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Screening and identification of a novel lipase producing *Aspergillus niger* Yz15 and optimization for lipase production

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ABSTRACT

A lipolytic fungus named Yz15 which produced extracellular lipase was isolated from the water and soil samples contaminated with oil. The 18S rRNA sequencing revealed it as a novel strain of Aspergillus niger. Lipases was particularly important due to the fact that they were used for different industrial processing. So in this study we carried out optimization of lipase production from Aspergillus niger Yz15 using statistical designs. The important factors influencing the lipase activity were identified by initial screening method of Plackett-Burman experimental design, which showed that olive oil, magnesium sulfate and initial pH were the significant variables ($P<0.05$). Box-Behnken design and response surface analysis were adopted to the further optimization. A maximum lipase activity of 4.55U/mL was obtained at 1.54% olive oil, 0.03% magnesium sulfate and initial pH 6.17. The the second order quadratic model for lipase production was capable of predicting the pratical fermentation with high determination coefficient (R^2) values of 0.9842. The average lipase production was observed to increase by about 67% using these statistical techniques of optimization of media and culture conditions.

Keywords: *Aspergillus niger* Yz15; lipase; Placket-Burman experimental design; Response surface methodology; Box-Behnken design

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INTRODUCTION

Lipases are special hydrolytic enzymes that catalyze the triacylglycerol into mono- and di-acyl glycerols, fatty acids and glycerol [1, 2]. This reaction is reversible and the enzyme also catalyses the synthesis of esters and transestrification in the oil water interface. Their ability to perform unique chemo-, region-, enantioselectivity has made them increasingly popular in industries , such as food processing, production of surfactants and fine chemicals [2, 3] .

Lipases are widely distributed in animals, plants and microorganisms [4, 5]. In particular, the lipase by microorganisms is very important because of their potential functions. Fungi [6, 7, 8] are regarded as the best lipases producers. Gwen Falony1 et al. [9] reported the mean lipolytic activity reached by fungi was about 5.4 and 2.8 times higher than the test bacterium and yeast. And *Aspergillus niger* showed more lipase activity among the strain tested. Interest in the immense potential of the *A. niger* lipase had increased.

The objective of the study was to isolate and identified a novel fungus from the water and soil samples contaminated with oil which produces extracellular lipase constitutively. An attempt was made to optimize medium constituents and culture conditions to increase the production of lipase by statistical method. Initially a Plackett-Burman design (PBD) was used to evaluate the effects of the culture medium components and the culture conditions on lipase sythensis. Finally, a Box-Behnken design (BBD) and response surface methodology (RSM) are performed for determining the relationships between the variables and the response, generally resulting in higher yields.

EXPERIMENTAL SECTION

Sample collection

Twenty-five samples were collected from the sewage and soil contaminated with oil near the dining rooms in Lanzhou University of Technology, and from the water and soil near the oil refineries.

Medium

Enrichment medium contained 200g potato and 20g glucose in the final volume of 1 L of distilled water. Rhodamine B agar medium contained 2.0g olive oil, 5g agar, 0.2g K_2HPO_4, 0.2g KH_2PO_4, 0.1g $MgSO_4.7H_2O$, 0.4g $(NH_4)_2S0_4$ and 1mL of Rhodamine B solution (0.001mol/L) were added. The original fermentation medium was composed of 0.5% peptone, 1% sucrose, 1% olive oil, 0.5% $(NH_4)_2S0_4$, 0.04% and 0.2% KH_2PO_4 per 100 milliliter of distilled water.

Screening of lipase producer

The water samples were diluted with sterile distilled water about 10-fold, then 5 mL of diluent was dissolved in 150 mL of enrichment medium and incubated at 45℃ on a rotary shaker at 150rpm for 3d. The 5.0g of soil sample was dispersed with 10 mL of sterile distilled water, then 1 mL of diluent was added to the 20 mL of enrichment medium which was incubated for 3d at 30℃. Then 1 mL of culture broth was diluted (10^{-2}, 10^{-6}, 10^{-7}) and the diluent were used for daubing to the Rhodamine B agar plates and incubated at 30℃ for 2-3d. Plates were irradiated with UV light 365 nm. The colonies were isolated according to orange fluorescent halo indicating the lipase production. The colonies with clear hydrolysis zones were collected and stored in PDA medium at 4℃.

Cloning and sequencing of the 18S rRNA

The 18S rRNA gene was amplified by polymerase chain reaction (PCR) using primers of NS1 (GTAGTCATATGCTTGTCTC) and NS8 (TCCTCCGCTTATTGATATGC). This work was performed by Inovogen Tech. Co.. The 18S rRNA gene sequence of the Yz15 was compared with those in the NCBI by using the BLAST program.

Lipase activity assay

Lipase activity was estimated with olive oil emulsion by the procedure of Gui Gao [10]. The olive oil emulsion was prepared by homogenizing 50 mL of olive oil and 100 mL of 4% polyvinyl alcohol solution in 0.04 mol/L of glycine-sodium hydroxide buffer solution, and the mixture was cooled before emulsification. The reaction mixture composed of 5 mL of olive oil emulsion and 4 mL of glycine-sodium hydroxide buffer solution of pH 9.4 was first incubated at 37℃ for 5 min, then 1ml of enzyme solution was added for an extra 10 min. The emulsion was destroyed by adding 15 ml of ethanol immediately after incubation, and then was diluted with 15 ml of distilled water. The liberated fatty acid was titrated against 0.05 mol/L of NaOH solution. One unit (U) of lipase activity was defined as 1 μmol of free fatty acid liberated per ml of enzyme per minute at 37℃.

Batch fermentation

A strain isolated as the methods mentioned above was first cultured in PDA medium to induce spore formation for 4 days, the fermentation medium was inoculated with a spore suspension of the strain in a 250 mL Erlenmeyer flask containing 100 mL fermentation medium, and then incubated at 28℃ with shaking at 150rpm for 60h.

Table 1 Eight independent variables and their levels used in PBD

Variable code	Variable	Low level	High level
A	Peptone (%)	0.5	0.75
B	Sucrose (%)	1	1.5
C	Dummy Ⅰ	-1	1
D	Olive oil (%)	1	1.5
E	Ammonium chloride (%)	0.3	0.45
F	Dummy Ⅱ	-1	1
G	Magnesium sulfate (%)	0.04	0.06
H	Dipotassium hydrogen Phosphate (%)	0.2	0.3
I	Dummy Ⅲ	-1	1
J	Initial pH	6	9
K	Shaking speed (r/min)	120	180

Optimization the components and conditions of fermentation by PBD

PBD was used to investigate and evaluate the effect of medium components and culture conditions [11]. This is a fraction of a two-level factorial design and allows the investigation of 'n-1' variables with at least 'n' experiments

[12]. Eight factors were screened in 12 combinations with three dummy variables which provide an adequate estimate of the error and all the trials were performed in duplicate. Among the nutrients, nitrogen and carbon sources were tested for their significance in lipase activity and the levels of temperature, initial pH, shaking speed were determined by changing one factor at a time while keeping the others constant. Each independent variables were tested at two levels, a high (+1) level and a low (-1) level (Table 1).

Optimization the components and conditions of fermentation by RSM

The best reaction conditions for lipase activity were established via RSM, an incomplete design of three-level and three-factor factorial with three central points were used, in which experimental points have been specially selected to allow an efficient estimation of coefficients in quadratic models [13]. The independent variables of the factors studied were olive oil (%, A), $MgSO_4 \cdot 7H_2O$ (%, B) and initial pH (C). The response (dependent variable) was lipase activity (U/mL). The setting of factors was determined in accordance with aforementioned method. The actual values of variables and the design matrix were shown in table 2 and table 3, respectively. The response surface model was confirmed by software Design-Expert 8.0.6.

Table 2 Levels of variables used for lipase activity optimization used in the RSM

Variable code	Variable	Coded level and actual value		
		-1	0	1
A (%)	Olive oil	1.0	1.5	2.0
B (%)	$MgSO_4 \cdot 7H_2O$	0.02	0.03	0.04
C	Initial pH	5	6	7

Table 3 Matrix and the results of BBD

Run	Variable and levels			
	A	B	C	Y (U/mL)
1	-1	0	1	2.80
2	0	0	0	4.55
3	-1	-1	0	2.014
4	0	-1	1	3.55
5	-1	1	0	1.875
6	1	1	0	3.25
7	0	-1	-1	2.25
8	0	0	0	4.48
9	0	1	-1	3.15
10	0	1	1	2.92
11	1	0	1	2.5
12	-1	0	-1	1.53
13	1	-1	0	2.08
14	1	0	-1	2.37
15	0	0	0	4.35

RESULTS AND DISCUSSION

Screening and isolation of lipase producer

A total of 35 isolates was initially obtained from samples with enrichment cultures, and 15 fungi showed obvious zone of orange fluorescent halo of lipase production on Rhodamine B agar plates. Among all of the isolates, the fungus of Yz15 showed the highest lipase activity.

The colonies of the strain Yz15 were up to 9 cm in diameter for 5 - 7 d at 28 ℃ on a PDA plates. The colony was light yellow in the early stage, and then became black in the later period, which indicated a large of spores were formed. Under the microscope, the heads of conidia were round and 200 μm in diameter, the top of sporangia were 20 -30 μm in diameter.

The phylogenetic tree (Figure 1) based on 18S rRNA gene sequences clearly indicated that the strain Yz15 was related to members of the *Aspergillus sp*. The 18S rRNA gene sequence of the strain Yz15 exhibited the highest homology to the sequence of *Aspergillus niger strain* CBS 513.88 clone F-wpysw4 in GenBank. This sequence date had been submitted to the GenBank databases under accession No.JX536387.1.

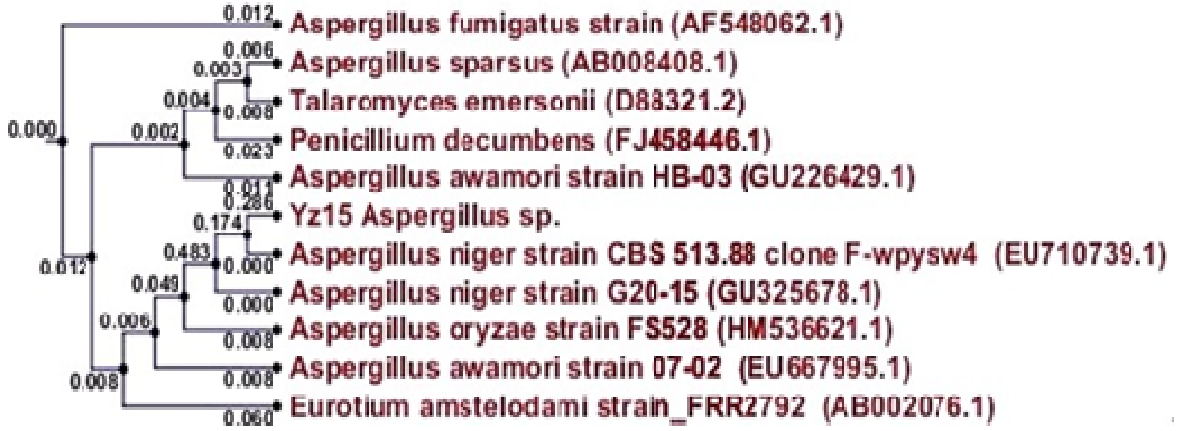

Figure 1 The phylogenetic tree resulting from analysis of the 18S rRNA sequences of Yz15

Optimization of the components and conditions of fermentation by PBD

The tests of PBD were performed with Minitab statistical software 16.1.0 (Minitab ® Statistical Software). Table 4 shows olive oil, magnesium sulfate and initial pH were the most significant factors affecting the production of lipase enzyme. Other variables had low confidence levels (P>0. 05) and were considered insignificant.

On the analysis of regression coefficient of variables, olive oil showed a positive effect for lipase activity, this revealed olive oil was essential for the production of lipase. It may be explained by the fact that lipases are inducible enzymes and are thus generally produced in the presence of a lipid source such as triacylglycerols, fatty acid. Veronica Leticia Colin et al [14] evidenced the maximum lipase activity was obtained from olive oil as an inducer by *Aspergillus niger* MYA 135. many similar studies for the production of extracellular lipases was obtained by other strains, which contained *Bacillus Sp* [15], *P. Camembert* [16], *Candida rugosa* [12]. In the present study, sucrose was found to have a confidence level of 91.2%, but the presence of sucrose in the cultivation medium depressed the production of lipase compared to olive oil. It is due to catabolic repression. In addition, Mobarak-Qamsari E, et al [17] reported the inhibition of lipase production by glucose, a similar result was obtained by Dalmau, J. et al[18].

Table 4 ANOVA for Plackett-Burman experimental design

Term	Effect	Coefficient	T- values	P-values
A	0.544	0.272	1.77	0.175
B	-0.769	-0.385	-2.50	0.088
D	1.077	0.539	3.50	0.039
E	-0.344	-0.172	-1.12	0.345
G	-4.344	-2.172	-14.13	0.001
H	0.889	0.445	2.89	0.063
J	-1.648	-0.824	-5.36	0.013
K	-0.539	-0.270	-1.75	0.178

A-Peptone B-sucrose D-olive oil E-Ammonium chloride G-Magnesium sulfate H-Dipotassium hydrogen phosphate J- Initial pH K-shaking speed

The variable MgSO4• 7H_2O had a negative effect on the production of *A. niger* lipase. Thus it was important to maintain lower concentration of MgSO4• 7H2O because an excess of MgSO4• 7H2O resulted in decreasing in lipase synthesis. Different behaviors were observed in effect of metallic icons on lipase production by *A. niger*. Fabiao Jares Contesini [2] reported Mg, Ca, Na, Mn, had no significant effect on the production of lipase by solid-state fermentation. While the results Ca, Fe substantially increased extracellular lipase production was obtained by Veronica Leticia Colin [14]. In short, the addition of micronutrients was essential in all the lipase fermentation process. Increasing the value of initial pH had a negative effect on lipase production. It seemed that an alkaline pH value was unappropriate for lipase production with the Aspergillus niger strain Yz15. Thus initial pH (5-7) was taken into consideration for the further optimization. In case of fungi, the majority of researchers has reported an acidic pH to be the most appropriate for enzyme production [19].

Box-Behnken design (BBD) and Response surface method

The results obtained with the method of BBD were presented in table 3. The levels of the variables for the BBD experiments were according to the results of the PB experiments. Regression analysis was performed to fit the response function with the experiment data [20]. The model can be explained by the following second-order polynomial equation:

$$Y (U) = 4.46 + 0.25A + 0.16B + 0.31C + 0.33AB - 0.28AC - 0.38BC - 1.41A^2 - 0.74B^2 - 0.75C^2 \quad (1)$$

Where Y is the response variable (lipase activity) and A, B and C are the coded values of olive oil, magnesium sulfate and initial pH. The goodness of the model can be checked by several parameters. The coefficient of determination, R^2 provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions [21]. The closer the R square value was to 1, the stronger the model was and the better it predicts the response. In the case, the R square implies that the sample variation of 98.42% for lipase enzyme production was attributed to the independent variables. The R^2 value also indicated that only 1.58% of the total variation was not explained by the model. The lack of fit was greater than 0.05 and indicated that the model was considered an adequate approximation of the true surface. In addition, the value of the adjusted R square (0.9558) was very high which advocates a high significance of the model. The corresponding analysis of variance (ANOVA) was shown in Table 5. The ANOVA

Table 5 ANOVA for the Box-Behnken design

Source	DF	Sum of squares	Mean square	F-value	P-value
Model	9	12.98	1.44	34.65	0.0006
A	1	0.49	0.49	11.79	0.0186
B	1	0.21	0.21	5.08	0.0738
C	1	0.76	0.76	18.33	0.0079
AB	1	0.43	0.43	10.29	0.0238
AC	1	0.32	0.32	7.81	0.0383
BC	1	0.59	0.59	14.06	0.0133
A^2	1	7.36	7.36	176.74	0.0001
B^2	1	2.04	2.04	49.72	0.0009
C^2	1	2.07	2.07	49.72	0.0009
Residual	5	0.21	0.042		
Lack of fit	3	0.19	0.062	6.07	0.1448
Pure error	2	0.021	0.010		
Total	14	13.19			

R-Squared 0.9842 C.V.% 7.01 Adeq Precisior 18.019 Adj R-Squared 0.9558

of the regression model demonstrated that the model was highly significant, as was evident from a very low P value ($P<0.001$). The significance of each variable was also determined by P value. A P value less than 0.05 indicated model terms were significant. This implied that A, C, AB, AC, BC, A^2, B^2, C^2 had a considerable influence on lipase enzyme production.

The response surface curves and its corresponding contour curves described by the regression models were illustrated in Figs. 2-4. Three-dimensional response surface plots graphically represented regression equations and were generally used to demonstrate relationships between the response and experimental levels of each variable [22]. The shape of the corresponding contour plots indicates whether the mutual interactions between the independent variables are significant or not [23].

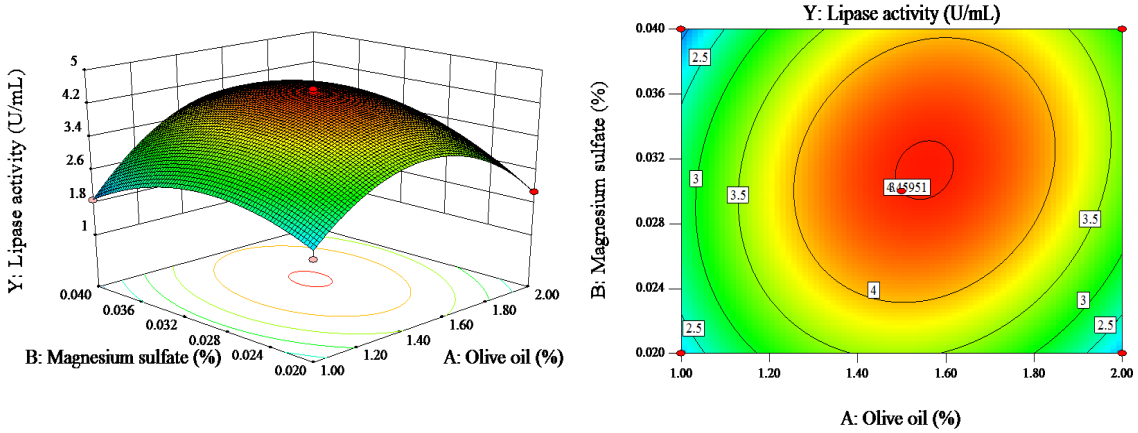

Fig. 2 the contour plot and response surface for lipase activity as a function of olive oil and magnesium sulfate

Figure 2 represented the combined effect of concentrations of olive oil (A) and magnesium sulfate (B) for the lipase activity by keeping the initial pH (C) as constant. It showed an increase in olive oil and magnesium sulfate resulted in an increase in lipase production, but after a certain point, a further increase in the concentrations of olive oil and magnesium sulfate resulted in a low lipase production. It also can be seen that, the lipase activity was sensitive when

the value of olive oil was increased in the range from 1% to 1.5%. But it sharply decreased beyond this level. So olive oil may play an important role in the process of fermentation. Figure 3 showed the effects of the concentration of magnesium sulfate (B) and initial pH (C) for the production of lipase, while olive oil was kept at an optimal level. The lipase activity increased up to a certain level with increasing in the concentration of magnesium sulfate and initial pH, then decreased with a further increase. It was obvious that there was a significant interaction between the concentration of magnesium sulfate and initial pH. Figure 4 showed the concentration of olive oil (A) and initial pH (C) affected on the lipase production at a concentration of 0.03% of magnesium sulfate.

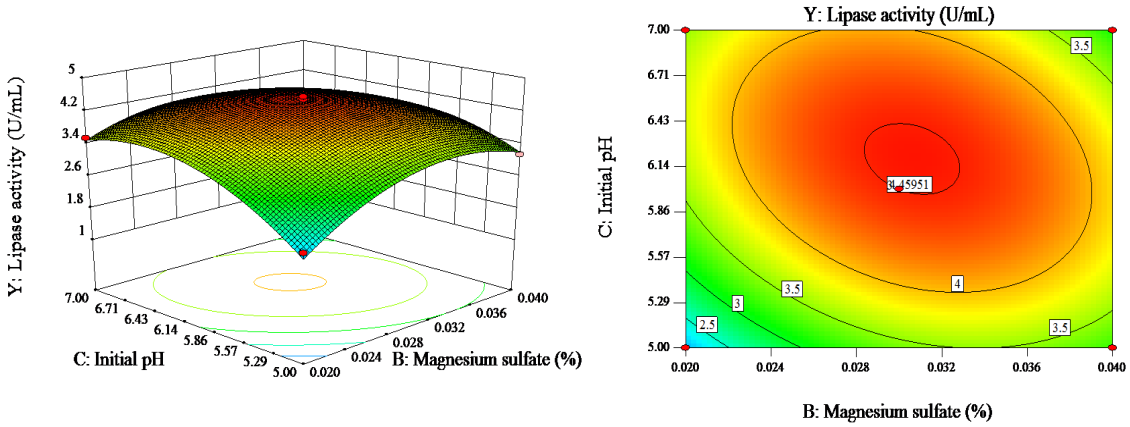

Fig. 3 the contour plot and response surface for lipase activity as a function of initial pH and magnesium sulfate

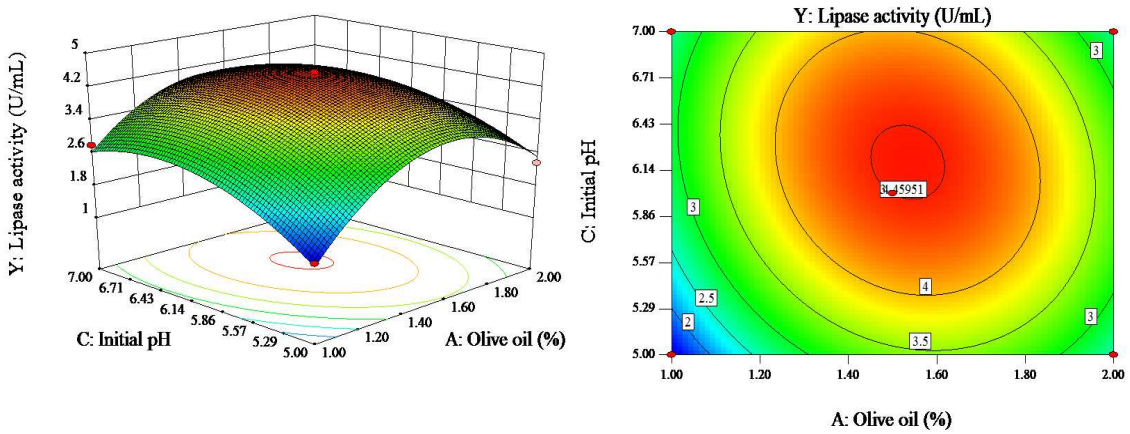

Fig.4 the contour plot and response surface for lipase activity as a function of initial pH and olive oil

Comparison of optimal results

A graphical multi-factor optimization technique was adopted to determine the optimum conditions for the production of lipase. In a comparative analysis for maximizing lipase production by A. niger Yz15, 67% increased lipase activity was observed in media supplemented with olive oil (1.54%), magnesium sulfate (0.03%) and initial pH 6.17.

There were several reports of lipase production by A. niger under solid state fermentation [2,9], lipase activity was improved significantly by statistical method. However, the solid state fermentation had some limitations such as the choice of microorganisms capable of growth under restricted conditions, and monitoring and controlling of parameters such as temperature, pH, humidity and air flow. In addition, industrial important enzyme had been obtained from submerged culture because of ease of handling environmental factors, such as temperature and pH.

Validation of the experimental model

In order to verify the optimal results and to validate the second order quadratic model, three replicate fermentation of the production of lipase was performed under optimal conditions. The maximum lipase activity of 4.55U/mL was obtained, which was close to the predicted value (4.5029U/mL). This discrepancy might be due to the slight variation in experimental conditions.

CONCLUSION

In this work, we reported a novel strain named Aspergillus niger Yz15 which constitutively produced extracellular lipase. Statistic methods were successfully applied to the lipase production and was proved to be a powerful tool for the optimization of the medium components and culture conditions. The optimal medium components were obtained using the following conditions: 0.63%peptone, 0.37% ammonium chloride, 1%sucrose, 1.54% olive oil, 0.03% magnesium sulfate and 0.2% dipotassium hydrogen phosphate. The optimal culture conditions was obtained at fermental temperature 28℃, fermental time 60h, shaking speed 150r/min and initial pH 6.17. The present study contributed towards the industrial process in terms of process optimization to enhance yields with minimal input in the batch cultures and conditions.

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