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Optimizations of α-amylase production by response surface methodology in immobilization *Bacillus amyloliquefaciens* ATCC 23350

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Abstract

Introduction: Production of an endogenous α-amylase from *Bacillus amyloliquefaciens* ATCC 23350 was studied and enhanced.

Materials and methods**:** Protein and carbon sources were analyzed for free and immobilized bacterial cells and number of beads was considered for immobilized cells via one factor at a time methodforα-amylase production by *Bacillus amyloliquefaciens*. Subsequently, optimization condition was employed solely for immobilized bacterial cells by response surface methodology (RSM).

Results: Peptone and rice starch showed to improve the α-amylase production in immobilized *Bacillus* cells. RSM generated a mathematical model explaining the optimum concentration of the efficient nutrients (139.35 g/l of rice starch and 80.00 g/l of peptone) leading to an optimum amylase production (205 U/ml).

Discussion and conclusion: The statistical advance displayed significant outcomes to optimize the process parameters for maximal α-amylase production using *Bacillus amyloliquefaciens* and gave permission to rapid screening of variables. RSM led to find out an immense improvement in enzyme activity (more than 90%: from 25 to 225 U/ml) for the first time.

Key words: *Bacillus amyloliquefaciens*, Enzyme production, Fermentation, Immobilization, Response Surface Methodology

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Introduction

Some microorganisms use secreted enzymes such as α-amylase to convert starch to glycosyl residues in order to generate sufficient energy for their survival (Table 1).

Species of *Bacillus* is well known for their α-amylase production (5 & 6) and in recent years product optimization has received great attention (7 & 8). It has been demonstrated that physicochemical properties of the medium have crucial roles in cell density and α-amylase production (9 & 10). Bacterial immobilization as cell factory/reactor has been considered useful for the production of industrially-important enzymes (11). The microbial cell and enzyme entrapment in polymer matrices such as agar, alginate, carrageenan, cellulose and their derivatives has been used as an efficient immobilization technique. Amongst which, alginate gel proved to be more popular due to its simple manipulation steps being set at milder conditionsand non-toxic character (12). Accordingly, we have examined secreted α-amylase production from *Bacillus amyloliquefaciens* in free and immobilized conditions and optimized the culture conditions with low cost substrates via implementing statistical analyses.

Materials and methods

Fermentation medium: Homemade starch from rice, wheat, corn, green bean and potato was prepared from 500g of each plant. The plant samples were washed, peeled, smashed, suspended in 1l of distilled water and cooked at 100 °C for 30 min. The juice was filtered through muslin cloth under vacuum. Starch content of filtered juice was measured according to Thivend *et al*'s method (13) and 60 g/l was used in fermentation medium. Protein content was obtained according to Lowry's method (14) and adjusted with either of the peptone, urea, free fat cotton seed and soy bean powder to have 30 g/l protein within the fermentation medium. Therefore, fermentation medium contained 60 g/l carbon sources (glucose, rice starch, wheat starch, corn starch, green bean starch and potato starch, Merck starch), 30 g/l protein sources (peptone, urea, free fat cotton seed and soy bean powder), 1 g/l KH_2PO_4,0.1 g/l $CaCl_2$, 0.7 g/l NaCl and 0.3 g/l $MgSO_4$ (15).

Table 1- *α*-Amylase production of *Bacillus* in various media with or without starch supplement by submerge fermentation.

Bacillus species	Media with or without supplement	Yield (U/mL)	Reference
B. amyloliquefaciens	wheat bran and groundnut oil cake (1:1)	41.4	(1)
B. halodurans	soluble starch	1.8	(2)
B.amyloliquefaciens 321S	soluble starch	150	(3)
B. subtilis KCC103	Sugarcane bagasse hydrolysate	144.5	(4)

Microorganisms and seed culture: *Bacillus amyloliquefaciens*ATCC 23350 was obtained from Persian Type Culture Collection (Tehran, Iran). The seeding medium contained 15 g/l glucose, 2 g/l of each of meat extract, yeast extract and 0.5 g/l NaCl (15). The medium was seeded with a single *Bacillus amyloliquefaciens* cells and incubated at 37°C for 48 h with a constant shake at 180 rpm. Cell suspension culture from seeding medium (5% v/v) was transferred into fermentation media with three replicates and incubated for 18 h at 37°C (15).

***Bacillus amyloliquefaciens* Immobilization:** The cells of the *Bacillus amyloliquefaciens* were inoculated into 50 ml of theseed culture media. The polyvinyl alcohol (PVA) borate cell beads (100, 300 and 500 beads) were formed according to Zhao et al (16) and the beads were transferred into 50 ml of the fermentation media.

α-Amylase Assay: The supernatant were collected after incubation in fermentation medium. The cultures were centrifuged at 7656 ×g for 20 min and the supernatant was used for enzyme production assay according to Bernfeld (17). A reaction mixture containing 520µl of 4% (w/v) soluble starch in 0.02 M phosphate buffer (pH = 5), 80µl of sample or crude enzyme (for calibration via standard curve) and 400 µl of the 0.15 M phosphate citrate buffer (pH =5) was incubated for 10min at 44°C. The reaction was incubated for 10 min at 90 C. The amount of liberated reducing sugar was determined by the dinitrosalicylic (DNS) acid method (18). 2 mL of DNSA (3, 5-dinitrosalicylic acid) reagent was added to the mixture, and then kept for 5 min in boiling water bath. Amount of reduced suger released from starch degradation was estimated by measuring OD at 540 nm.

The assay was followed by “one factor at a time” method considering number of beads (100, 300 and 500), protein and carbon sources as stated above. For continuation of our study, the demonstrated maximum enzyme production at each specific condition (bead number, protein content and starch source) was considered.

Optimization of medium components by RSM: RSM using Composite Central Design (CCD) was used to establish the concentration of starch and protein sources for the maximum production of α-Amylase. The statistical software package Design-Expert 6.0 (Stat- Ease Inc, Minneapolis, USA) was used to produce a regression model for predicting the result of combined variables on α-amylase production.

Results

Effects of number of beads, nitrogen and carbon sources on the α-amylase production by "one factor at a time" method: Analysis of the number of bead (100, 300, and 500) showed that the highest α-amylase production was achieved at 300 beads in the following condition: 37°C, pH = 7.0 and 18 h of incubation in green bean starch (60 g/l) as the carbon source and cotton seed powder (30 g/l) as a nitrogen source on a rotary shaker. Conversely, the lowest production was achieved in free cell culture (Fig. 1).

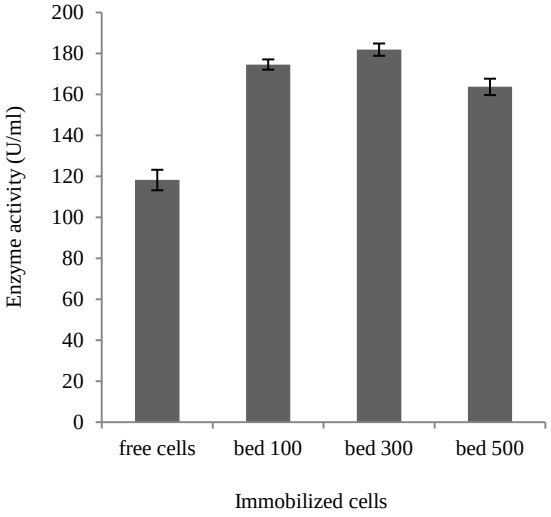

Fig 1- Effect of number of bed on α-amylase production

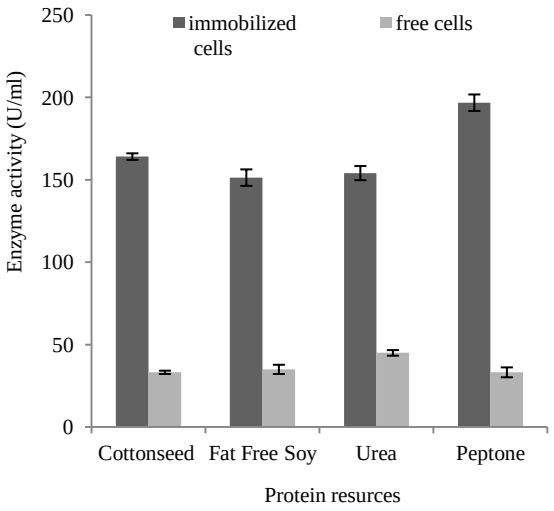

Fig 2- α-amylase production of the free and the immobilized cell on difference protein sources

By defining the optimum number of beads (*i.e*. 300), contribution of nitrogen sources was evaluated. The results showed that the lowest (33 U/ml) and highest (197 U/ml) activities were achieved in the presence of peptone in free and immobilized cells, respectively. In contrast, urea demonstrated to act in the opposite direction to the peptone (Fig. 2). At this stage, peptone (30 g/l) was chosen amongst tested nitrogen sources.

As the final step carbon sources were analyzed for α-amylase production in the availability of 300 beads and 30 g/l peptone. Free and immobilized cells responded differently to glucose and rice starch. In free cell condition, glucose demonstrated the highest activity (136 U/ml). Whereas rice starch showed to be the most efficient carbon source (170 U/ml), once the cells were immobilized (Fig. 3).

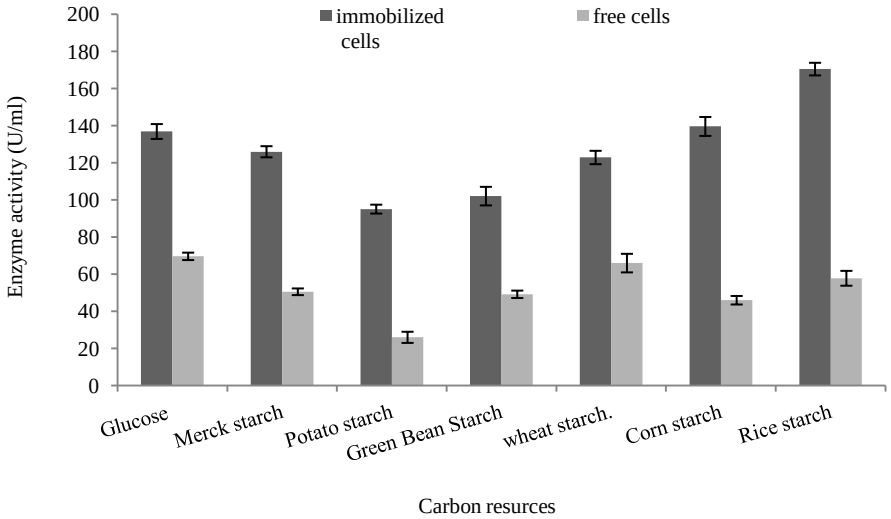

Fig 3- α-amylase production of the free and the immobilized cell on difference carbon resources

Optimization by RSM: By defining the suitable carbon and nitrogen sources and number of beads, RSM was used to define the optimum concentrations of rice starch and peptone and their interactions. Accordingly, the high and low values of selected nitrogen and carbon sources were defined for Design-Expert 6.0. The software package introduced more coded values (Table 2) and RSM analysis was performed to generate an equation describing the α-amylase production, considering ranges of rice starch and peptone.

RSM followed by multivariate regression analysis, resulted the following equation:

$$Y_1=+124.47901-\ 0.40919\ \times C-\ 1.51742\ \times N+0.030473\ \times C\ \times N+3.24755E^{-003}\times C^2+9.54545E-003\ \times N^2-\ 1.31590E^{-004}\times C^2\times N \quad (R^2 = 0.990371,\ CV = 4.06,\ P < 0.05)$$

Where, Y_1 is the presented α-amylase production, C and N correspond to starch and peptone respectively. Further analysis revealed that the fitted model well explain the α-amylase production. In this regression model, the interaction between carbon and nitrogen sources (C × N) and N^2 were not statistically significant ($P < 0.05$, Table 3).

Table 2- Results of Face Centered Central Composite Design (FCCCD) using two variables demonstrating observed and predicted responses

	Rice starch (g/l)	Peptone (g/l)	α-Amylase activity (U/ml)	
Run	(C)	(N)	Observed	Predicted
1	1 (200)	1 (80)	178.72	179.54
2	0 (125)	0 (55)	165.91	170.45
3	1 (200)	-1 (30)	160.54	161.36
4	1.4 (231.1)	0 (55)	149.59	147.73
5	-1 (50)	1 (80)	147.41	143.18
6	0 (125)	-1.4 (19.65)	132.38	134.10
7	-1 (50)	-1 (30)	111.04	106.82
8	0 (125)	0 (55)	160.54	161.36
9	-1.4 (18.9)	0 (55)	92.45	97.72
10	0 (125)	1.4 (90.35)	223.29	225.00

Table 3- Model coefficients assessed by multivariate regression and significance of regression coefficient for α-amylase.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F	
Model	11886.5	6	1981.084	51.42863	0.0041	significant
C	3265.119	1	3265.119	84.76197	0.0027	
N	4132.231	1	4132.231	107.2721	0.0019	
CN	82.64463	1	82.64463	2.145442	0.2392	
C^2	2302.612	1	2302.612	59.77545	0.0045	
N^2	162.7066	1	162.7066	4.223838	0.1321	
C^2N	684.8592	1	684.8592	17.77884	0.0244	
Residual	115.5631	3	38.52103			
Lack of Fit	74.24079	2	37.12039	0.898314	0.5980	not significant
Pure Error	41.32231	1	41.32231			
Core Total	12002.07	9				

The model predicts that the maximum α-amylase activity (that is up to 205.51 U/ml) could be gained once 139.35 g/l of rice starch and 80.00 g/l of peptone were included in production medium. Table 3 demonstrates that the nitrogen source has the highest effect on enzyme production. The presented data in Table 2 shows with constant amount of C source, increasing the amount of N source would cause a significant incline in enzyme production. This boost was higher when the carbon source is close to its optimum level (*i.e.*139.35 g/l and here and within table is 125 g/l, Table 2). The lowest N source effect was noted where the C source was in highest value (200 g/l, Table 2). On the other hand, the increase in C source up to 160 g/l would increase the enzyme production (Fig. 4).

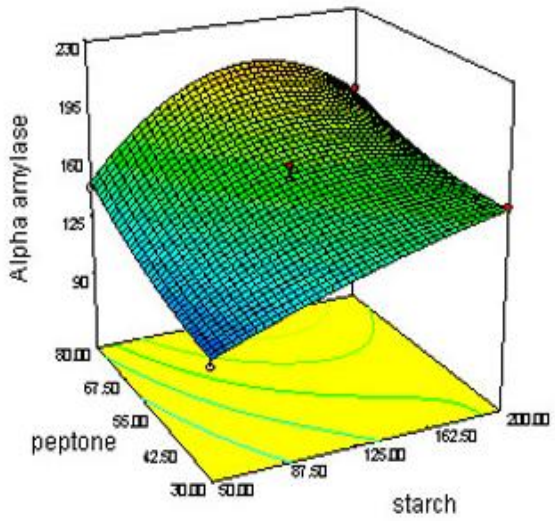

Fig 4- RSM for α-Amylase activity (U/ml) by *Bacillus amyloliquefaciens*

Verification of optimum conditions: In order to check the efficacy of model in prediction of enzyme production, the proposed optimum C and N source values were considered for α-amylase production. Results were illustrative of insignificant difference between the predicted (205.51 U/ml) and observed (207.45 U/ml) values (Fig. 5).

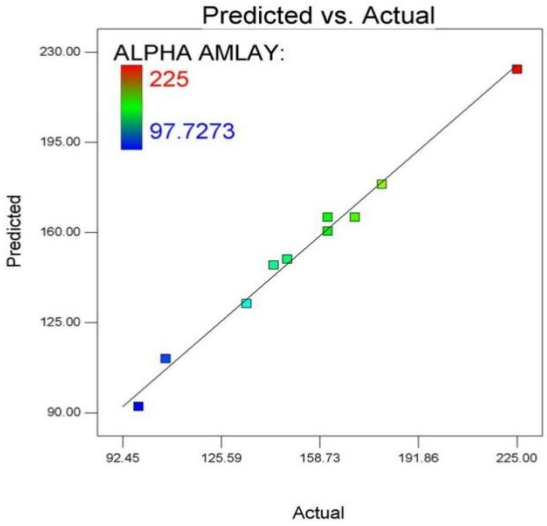

Fig. 5- The observed production of α-amylase versus the predicted production of α-amylase under the experimental conditions.

Discussion and conclusion

Enzyme biotechnology has many applications in industry, including but not limited to food science. Amongst useful enzymes, α-amylases have shown to be important enzymes in conversion of poly- and oligosaccharides into reducing sugars that can be used as an additive or energy source in other dependent industries. The great emphasis has put on α-amylases, isolated or secreted from varieties of *Bacillus* species (Table 1). In any case, the native enzymes need to be functionally characterized and the optimum conditions for their production to be determined. Moreover, enzyme production may be improved via ranges of physical, chemical and biological techniques. On the same

note, physical changes of an α-amylase secreted from *Bacillus amyloliquefaciens* was considered through immobilization of the bacterial cell using alginate + polyvinyl alcohol and compared with free cells. Moreover, the enzyme production was optimized considering varieties of nitrogen and carbon sources implementing the statistical analyses techniques including "one-factor at a time" and RSM.

Earlier studies are indicative that bacterial cell immobilization can improve α-amylase activity up to 2 fold (19). In these studies varieties of immobilization factors such as channeled porous alumina (20), carrageenan gel (21) calcium alginate (22), alginate/agar [19] and macro-reticular anionic exchange resin (23 & 24) were used. Similar improvement was achieved once alginate + polyvinyl alcohol was used, but this time the improvement was even much higher and reached to 9 fold higher than free cell condition.

Although glucose and lactose are being considered as simple sugars, they appear to be expensive to use. As a result soluble starch (1-2% w/v) is the common carbon source in culture media (25 & 26). Interestingly, the soluble or extracted starch from agricultural products such as white corn (26), barley pearl millet, rice and wheat (26) has demonstrated promising α-amylase production once incorporated in bacterial cell culture. Amongst carbon sources that were used, rice starch showed an improvement in the α-amylase production in immobilized *Bacillus* cells that supports the earlier findings. Similar to earlier results (27 & 28), in free cell condition use of glucose improved the enzyme production. Interestingly, in *Bacillus subtilis* ATCC-21556 in the presence of glucose, α-amylase production was improved in immobilized cells while the production repressed in free bacterial cell system due to negative effect of glucose on the enzyme in this latter condition (28), similar to our findings.

The common nitrogen source in bacterial culture medium is either of the following peptide derivatives including peptone (23), beef extract (29) and triptone (22) that are capable to improve the α-amylase production or can be used as the enzyme inducer (30, 8 & 31).

In almost all earlier studies, peptone appeared to be the most prominent nitrogen source for α-amylase production. Since peptone provides soluble amino acids necessary for bacterial growth and survival, therefore providing a suitable milieu for enzyme production and production was included but was not limited to α-amylase (32). In our study, peptone appeared to improve enzyme production in immobilized cells.

Application of RSM has proven to provide solid data in terms of enzyme product optimization, being less labor intensive and time saving (8). In our study, RSM led to find out the proper nitrogen and carbon level in α-amylase production and enzyme response to changes in provided conditions. Above 160 g/l rice starch over constant nitrogen level, enzyme production reduced significantly similar to Gangadharan et al. 2008[8]. It was found that high starch concentration had an

inhibitory effect on α-Amylase production. Earlier results were indicative that the enzyme tolerance upon the increase of the substrate was in the ranges of 1-12.5 percent of starch (8 & 33). However, our data shows a tolerance up to 16% that is enormously higher than the earlier results. This can be considered as an advantage in food industry that addition of more enzymes would be less required in the availability of higher substrate, *i.e*., starch in this case. To verify the model acceptability, α-amylase production was checked using the calculated optimal culture condition (starch 139.35 g/l, peptone 80.00 g/l). The results demonstrated that the highest α-amylase production was achieved after 18 h of immobilized cell fermentation. The mean value of α-amylase was 207.45, which best complied with the predicted value (205.51 U/ml). Considering earlier studies (34) the difference between observed and predicted values was minimal (Fig. 5). Accordingly, it can be said that the model was trustworthy for forecasting the production of α-amylase by immobilized *Bacillus amyloliquefaciens.*

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بهینه‌سازی تولید آلفا آمیلاز از گونه تثبیت شده *باسیلوس آمیلوفاسینس* ATCC 23350

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چکیده

مقدمه: در این پژوهش تولید آلفا امیلاز از گونه باکتری *باسیلوس آمیلوفاسینس* ATCC23350 بررسی و بهینه شد.

مواد و روش‌ها: منابع کربنی، پروتئینی و تعداد سلول‌های تثبیت شده در تولید آلفا امیلاز به روش یک آزمون در یک مرحله (One factor at a time) بررسی شد. در مرحله بعد تولید آنزیم از سلول‌های تثبیت شده به روش آماری سطح پاسخ (Response surface methodology) بهینه‌سازی شد.

نتایج: فعالیت آنزیم آلفا آمیلاز از سلول‌های تثبیت شده *باسیلوس* در محیط کشت پپتن و نشاسته برنج افزایش یافت. با بهینه‌سازی به روش سطح پاسخ یک مدل ریاضی به‌دست آمد که نشان داد در سطح ۱۳۹/۳۵ گرم در لیتر نشاسته برنج و ۸۰ گرم در لیتر پپتن بهینه فعالیت آلفا آمیلاز ۲۰۵ واحد در میلی‌لیتر به‌دست آمد.

بحث و نتیجه‌گیری: مدل آماری نتیجه در خور توجهی در بهینه‌سازی عوامل در تولید حداکثری آنزیم آلفا آمیلاز از گونه *Bacillus amyloliquefaciens* داشت و نشان داد که حداکثر متغییرها در حداقل آزمون با این روش آماری قابل انجام است. بهینه‌سازی باعث افزایش در خور توجه در فعالیت آنزیمی داشت؛ به طوری‌که فعالیت آنزیمی برای نخستین بار نسبت به حالت پایه ۹۰ درصد و از ۲۵ به ۲۲۵ واحد در میلی‌لیتر محیط کشت افزایش یافت.

واژه‌های کلیدی: *باسیلوس آمیلولیکویی* فاشینز، فعالیت آنزیمی، تثبیت، محیط کشت، سطح پاسخ

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