation using RSM.

Materials and Methods

Microorganism

Psychrotrophic Streptomyces 4 Alga was isolated from Antarctic vegetation samples from Progress Lake 2 (East Antarctica) [25]. The culture was maintained on Gause-agar medium (pH 7.0) containing (% w/v): starch 2.0; K_2HPO_4 0.5; $MgSO_4 \cdot 7H_2O$ 0.5; KNO_3 1.0; NaCl 0.5; $FeSO_4 \cdot 7H_2O$ 0.01; agar 25.0.

Enzyme production

Each 250 ml Erlenmeyer flask containing 100 ml of a basal medium (% w/v): $CaCO_3$ 0.8; NaCl 0.5 and soybean oil 0.02 ml, pH 7.0 with the carbon and nitrogen sources varied according to the experimental design was inoculated with 2% (v/v) spore suspension and cultivated nine days at 20°C and 7.276xg. The cell free supernatant centrifuged at 4952xg for 15 min at 4°C was assayed for enzyme activity.

Enzyme assay

Alpha-amylase assay was determined using a method based on the difference of the hydrolysis products in 0.1 N Lugol solution. One unit of α-amylase (UA) was defined as the amount of enzyme which generates a 0.05 decrease in the optical density, for one min, measured at OD_{610} nm, of the color iodine-starch complex, into a 1% starch solution, at pH 7.0 and 20ºC [26].

Beta-amylase assay using Merck method was achieved. One β-amylase unit (UA) represents the amount of maltose (in mg) produced by one ml cell free extract by using 1% starch as substrate, at 20ºC and pH 7.0, for 1 min. To measure the maltose the Shaffer-Somogyi method was used [27].

Protease assay was determined via modified Anson method using 2% casein as substrate [28,29] and protease activity was expressed as Anson units (UA). One Anson unit is the amount of enzyme which, under the analytical specified conditions (2% casein as substrate, pH 7.0; for 15 min, at 20°C) hydrolyzed the casein at a speed that facilitates release, in one minute, the hydrolysis products soluble in the trichloroacetic acid; this provides coloration equivalent, measured at OD_{670} nm, to one μmol of tyrosine, in the presence of the Folin-Ciocalteau reagent by using a tyrosine standard curve over the range 0.02-0.24 μmol/ml [30].

Experimental design and optimization

The conventional one-factor-at-a-time method was used

to select the effective factors and the initial test range of each of four variables: starch (A), glycerol (B) as carbon sources and sodium caseinate (C) and urea (D) as nitrogen sources. Taking into consideration these factors a response surface methodology using central composite design was adopted for evaluating cold-adapted amylase and protease biosynthesis from *Streptomyces* 4 Alga.

The statistical software package Design-Expert® 7.1.6 (Stat-Ease Inc., Minneapolis, USA) was used to analyze the experimental design. A 2^4 factorial central composite experimental design, with four factors and six replicates at the center point, leading to a set of 30 experiments, was achieved to optimize the production of cold-adapted amylase and protease from *Streptomyces* 4 Alga. All the variables were taken at a central coded value considered as zero. The minimum and maximum ranges of variables investigated, and the full experimental plan with respect to their values in actual and coded form are listed in Table 1. Productions of cold-adapted amylase (α, β amylase) and protease (Y_1, Y_2, Y_3, UA) were used as the dependent output variable.

Statistical analysis of the model was performed using the analysis of variance (ANOVA). The optimal concentrations of the medium components were obtained by numerical optimization procedure using Design-Expert® 7.1.6 (Stat-Ease Inc., Minneapolis, USA).

Results and Discussion

The results of CCD experiments for studying the effect of four independent variables were presented along with the mean predicted and observed responses in Table 2. The regression equation obtained after the ANOVA gave the level of production of cold-adapted hydrolase as a function of the initial values of starch, glycerol, sodium caseinate and urea. The regression equation for cold-adapted amylase and protease biosynthesis were given below:

$$Y_1 = +1.47 + 0.19\ A + 0.21\ B + 0.57C - 0.10D + 0.23AB + 0.34\ AC - 0.27\ AD + 0.45\ BC - 0.38\ BD - 0.33\ CD,$$

$$Y_2 = +12.97 - 0.15\ A + 4.63\ B + 0.035\ C + 1.79\ D - 3.10\ AB - 1.86\ AC - 2.07\ AD + 1.59\ BC + 3.18\ BD + 4.18\ CD - 2.18\ A^2 + 0.18\ B^2 - 1.61\ C^2 - 1.75\ D^2 - 4.07\ ABC - 4.21\ ABD - 3.02\ ACD + 3.19\ BCD - 3.47\ A^2B + 3.55\ A^2C + 0.94\ A^2D - 1.21\ AB^2$$

$$Y_3 = 0.0055 + 0.00\ A + 0.00\ B - 0.0006\ C + 0.00\ D - 0.015\ AB - 0.015AC - 0.015\ AD + 0.015\ BC + 0.015\ BD + 0.014\ CD + 0.0018\ A^2 + 0.0018\ B^2 + 0.014\ ABC + 0.014\ ABD + 0.014\ ACD - 0.015\ BCD - 0.014\ A^2B - 0.013\ A^2C - 0.014\ A^2D + 0.013AB^2$$

where the predicted response (α-amylase, UA, β-amylase, UA, and protease, UA) and the coded value of variable starch, glycerol, sodium caseinate and urea were shown as Y_1, Y_2, Y_3, A, B, C and D, respectively.

The regression equation indicated that coefficient of determination (R^2) was 0.8379 (a value of $R^2 > 0.75$ indicates the aptness of the model) for cold-adapted α-amylase biosynthesis and thus the model could explain more than 83.79% of variability in the response (Table 3). The experimental data was statistically analyzed using Fischer's statistical test for ANOVA and the results indicated that the model was highly significant, as the F-value for the model was 9.82. There was only a 0.01% chance that a 'Model F-Value' this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant. In cold-adapted α-amylase biosynthesis B, C, AC, AD, BC, BD, CD are significant model terms (Table 4). Furthermore, the linear effect of sodium caseinate is more significant than other factors.

The parity plot showed a satisfactory correlation between the values of experimental values and predictive values (Fig. 1), wherein, the points cluster around the diagonal line which indicates the good fit of the model, since the deviation between the experimental and predictive values was less.

The mathematical model for cold-adapted β-amylase gave the "Model F-value" of 0.71 implies the model is not significant relative to the noise. There is a 74.77% chance that a "Model F-value" this large could occur due to noise (Table 5). In this case there are no significant model terms.

For protease the model lead a $R^2 = 0.9080$, and also the Model F-value of 4.44 implies the model is significant. There is only a 1.32% chance that a "Model F-Value" this large could occur due to noise (Table 6).

Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case AB, AC, AD, BC, BD, CD, ABC, ABD, ACD, and BCD are significant model terms (Table 7).

Table 1. Experimental range and levels of four independent variables studied using CCD in terms of actual and coded factor

Variable	Symbol code	Coded level of variables				
		-α	-1	0	+1	+ α
Starch, (% w/v)	A	0	0.25	0.50	0.75	1
Glycerol, (% v/v)	B	0	0.25	0.50	0.75	1
Sodium caseinate, (% w/v)	C	0	0.25	0.50	0.75	1
Urea, (% w/v)	D	0	0.10	0.20	0.30	1

Table 2. Central composite design matrix with experimental and predicted values of enzymes yield

Trial no.	Variables				α-amylase yield, (UA) (Y_1)		β-amylase yield, (UA) (Y_2)		protease yield, (UA) (Y_3)	
	Starch, (% w/v) (A)	Glycerol, (% v/v) (B)	Sodium caseinate, (%w/v) (C)	Urea, (% w/v) (D)	Actual	Predicted	Actual	Predicted	Actual	Predicted
1	-1	-1	-1	-1	0.58±0.0608	0.62	10.53±0.5655	11.51	0±0	0.0073
2	+1	-1	-1	-1	0.7±0.01	0.40	2.88±0.1539	0.28	0.23±1.2773	0.21
3	-1	+1	-1	-1	0.73±0.0458	0.46	2.92±0.2640	0.32	0±0	-0.020
4	+1	+1	-1	-1	1.18±0.0251	1.15	8.80±0.2645	9.78	0±0	0.0073
5	-1	-1	+1	-1	0.76±0.03511	0.86	5.67±0.3197	3.07	0±0	-0.020
6	+1	-1	+1	-1	2.37±0.1484	2.00	11.76±0.3955	12.74	0.0005±4.73E-05	0.0078
7	-1	+1	+1	-1	2.94±0.1527	2.48	0.77±0.1637	1.75	0.0058±0.0030	0.013
8	+1	+1	+1	-1	4.88±0.01	4.54	2.16±0.1350	-0.44	0±0	-0.020
9	-1	-1	-1	+1	2.5±0.0624	2.38	0.91±0.1814	-1.69	0±0	-0.020
10	+1	-1	-1	+1	1.01±0.0721	1.10	6.72±0.1757	7.70	0±0	0.0073
11	-1	+1	-1	+1	0.7±0,0503	0.70	2.94±0.1852	3.92	0.0054±0.0006	0.013
12	+1	+1	-1	+1	0.88±0.0655	0.32	2.92±0.1552	0.32	0±0	-0.020
13	-1	-1	+1	+1	1.63±0.0802	1.29	4.92±0.0808	5.90	0.004±0.0013	0.011
14	+1	-1	+1	+1	1.56±0.0665	1.37	14.64±0.1069	12.04	0±0	-0.020
15	-1	+1	+1	+1	1.55±0.0608	1.39	49.51±0.2107	46.91	0.0067±0.0006	-0.014
16	+1	+1	+1	+1	2.79±0.0251	2.38	6.56±0.1106	7.54	0±0	0.0073
17	-2	0	0	0	0.59±0.0351	1.08	2.94±0.0404	4.56	0±0	0.013
18	+2	0	0	0	0.9±0.0529	1.85	2.33±0.1777	3.95	0±0	0.013
19	0	-2	0	0	1.27±0.0152	1.04	2.79±0.1497	4.41	0±0	0.013
20	0	+2	0	0	1.55±0.0173	1.89	21.31±0.6964	22.93	0±0	0.013
21	0	0	-2	0	0.19±0.0152	0.32	4.82±0.1385	6.44	0.0024±0.0008	0.0067
22	0	0	+2	0	1.95±0.0757	2.61	4.96±0.2608	6.58	0±0	0.0043
23	0	0	0	-2	1.43±0.0692	1.67	0.77±0.1473	2.39	0±0	0.0055
24	0	0	0	+2	0.99±0.0529	1.27	7.92±0.0929	9.54	0±0	0.0055
25	0	0	0	0	0.97±0.0655	1.47	5.00±0.07	12.97	0±0	0.0055
26	0	0	0	0	1.95±0.0529	1.47	14.49±0.2463	12.97	0.00019±3.21E-05	0.0055
27	0	0	0	0	1.86±0.0655	1.47	40.80±0.5036	12.97	0±0	0.0055
28	0	0	0	0	1.22±0.0513	1.47	3.78±0.1331	12.97	0±0	0.0055
29	0	0	0	0	1.21±0.0472	1.47	5.04±0.0873	12.97	0±0	0.0055
30	0	0	0	0	1.12±0.1039	1.47	8.70±0.0680	12.97	0.0009±5.69E-05	0.0055

Table 3. ANOVA for liniar model for cold-adapted α-amylase production

Source	SS	DF	MS	F-value	P>F
Model	21.15	10	2.12	9.82	< 0.0001
Residual (error)	4.09	19	0.22		
Lack of fit	3.25	14	0.23	1.37	0.3866
Pure error	0.85	5	0.17		
Total	25.25	29			

R^2 = 0.8379; Adj R^2 = 0.7526; Coefficient of variance = 31.67%; SS, sum of squares, DF, degrees of freedom; MS, mean square.

Table 4. The least-squares fit and parameter estimates (significance of regression coefficient) for cold-adapted α-amylase production

Term	Coefficient[a]	F-value	P-value P>F
Constant	1.47	9.82	< 0.0001
Starch	0.19	4.09	0.0573
Glycerol	0.21	5.03	0.0370
Sodium cazeinate	0.57	36.42	< 0.0001
Urea	-0.10	1.11	0.3044
Starch x glycerol	0.23	3.85	0.0647
Starch x sodium cazeinate	0.34	8.65	0.0084
Starch x urea	-0.27	5.27	0.0333
Glycerol x sodium cazeinate	0.45	14.80	0.0011
Glycerol x urea	-0.38	10.80	0.0039
Sodium cazeinate x urea	-0.33	8.21	0.0099

[a] Estimated real value of parameters.

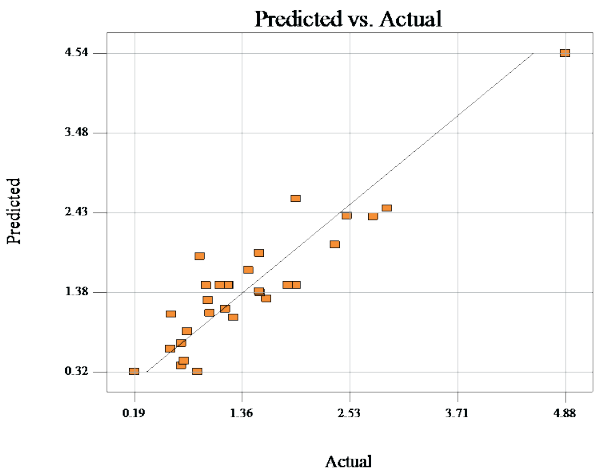

Fig. 1. Parity plot showing the distribution of experimental vs. predicted values of cold-adapted α-amylase activity.

Table 5. ANOVA for cubic model for cold-adapted beta-amylase production

Source	SS	DF	MS	F	P>F
Model	2432.43	22	110.57	0.71	0.7477
Residual (error)	1088.65	7	155.52		
Lack of fit	82.73	2	41.37	0.21	0.8207
Pure error	1005.91	5	201.18		
Total	3521.08	29			

R^2 = 0.6908; Adj R^2 = -0.2809; Coefficient of variance = 3143.75%; SS, sum of squares, DF, degrees of freedom; MS, mean square.

Table 6. ANOVA for cubic model for cold-adapted protease production

Source	SS	DF	MS	F	P>F
Model	0.046	20	0.0023	4.44	0.0132
Residual (error)	0.0046	9	0.0011		
Lack of fit	0.0046	4	0.0011	8842.99	< 0.0001
Pure error	6.611E-007	5	1.322E-007		
Total	0.051	29			

R^2 = 0.9080; Adj R^2 = 0.7036; Coefficient of variance = 267.25%; SS, sum of squares, DF, degrees of freedom; MS, mean square.

Table 7. The least-squares fit and parameter estimates (significance of regression coefficient) for cold-adapted protease production

Term	Coefficient[a]	F-value	P-value P>F
Constant	5.551 E-003	4.44	0.0132
Starch	0.0	0.0	1.0
Glycerol	0.0	0.0	1.0
Sodium cazeinate	6.025E-004	5.58E-003	0.9420
Urea	0.0	0.0	1.0
Starch x glycerol	0.015	7.18	0.0252
Starch x sodium cazeinate	0.015	6.96	0.0270
Starch x urea	0.015	6.97	0.0269
Glycerol x sodium cazeinate	0.015	6.51	0.0312
Glycerol x urea	0.015	6.52	0.0310
Sodium cazeinate x urea	0.014	6.31	0.0332
Starch x starch	1.862E-003	0.19	0.6733
Glycerol x glycerol	1.862E-003	0.19	0.6733
Starch x glycerol x sodium caseinate	0.014	6.16	0.0348
Starch x glycerol x urea	0.014	6.26	0.0337
Starch x sodium caseinate x urea	0.014	6.36	0.0326
Glycerol x sodium caseinate x urea	0.015	6.81	0.0283
Starch x starch x glycerol	0.014	1.88	0.2035
Starch x starch x sodium caseinate	0.013	1.75	0.2189
Starch x starch x urea	0.014	1.94	0.1967
Starch x glycerol x glycerol	0.013	1.74	0.2192

[a] Estimated real value of parameters.

The three dimensional response surfaces were plotted to study the interaction among the various factors selected and to determine the optimum concentration for attaining maximum cold-adapted α-amylase production. The plots were generated by plotting the response using z-axis against two independent variables while keeping the other independent variables at their 0-level. The coordinates of the central point within the highest contour levels in each of the figures correspond to the optimum concentrations of the respective components.

Figs. 2-4 show the response for the interaction of starch (A) with glycerol (B) (Fig. 2), sodium caseinate (C) (Fig. 3) and urea (D) (Fig. 4). From Figs. 2-3, it can be seen that the cold-adapted α-amylase yield gradually increased upon increasing the concentrations of starch, glycerol and sodium caseinate, respectively. Therefore, at 0.75% starch, glycerol and sodium caseinate concentrations, while other variables were kept constant, the maximum production of cold-adapted α-amylase activity (2.77 UA) was achieved. The importance of starch, glycerol and sodium caseinate on the production of α-amylase is emphasized in the literature [2,6,7,31,32]. The effect of glycerol can result from conversion of glycerol into dihydroxy acetone by entering to glycolytic pathway for formation of metabolic energy [21].

Fig. 4 represents the isoresponse surface plot on behalf of the effect of starch and urea on the cold-adapted α-amylase production. An increase in the concentration of starch up to 0.75% and increase the urea level up to 0.26% had the effect of obtaining the maximum cold-adapted α-amylase production (2.77 UA).

In Figs. 5-7 it can be seen that the activity of α-amylase increased upon the maximum concentration of glycerol and sodium caseinate (Fig. 5). Increasing the concentration of glycerol and sodium caseinate at higher limit and urea at central level resulted in maximum yield of cold-adapted amylase (Figs. 6-7).

A linear model and response surface method showed the optimum conditions for maximizing the cold-adapted α-amylase production (starch 0.75% (w/v), glycerol 0.75% (v/v), sodium caseinate 0.75% (w/v) and urea 0.26% (w/v)).

To validate and confirm these predictions, 3 experiments were designed with random levels of nutrients. The model was successfully validated as the values predicted by the model were in good agreement with the results obtained on validation for different levels of starch, glycerol, sodium caseinate and urea (Table 8). Close results were observed between the predicted and experimental results that reflected the accuracy and applicability of RSM to optimize the fermentation medium.

The time course of α-amylase for both cases; that is, before and after optimization, is also depicted in Fig. 8.

A linear model and response surface method showed that the optimum conditions for maximizing the cold-adapted α-amylase production (starch 0.75% (w/w), glycerol 0.75% (v/w), sodium caseinate 0.75% (w/w) and urea 0.26% (w/w)) results in 1.71-fold improvement in cold-active α-amylase production (4.25 UA) as compared to initial level (2.47 UA) (data not shown), after 24 h of submerged cultivation, at 7.276xg. After 96 h of submerged cultivation the biosynthesis process should be stopped for economical reasons.

Using the response surface methodology did not achieve a higher cold-adapted beta-amylase and protease production comparing with the initial medium (data not shown), although for cold-adapted protease production the coefficient of the determination (R^2) was 0.9080. Instead, for cold-adapted beta-amylase the mathematical model did not include the significant factors. In general, organic nitrogen (urea) reduced the proteolytic production greatly (80%-95%, approximately) [33,34]. Boominadhan et al. (2008) [35] related that at different species of *Bacillus* (*Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus megaterium* and *Bacillus licheniformis*) urea

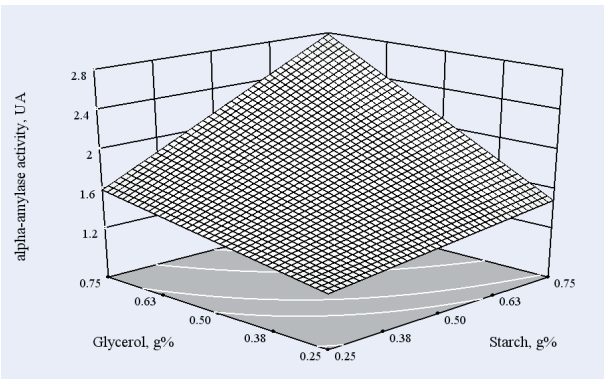

Fig. 2. Response surface plot showing the mutual effect of glycerol and starch concentrations on the production of cold-adapted α-amylase. Sodium caseinate and urea were held at zero level.

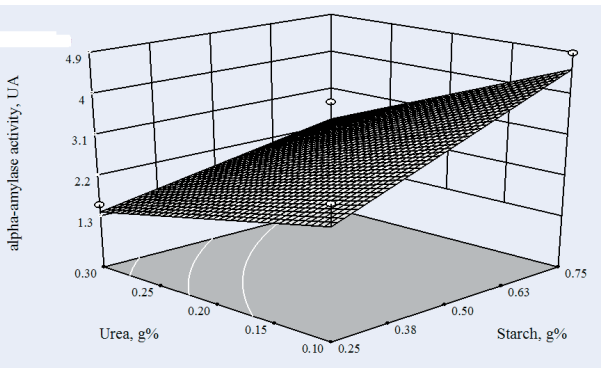

Fig. 4. Response surface plot showing the mutual effect of starch and urea concentrations on the production of cold-adapted α-amylase. Glycerol and sodium caseinate were held at zero level.

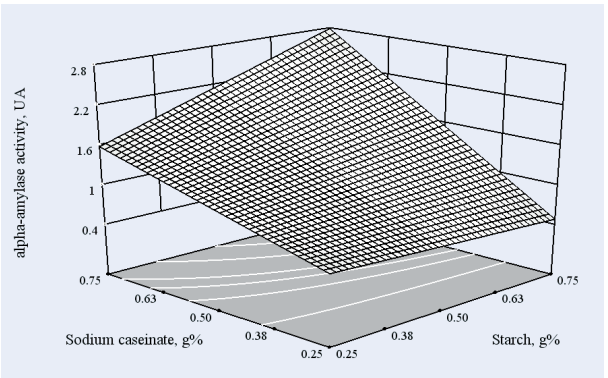

Fig.3. Response surface plot showing the mutual effect of sodium caseinate and starch concentrations on the production of cold-adapted α-amylase. Glycerol and urea were held at zero level.

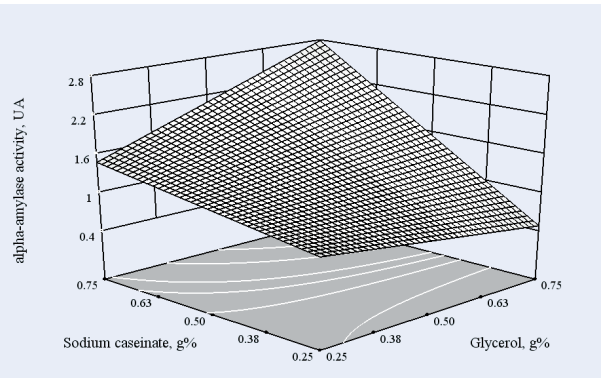

Fig.5. Response surface plot showing the mutual effect of glycerol and sodium caseinate concentrations on the production of cold-adapted α-amylase. Starch and urea were held at zero level.

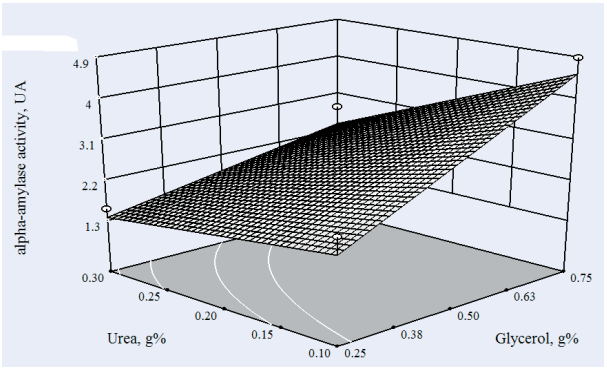

Fig. 6. Response surface plot showing the mutual effect of urea and glycerol concentrations on the production of cold-adapted α-amylase. Sodium caseinate and starch were held at zero level.

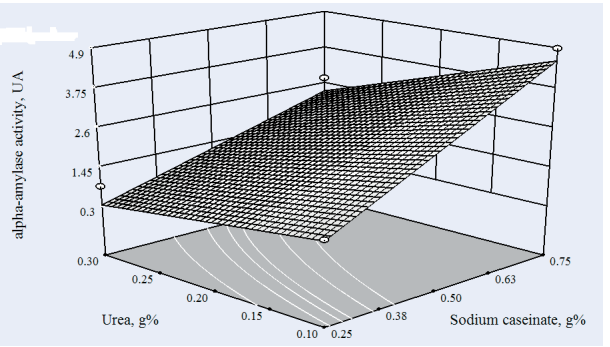

Fig. 7. Response surface plot showing the mutual effect of urea and sodium caseinate concentrations on the production of cold-adapted α-amylase. Glycerol and starch were held at zero level.

Table 8. Validation of response surface linear model for cold-adapted α-amylase production

Experiment No	Starch (% w/v)	Glycerol (% v/v)	Sodium caseinate (% w/v)	Urea (% w/v)	Experimental value (UA)	Predicted value (UA)
1	0.3	0.3	0.3	0.05	2.9562±0.3631	1.6678
2	0.6	0.6	0.6	0.15	4.3058±0.0578	2.088
3	0.75	0.75	0.75	0.3	4.628±0.0973	2.5206

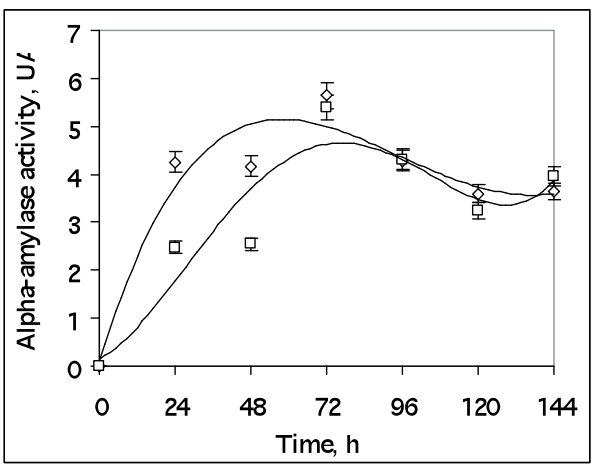

Fig. 8 Time course of cold-adapted α-amylase production in both optimized (◊) and non-optimized (□) medium.

had an inhibitor effect on protease activity. This can be the reason for not obtaining higher protease production.

Over the last few decades, even though several papers regarding optimization of cold-adapted amylase production have been reported, little information about the optimization of this enzyme production using *Streptomyces* sp. by submerged fermentation is available in the scientific literature. The use of statistical methods for the optimization of α-amylase produce by *Bacillus* sp. in submerged cultivation was previously reported [21]. The optimal combinations of media constituents for maximum α-amylase production were determined as 17.58 g/L starch, 12.37% (v/v) glycerin, 8.77 g/L peptone and 0.00 g/L YE.

A response surface method with 2^4 factorial designs has been used to optimize the medium components for maximum α-amylase production in solid substrate fermentation by *Bacillus amyloliquefaciens* NRRL B-645. Hazelnut cake (HC) was found to be a good substrate for the production of α-amylase. The highest α-amylase activity (4895 IU) was measured when the HC, peptone, YE and $(NH_4)_2SO_4$ concentrations in the medium were 22.62, 5.20, 1.62, and 6.81g/L, respectively [21].

Conclusions

The one-factor-at-a-time is the most frequently used operation in optimization process. This technique is based on changing one parameter at a time, while keeping the others at fixed levels is laborious and time consuming. This method requires a complete series of experiments for every factor of interest. Moreover, such a method does not provide means of observing possible factors interactions. In contrast, CCD offers a number of important advantages. For instance, the researchers could easily determine factor effects with considerably less experimental effort, identify factors, find optima, offer greater precision and facilitate system modeling.

Thus, the present study using the RSM with CCD enables to find the importance of factors at different levels. A high similarity was observed between the predicted and experimental results, which reflected the accuracy and applicability of RSM to optimize the production of cold-adapted amylase. The results of this study have clearly

indicated RSM is an effective method for maximum production of amylase using SmF with *Streptomyces* 4 Alga. Further experiments will focus to discover the most important factors for cold-adapted beta-amylase and protease in order to obtain higher enzymes production.

Acknowledgements

The Bioaliment Platform of Dunarea de Jos University, Galati Romania has been provided high scientific conditions of researches.

Conflict of Interest: authors have no conflict of interest.

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