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High Level Amylase Production by *Pseudomonas stutzeri* ML-18; Isolation, Identification and Media Optimization in a Fermentation System using Response Surface Methodology (RSM)

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The bacterium *Pseudomonas stutzeri* ML-18 was isolated from the kitchen waste water of a sweet shop. The production of amylase by this bacterium was investigated and optimized using statistical approaches (Response Surface Methodology (RSM); Box–Behnken design). The optimized medium was obtained with these conditions; (% w/v) KH_2PO_4 0.1, Na_2PO_4 0.25, NaCl 0.1, KNO_3 0.1, $CaCl_2$ 0.005, tryptone 0.2, $MgSO_4.7H_2O$ 0.005, starch 1 and pH 8. The amylase activity in optimized medium was 243.614 U/ml. furthermore, high level of amylase production (245.612 U/ml) was observed by using fermentor system.

Key words: Amylase, *Pseudomonas*, RSM, optimization.

Amylases are important enzymes, which break down glycogen or starch molecules to give low molecular weight products, such as maltose, glucose and smaller polymers compound of glucose units[1-2] they have been divided into two major groups; endoamylases and exoamylases, that hydrolyse interior and the non-reducing end of the starch molecule, respectively[2]. Amylases have wide applications in different fields such as bread and baking industry, starch liquefaction and saccharification, textile desizing, paper industry, detergent applications, fuel alcohol production and pharmaceutical industry[2-3].

Amylases have been distributed in many species of plants, animals and microorganisms such as bacteria, fungi and etc[2, 4]. Using microorganisms for production of enzymes are most interesting, because of the short growth period and their simple features to manipulate of enzyme producing with desired characteristics[2, 5]. There are many reports of the isolation of amylolytic bacteria such as *Bacillus flexus*, *Exiguobacterium* sp., *Sediminibacillus halophilus*, *Thalassobacillus* sp., *Bacillus selenatarsenatis*, *Oceanobacillus picturae*[6], *Pseudoalteromonas* sp[7], *Thalassobacillus* sp. LY18[8], *Anoxybacillus flavithermus*[9]. It seems that, isolation of microorganisms to find novel amylases with different features have been continued yet.

In this work we study the isolation and identification of some Amylase producing bacteria from the kitchen waste water of a sweet shop, using 16S rDNA gene sequencing, followed by RSM was employed to optimize the components of a nutritional medium for amylase production and attained a higher amylase yield of selected

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bacterium *Pseudomonas stutzeri* ML-18 which exhibited the highest amylolytic activity. The production of amylase by *Pseudomonas stutzeri* ML-18 using optimized medium in a kind of stirred tank fermentor was also obtained.

MATERIALS AND METHODS

Screening and isolation of strains for amylolytic activities

The amylase-producing microorganisms were isolated from the kitchen waste water of a sweet shop. Microorganisms were screened based on the formation of a clear zone on starch plates with the following composition (gr/L); (yeast extract 5.0, KCl 10.0, tryptone10, starch 10, agar 15 and pH 8) at 37°C with flooded Gram's iodine for 3 to 5 minutes.

Amylase production and amylase activity assay

For amylase production, isolated strains were grown in 250 ml erlenmeyer flasks each containing 50 ml medium with composition of (gr/L): KH_2PO_4 1, Na_2HPO_4 2.5, NaCl 1, $(NH_4)_2SO_4$ 2, $CaCl_2$ 0.05, tryptone 2, $MgSO_4.7H_2O$ 0.05, starch 5 and pH 8. The production medium was inoculated with 1 ml bacterial suspension of initial OD 600 of 0.6 from the LB preculture medium and incubated at 37°C under shaking (140 rpm, 36 h) conditions. Amylase activities were determined according to the DNS (3, 5-dinitrosalicylic acid) method, as described by Özdemir S, et al. with some modifications. One unit of amylase activity was defined as the amount of enzyme releasing one µ mol reducing sugar per mL, per minute under the defined conditions[9]

Identification of amylase-producing strains

Amylase producing strains were identified based on some conventional morphological studies [gram stain, motility, spore stain and cell shape (data not shown)] and molecular identification method. The 16S rDNA genes of selected strains were amplified using the universal prokaryotic primers (Table 1) [10].

Optimization of amylase production medium

After obtaining of amylase activity (in triplet repeats), the production medium of the highest amylase producing bacterium was optimized, at first by one factor at a time model. Finally, RSM was managed to optimize the medium compositions of amylase production[11-12].

Selection of the best nitrogen sources

Based on one factor at a time model, the rate of amylase productions was achieved using different nitrogen sources (NaNO3, NH_4Cl, KNO_3, peptone, yeast extract, arginine, tryptone) (Table 2).

RSM; Box–Behnken design

Among of the response surface in the experimental region, a Box–Behnken design was used to enhance a mathematical relation between three variables on production of amylase.Due to given the results of one factor at a time model, table 2 shows, the parameters of the highest assurance levels were given into three levels [tryptone (X1), KNO_3 (X2) and starch (X3)], and designated as +1, 0 and -1 (coded), for high, middle and low rates (or values), separately. Table 3 represents the design matrix of a 15 trials experiment. The equation of the effectors (three factors) is:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

Where Y is the predicted response, β_0 is model constant; β_1, β_2 and β_3 are linear coefficients; β_{11}, β_{22} and β_{33} are the quadratic coefficients; β_{12}, β_{13} and β_{23} are cross product coefficients and X_1, X_2 and X_3 independent variables. The MINITAB (version 15, PA, USA) statistical software was used to analysis optimal working parameters and to generate response surface graphs[11, 13].

Fermentation system

The same as authors previous investigation, fermentation conditions was considered for the growth and amylase production and done in a 10 L fermentor (BioTron, Southern Korea) consisted of a cylindrical culture vessel with a working volume of 7 L. The fermentation system was managed by a computer for monitoring pH, agitation speed and temperature. The fermentor was provided with two rushton-type impellers fitted with six blades, pH electrode, dissolved oxygen (DO) probe and four peristaltic pumps for antifoam, feed, base and acid mode. The aeration system was equipped with an air pump, connected to a ring sparger with filter and air-flow meter. Verify model which was achieved by Box–Behnken design in erlenmeyer flasks, was experimented in this fermentor. Fermentation process was done using inoculum volume of 5%

of the total volume (of initial OD 600 of 0.6 from the LB preculture medium) with initial pH of 8 (it was kept at 8 by the addition of HCl and NaOH), the agitation rate of 50 rpm, DO at 30 % and temperature of 37 °C**,** 36 hours[14].

RESULTS AND DISCUSSION

Isolation and identification of amylase producing bacteria

41 strains were isolated from the kitchen waste water of a sweet shop, among them 15 isolates had clear zones on starch agar plates, which had selected for further studies. The amylase activity of isolated strains were tested in triplet repeats using the colorimetric method, among them the bacterium *Pseudomonas stutzeri* ML-18 with the highest amylolytic activities was selected for the optimization of amylase production medium table1.

All 15 strains were identified based on molecular 16S rDNA gene identification and published in NCBI under specific accession numbers (Table 1). The 16S rDNA sequences were edited and compared as queries in BLASTN searches to determine the homologous sequences in the complete GenBank nucleotide database. The contrast of 16S rDNA gene sequence displayed that the sequences of the strains have about 98-100% similarity with the other present bacteria in GenBank.

Optimization of amylase production medium

Selection for the best organic and inorganic nitrogen sources

Amylase production from *Pseudomonas stutzeri* ML-18 was performed through various sources of organic and inorganic nitrogen. In table 2, results indicates that the highest amylase activity (185.208 U/mL) was obtained when tryptone was used as an organic nitrogen source, followed by yeast extract (62.320 U/mL), peptone (50.471 U/mL) and arginine (8.116 U/mL). In terms of the effect of mineral nitrogen sources on the amylase activities of *Pseudomonas stutzeri*, the highest amylase activity (205.680 U/mL) was achieved when KNO_3 was used as an inorganic nitrogen source, whereas, lower amylase activities were observed when $NaNO_3$ (195.550 U/mL) and NH_4Cl (64.340 U/mL). Therefore, the achieved amylase production medium composed contains; (% w/v): KH_2PO_4 0.1, Na_2PO_4 0.25, NaCl 0.1, KNO_3 0.2, $CaCl_2$ 0.005, tryptone 0.2, $MgSO_4.7H_2O$ 0.005, starch 0.5 and pH 8.

The influences of organic and inorganic nitrogen sources on amylase production have been studied in details by several authors[9, 15-17]. Yeast extract has been used as a good organic nitrogen source in the production of amylase from *B. subtilis* KCC103[16], *Bacillus* sp. IMD 435, *Streptomyces* sp. and *Halomonas meridiana*[15]. As reported earlier, yeast extract, because of contains free amino acids, growth factors and short peptides could be an essential organic nitrogen source[17]. Tryptone is known to increase enzyme production in *Bacillus amyloliquefaciens* P-001,[15-16]. It has been reported that peptone also used to influence amylase production in *Bacillus* sp. and *Bacillus thermooleovorans*[9]. Using mineral nitrogen sources have also been reported in details[9, 15]. Özdemir S, *et al*. (2012) have been investigated the effects of ammonium sulphate, ammonium nitrate and ammonium chloride in amylase production by *Anoxybacillus flavithermus* sp. nov., which had no influence on the production of á-amylase[9]. Gupta R, et al. (2003) have been reported the significant effects of mineral nitrogen sources on production amylase[15]. However, in this study, results verified the high effects of tryptone as an organic and KNO_3 as an inorganic nitrogen sources on amylase activity by *Pseudomonas stutzeri* ML-18 (Table 2).

Optimization of medium constituents

Box–Behnken design

According to the results achieved in table 2, tryptone and KNO_3 were chosen as an organic and inorganic nitrogen sources, respectively and as more effective factors on amylase production. Therefore tryptone, KNO_3 and starch were selected to define the influences of the concentrations of medium composition on amylase activity in *Pseudomonas stutzeri*.

The random combinations of tryptone, KNO_3 and starch were designed using Box–Behnken design. Results indicate the comparisons of amylase production in various media along with predicted and observed responses (Table 3). The derived regression equation for the optimization of medium constituents indicated that the amylase activity (Y, U/mL) is a function of the concentration of tryptone (X_1), KNO_3 (X_2) and starch (X_3). The

multinomial model giving the relation between the 3 factors and amylase activity could be shown as follows:

$$Y = 30.8167 + 5.7325X_1 - 0.9850X_2 - 8.6400X_3 - 1.0075X_1X_2 + 1.9275X_1X_3 - 2.2625X_2X_3 + 3.2929X_1^2 - 1.6021X_2^2 - 1.5071X_3^2$$

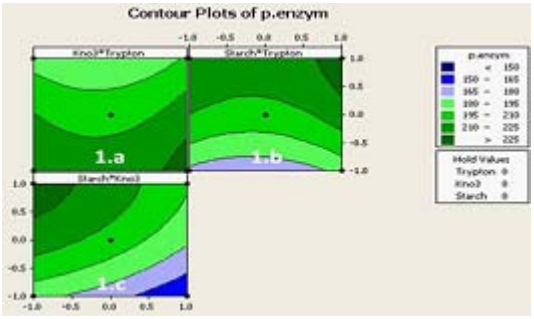

Fig. 1. a-c- Contour plots of the dependent variable amylase production**.**

The highest enzyme production was obtained the medium in trial 7, after that the medium in trial 11 and 14 (Table 3). Therefore, the best medium composed was achieved with these conditions; (% w/v) KH_2PO_4 0.1, Na_2PO_4 0.25, NaCl 0.1, KNO_3 0.1, $CaCl_2$ 0.005, tryptone 0.4, $MgSO_4.7H_2O$ 0.005, starch 1 and pH 8. Amylase activity varied from 205.680 to 232.027 U/mL, which are 26.347 units more than that resulted in the basal medium. Due to these results, this variation indicates the importance of medium optimization to get higher efficiency. The results of Box-Behnken design experiments for three variables parameter on amylase production are shown in Table 4. It indicates the calculated *P*-values and the interaction strength between each independent variable which found starch and KNO_3 to be the

Table 1. Shows the amylase activity (U/mL), the accession numbers of the DNA published sequences at the NCBI

Row	Accession number	Strains Name	amylase activity (U/ml)
1	JQ435755	*Basillus* sp. ML-01	29.563
2	JQ435756	*Basillus* sp. ML-03	20.363
3	JQ435757	*Basillus* sp. ML-04	72.463
4	JQ435758	*Basillus* sp. ML-05	88.303
5	JQ435760	*Basillus* sp. ML-06	78.733
6	JQ435759	*Basillus* sp. ML-07	52.663
7	JQ435761	*Basillus* sp. ML-08	82.363
8	JQ435762	*Acinetobacter* sp. ML-09	55.963
9	JQ435763	*Esherichia* sp. ML-11	65.203
10	JQ435764	*Basillus* sp. ML-12	84.013
11	JQ435765	*Basillus* sp. ML-14	93.583
12	JQ435766	*Bacillus* sp. ML-15	87.313
13	JQ435767	*Bacillus* sp. ML-16	92.263
14	JQ435768	*Pseudomonas stutzeri* ML-18	101.503
15	JQ435769	*Bacillus* sp. ML-19	95.233

Table 2. Medium ingredients and amylase activity for optimization of the amylase production by *Pseudomonas* stutzeri ML-18

Trial no.	Constant parameters	Variable parameters (nitrogen sources)	Amylase activity (U/mL)
1	[KH_2PO_4, Na_2PO_4, NaCl, (NH_4)	Tryptone	185.208
2	$_2SO_4$, $CaCl_2$, $MgSO_4.7H_2O$, starch]	Peptone	50.471
3		Yeast extract	62.320
4		Arginine	8.116
5	[KH_2PO_4, Na_2PO_4, NaCl, Tryptone,	KNO_3	205.680
6	$CaCl_2$, $MgSO_4.7H_2O$, starch]	$NaNO_3$	195.550
7		NH_4Cl	64.340

most significant parameters effecting on amylase activity.

The adequacy of the model could be obtained by The R^2 (the coefficient of multiple determinations). The R^2 rate is always between 0 and 1. Thus, the value of R^2 of the model nearer to 1.0 is strong enough to predict the response[11]. In this study, the value of the R^2 is 0.9549, suggesting that 95.49% of the variability in the response could be clarified by the model. This result indicated a good correlation between the predicted values and experimentally obtained results. Therefore, using the model to analyze of the response trend was considered to be reasonable (Table 4).

The contour plots of the variable elements on the amylase activity were computed and reported in fig (1.a-c). The contour plots of tryptone and KNO_3 showed that the amylase activity increased up when KNO_3 was used at the first point of concentrations (-1) and tryptone at the end point of concentrations (+1) (Fig 1.a). The contour plots indicated that at the end point of tryptone (+1) and starch (+1), amylase activity was the highest (Fig 1.b). The activity was the highest in contour plots at the low level of KNO_3 (-1) and high level of starch (+1) (Fig 1.c). According to analysis of the contour plots of the variables; the bacterium *Pseudomonas stutzeri* ML-18 was produced more amylase when

Table 3. The Box-Behnken Design for the three independent variables

Trial no.	Tryptone (X_1)	KNO_3(X_2)	Starch (X_3)	Amylase activity (U/mL) Observed Values	Predicted by RSM
1	0.4 (0)	0.2 (0)	0.75 (0)	200.567	203.706
2	0.2 (-1)	0.3 (1)	0.75 (0)	191.151	183.495
3	0.4 (0)	0.2 (0)	0.75 (0)	201.051	203.706
4	0.4 (0)	0.1 (-1)	0.5 (-1)	194.143	186.003
5	0.2 (-1)	0.1 (-1)	0.75 (0)	226.307	229.189
6	0.6 (1)	0.1 (-1)	0.25 (0)	218.519	226.175
7	0.4 (0)	0.1 (-1)	1 (1)	232.027	229.629
8	0.4 (0)	0.3 (1)	1 (1)	189.875	198.015
9	0.4 (0)	0.2 (0)	0.75 (0)	209.499	203.706
10	0.6 (1)	0.3 (1)	0.75 (0)	205.011	202.129
11	0.2 (-1)	0.2 (0)	1 (1)	231.851	231.367
12	0.2 (-1)	0.2 (0)	0.5 (-1)	164.619	169.877
13	0.6 (1)	0.2 (0)	0.5 (-1)	191.811	192.295
14	0.6 (1)	0.2 (0)	1 (1)	229.827	224.569
15	0.4 (0)	0.3 (1)	0.5 (-1)	145.479	147.877

Table 4. Results of regression analysis of Box-Behnken design for amylase production by Pseudomonas stutzeri ML-18.

Variables	Coefficients	SE-statistics	t-statistics	P-value
Constant	203.706	5.06	40.257	0
Tryptone	3.905	3.099	1.26	0.263
KNO_3	-17.435	3.099	-5.627	0.002
Starch	23.441	3.099	7.565	0.001
Tryptone*Tryptone	10.344	4.561	2.268	0.073
KNO_3 * KNO_3	-3.802	4.561	-0.834	0.442
Starch * Starch	-9.522	4.561	-2.088	0.091
Tryptone * KNO_3	5.412	4.382	1.235	0.272
Tryptone * Starch	-7.304	4.382	-1.667	0.156
KNO_3* Starch	1.628	4.382	0.372	0.725

tryptone 0.2, KNO_3 0.1 and starch 1 were used in the medium.

In this study the RSM was used to obtain optimum values of the parameters and also to detect a good location in the design space. The same as our work Sumrin A, et al. (2011) had optimized the production of amylase from *Bacillus subtilis* 168. They had reported the optimal levels of the significant factors (yeast extract 8.4 g/l, sodium, chloride 8.1%, starch 2.55 g/l) and found the maximum amylase yield under optimal conditions (639.7 IU/ml)[19]. They had also suggested that starch as carbon source showed highly significant factor in amylase production by *Bacillus subtilis* 168. In our work, starch as carbon source also showed highly significant factor. Therefore, this result confirmed the suggestion of Sumrin A, et al. (2011) that concentration of starch has a significant effecting on amylase production[19].

As described by Sumrin A, et al. (2011) yeast extract as an Organic nitrogen sources produce high á-amylase yield as compared to inorganic nitrogen sources[19]. In compare with our work composition of organic (tryptone) and inorganic (KNO_3) nitrogen sources could facilitate the production of amylase by *Pseudomonas stutzeri* ML-18.

Therefore, determining of the optimized conditions for producing amylase with special features could be helpful for further study.

Verification of model

The final optimum composition of the three variables and amylase conditions were designed using the MINITAB software (version 15, PA, USA), which were found to be; tryptone 0.2, KNO_3 0.1 and starch 1. This model also predicted amylase activity of 248.784 U/ml. The high relation between predicted and observed values of the Box–Behnken explains the accuracy of the existence and the response model of an optimum point. Verified model of medium compositions were used and validated by experiments, and 243.614 U/ml of amyalse was produced from *Pseudomonas stutzeri* ML-18. High degree of the accuracy was revealed (97.92 %), which is an evidence for the model validation under the investigated circumstances. Result indicates reliability of model for production of maximal amylase by *Pseudomonas stutzeri* ML-18.

Using optimized medium in bioreactor:

After using verified model conditions in the fermentor, 245.612 U/mL amylase activities were achieved. The verification created a high level of the precision of the model (98.72%), which is an instrument for the model validation under the studied circumstances. Compared to the erlenmeyer flask, the fermentor produced more enzyme, which could be prior to the high level production of industrial enzymes.

CONCLUSION

Here we studied the isolation and identification of some Amylase producing bacteria from the kitchen waste water of a sweet shop. Among them the bacterium *Pseudomonas stutzeri* ML-18 produced high yield of amylase which was optimized the amylase production medium by using RSM. Medium optimization by RSM effectively enhanced amylase production. Thus, RSM as a kind of statistical approach could be design a suitable strategy for obtaining of key medium parameters for amylase production. High level of amylase production by *Pseudomonas stutzeri* ML-18 in fermentor shows high capacity of this bacterium, which could be applied in industrial processes.

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