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Bioprocess technology for LasB protease (elastase) production from *Pseudomonas aeruginosa* MCCB 123

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LasB protease, also known as the elastase enzyme was purified from an environmental isolate of *Pseudomonas aeruginosa* MCCB 123. The enzyme production was optimized by response surface methodology (RSM) which yielded 24754.17 U/mL protease activity and 654.14 U/ml elastase activity. The yields were 1.83 fold and 2.39 fold higher for protease and elastase activities, respectively. Scale up study of enzyme production was carried in a 5 L bioreactor. Bioprocess technology for enzyme production was successfully developed.

Keywords: *Pseudomonas aeruginosa*, LasB protease, elastase, optimization

Introduction

Elastase has been defined as a proteinase that is capable of solubilizes and degrades insoluble elastin fibers[1]. During the reaction in which elastase breaks down the substrate elastin, two phases occur: a burst phase during which the amino side of the peptide bond is released, and a steady-state phase, in which the acyl side of the substrate is released. The elastin is positioned on the enzyme elastase so that the catalytic triad has access to the peptide bond. After this occurs, serine nucleophilically attacks the carbonyl of the peptide bond. A tetrahedral intermediate is formed and decomposed. Water enters the active site and attacks the reaction, causing the nitrogen terminus to leave. Water then attacks the acyl-enzyme intermediate and causes the release of the carboxylic acid component[2]. LasB protease, which is known as the elastase of *Pseudomonas aeruginosa* is a 33 kDa zinc metalloendopeptidase. Encoded by lasB, elastase is synthesized as a pre proenzyme (53.4 kDa) with a classical signal peptide and a covalently linked 18 kDa amino-terminal propeptide. The 2.4 kDa signal sequence is removed upon passage through the inner membrane into the periplasm, where the propeptide is rapidly cleaved off by autoproteolysis[3].

Bacterial elastase is identified as an enzyme of great biotechnological significance. Elastase from *Lactobacillus fermentii* is reported to as drug molecule in the treatment of Alzheimer's disease[2]. Elastase of *Pseudomonas aeruginosa* is identified to have potential application as depilating protease with potential application in the leather industry[4-5] and in the deproteinization of shrimp shell waste[5].

However, a few reports exist on the purification, characterization and optimization process on bacterial elastases[1,2,6-9]. Development of bioprocess technology is essential for the industrial survival of an enzyme. Therefore, the present work focuses on bioprocess optimization of LasB protease (elastase) from *Pseudomonas aeruginosa* MCCB 123, an organism isolated from a coir retting ground located at Chellanum North, Kerala, India. The enzyme production was optimized in terms of protease activity, since protease activity of *Pseudomonas aeruginosa* far exceeds its elastase activity[10].

Materials and Methods

Molecular Characterization of Isolate

Total genomic DNA was extracted by phenol - chloroform method[11]. The bacterium was identified by 16S rRNA gene sequence analysis. PCR amplification and sequencing of 16S rRNA gene was carried out according to Reddy *et al* 2000[12] in a 25 µL reaction mixture using universal primers 16 S1 (GAG TTT GAT CCT GGC TCA) and 16 S2 (ACG GCT ACC TTG TTA CGA CTT) with the following conditions: initial denaturation at 95°C for 5 min followed by 34 cycles of denaturation at 94°C for 20s,

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annealing at 58°C for 30s and extension at 68°C for 2 min followed by a final extension at 68°C for 10 min. The amplification was performed using DNA Thermal cycler (Eppendorf). The amplified 16S rRNA gene (1500 bp) was separated on 1% agarose gel and was then purified using QIAEX II gel purification kit (Qiagen) and cloned into pGEM-T Easy Vector (Promega, USA). The PCR products were sequenced using primer walking service of Microsynth AG, Switzerland. The sequences obtained were matched with the GenBank database using basic local alignment search tool (BLAST) algorithm and the ribosomal database project (RDP) release 9[13-14].

Enzyme Production

For enzyme production, the isolate was grown in a medium composed of (g/l): glucose, 10; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 0.2; yeast extract, 0.1, casein 10, pH 7.0. Erlenmeyer flasks containing 50 mL of medium were inoculated with 1% v/v of 18 hold culture (O.D. 0.1 at Abs_{600}). The flasks were incubated at 28°C for 48 h. Uninoculated media served as control. The cultures were harvested by centrifugation at 12,000 rpm for 15 min and the cell free supernatant was used for estimating the extracellular enzyme activity.

Elastase Assay

Elastase activity was determined according to Kessler *et al*[15]. One mL of enzyme solution, 5 mg elastin Congo red and 1 ml 50 mM Tris-Cl buffer (pH 8) were combined and incubated for 3 h at 40ºC. Control was maintained without the addition of enzyme solution. The reaction was stopped by the addition of 0.1 mL of 100 mM EDTA. After the termination of the reaction, the mixture was centrifuged at 12,000 rpm for 15 min at 4ºC. The amino acids released during the degradation of elastin were determined by measuring the absorbance at 495 nm.

Protease Assay

Protease assay was carried out according to Khembavi *et al*[16]. Half mL of enzyme solution and with half mL substrate (1% Hammerstein casein in 50 mM Tris-Cl buffer, at pH 9) were combined and incubated for 30 min at 60ºC. The reaction was stopped by the addition of 0.5 mL of 20% trichloro acetic acid (TCA). After the termination of the reaction, the mixture was centrifuged at 12,000 rpm for 15 min at 4ºC. The amount of tyrosine released during the reaction was determined by measuring the absorbance at 280 nm. One unit of protease activity is defined as the amount of enzyme needed to liberate 1 µg tyrosine per mL per min.

Optimization of LasB Protease (elastase) Production by Response Surface Methodology

Medium and Culture Conditions

For enzyme production synthetic mineral based medium was used [1% (w/v) glucose, 0.2% (w/v) yeast extract, 1% (w/v) $(NH_4)_2HPO_4$, 1% (w/v) Na_2HPO_4, 0.02% (w/v) KH_2PO_4, 0.02% (w/v) $MgSO_4.7H_2O$, 0.05% (w/v) $CaCl_2$, 0.00025% (w/v) $ZnCl_2$, 1% (w/v) casein. The pH was maintained at 7.0. Erlenmeyer flasks containing 50 ml of the medium were inoculated with 1% v/v of 18 h old culture (O.D. 0.1 at Abs 600) of *Pseudomonas aeruginosa* MCCB 123. The flasks were incubated at 28°C for 48 h. Uninoculated media served as control. The cultures were harvested by centrifuging at 12,000 rpm for 15 min at 4°C and the cell free supernatant was used for estimating extracellular enzyme activity.

One Dimensional Screening

Shake flask experiments were carried out in order to determine the range of the media for further optimization experiments. For one dimensional screening, various media parameters were ranged in synthetic mineral medium [0.25 - 2% (w/v) casein, 0.2 - 2% (w/v) yeast extract, 0.2 - 2% (w/v) $(NH_4)_2HPO_4$, 0.05 - 1% (w/v) Na_2HPO_4, 0.05 - 1.5% (w/v) KH_2PO_4, 0.05 - 0.2% (w/v) $MgSO_4.7H_2O$, 0.01 - 0.1 % (w/v) $CaCl_2$, 0.0005 - 0.1% (w/v) $ZnCl_2$, pH, 6 - 9, temperature, 25 - 40°C.

Screening of Important Components Using Plackett and Burman Design

Plackett and Burman design[18] was used to find out the significant components for enzyme production. The design consists of a set of 12 experiments. The total number of experiments to be carried out according to Placket-Burman design is $n + 1$, where n is the number of variables. Each variable is represented at two levels, high and low, denoted by (+) and (-), respectively. The number of positive and negative signs per experiment or trial is (n + 1) / 2 and (n) 1 / 2, respectively. Each column should contain equal number of (+) and (-) signs. A total of 11 medium components were screened in 12 trials. The design was developed using Design Expert (version 6.0.9, Stat-Ease Inc, Minneapolis, MN, USA).

Face Centered Central Composite Design for Optimization of Protease Production

Response surface methodology using central composite design was applied to determine the optimum levels of significant variables (casein, $NH_4H_2PO_4$, KH_2PO) identified by Plackett – Burman design, and their interaction effects on enzyme

production. A total of 20 experiments were carried out. Each independent variable was studied at three different levels (low, medium and high, coded as -1, 0 and +1, respectively. The centre point of the design was replicated eight times for the estimation of errors. The experimental design was shown in Table 3. The software, Design Expert (version 6.0.9, Stat-Ease Inc, Minneapolis, MN) was used for the experimental design, data analysis and quadratic model building. Each run was performed in triplicate and the average was taken as the experimental value of the dependent variable or response (Y), while predicted values of the response were obtained from the quadratic model fitting. A multiple regression analysis of the data was carried out to define the response in terms of independent variables. The response surface graphs were obtained to understand the effects of the variables individually and in combination and to determine their optimum levels for maximum enzyme production.

Validation of the Model

The experiments were conducted at the optimum concentrations of media components. The experimental values were subsequently compared with the predicted values obtained from the model equations.

Scale up of Enzyme Production in 5 L Fermenter

Inoculum was grown in 100 mL of optimized medium [0.97% (w/v) casein, 1.28% (w/v) $NH_4H_2PO_4$, 0.31% (w/v) KH_2PO_4 for 18 h at 28°C. The optical density (OD) of this seed culture was again adjusted to 0.1 Abs_{600} and 1% of the inoculum were transferred into 5 L fermenter (Biostat-B-Lite bench top fermenter, Sartorius, Germany) containing 3 L fermentation medium. Fermentation was carried out at pH 7.0, 28°C and 300 rpm and sterile air supplied at the rate 2.5 $L^{-1}$and the pH maintained at 7.0 ± 0.05. Protease activity and OD were monitored at regular intervals of 6 h for 72 h.

Results and Discussion

Identification of the Bacterial Isolate

Based on phenotypic characteristics, the organism was identified as *Pseudomonas aeruginosa*. The 1500 bp fragment of 16S rRNA gene sequences when compared with the GenBank database using the BLAST algorithm showed 99% similar to the 16S rRNA gene sequence of *Pseudomonas aeruginosa*. The nucleotide sequence was deposited in the GenBank and assigned the following accession number FJ 665510.

First Step Optimization

One Dimensional Screening

One-dimensional screening results were used to find out the range of each parameter to be used for further optimization experiments. Regression analysis was done for all the factors and wherever the regression was found highly significant, the factors were taken for further screening by Plackett-Burman design.

Screening of Parameters Using Plackett-Burman Design

In order to bring down the number of parameters for further analysis, critical medium components affecting enzyme production were screened using Plackett-Burman design. The design used for screening 11 selected variables along with their corresponding experimental and predicted values is shown in Table 1.

Table 1 — Plackett Burman matrix for screening factors influencing protease production

Run	A	B	C	D	E	F	G	H	J	K	L	Protease activity	
												Observed	predicted
1	1	-1	1	-1	-1	-1	1	1	1	-1	1	2100	3182
2	1	1	-1	1	-1	-1	-1	1	1	1	-1	23012.5	22847.05
3	-1	1	1	-1	1	-1	-1	-1	1	1	1	7750	6667.88
4	1	-1	1	1	-1	1	1	-1	-1	1	1	24050	23230.03
5	1	1	-1	1	1	1	-1	1	-1	1	1	21950	22115.45
6	1	1	1	-1	1	1	1	1	-1	1	-1	25037.5	25857.47
7	-1	1	1	1	-1	1	1	-1	1	1	-1	20608.3	21035.94
8	-1	-1	1	1	1	1	1	1	-1	1	-1	18231.3	17803.65
9	-1	-1	-1	1	1	1	-1	1	1	1	1	17956.3	18776.22
10	1	-1	-1	-1	1	1	1	-1	1	1	-1	19000	17917.88
11	-1	1	-1	-1	-1	1	1	1	1	1	1	20837.5	20672.05
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	1	-1	17743.8	18171.35

A : Glucose, B : Casein, C : Yeast extract, D : $NH_4H_2PO_4$, E : Na_2HPO_4, F : KH_2PO_4, G : $MgSO_4.7H_2O$, H : $ZnCl_2$, J : $CaCl_2$, K : pH, L : Tempreature

Table 2 — Actual level of variables tested on Plackett-Burman design and their effects on protease production

Code	Parameter	Low level (-1)	High level (+1)	Coefficient	F value	P value
A	Glucose	0.25	1	1001.91	5.87	0.0940
B	Casein	0.1	0.5	1676.22	16.42	0.0271
C	Yeast extract	0.25	0.5	-1893.58	20.95	0.0196
D	NH_4H_2PO4	0.1	1	2778.30	45.11	0.0067
E	Na_2HPO4	0.1	1	n	n	n
F	KH_2PO4	0.2	3058.51	54.66	0.0051	0.0051
G	MgSO4.7H2O	0	0.1	-1068.58	6.67	0.0816
H	$ZnCl_2$	0	0.032	n	n	n
J	$CaCl_2$	0	0.001	-3118.58	56.83	0.0048
K	pH	7	8	n	n	n
L	Temperature	25	30	-2415.80	34.10	0.0100
n	Terms not included in the model					
	Coefficient of determination (R^2) = 0.9823					
	Confidence level above 95%					

Table 3 — Experimental design and results of face centered central composite design (FCCD)

Run no.	Casein	NH_4HPO_4	KH_2PO_4	Protease activity	
				Actual	Predicted
1	-1	-1	-1	23800	23804.10
2	1	-1	-1	23633.3	23572.84
3	-1	1	-1	19745.8	19858.30
4	1	1	-1	24291.7	24260.38
5	-1	-1	1	22570.8	22614.51
6	1	-1	1	21037.5	20937.43
7	-1	1	1	20904.2	20977.01
8	1	1	1	23925	23933.28
9	-1	0	0	23525	23291.89
10	1	0	0	24470.8	24654.40
11	0	-1	0	21833.3	21946.02
12	0	1	0	21633.3	21471.04
13	0	0	-1	22041.7	22016.92
14	0	0	1	21283.3	21258.57
15	0	0	0	22733.3	22412.35
16	0	0	0	22200	22412.35
17	0	0	0	22254.2	22412.35
18	0	0	0	22962.5	22412.35
19	0	0	0	21587.5	22412.35
20	0	0	0	22637.5	22412.35

Table 2 shows the coefficient of each variable, degrees of freedom, standard error and prob > F value. When the value of the concentration effect of the tested variable was positive, the influence of the variable was higher at higher tested concentration, and when negative, the influence was higher at lower concentration. A prob > F value less than 0.05 indicate that the model terms are significant. Therefore, considering the prob > F value and positive effect, casein, $NH_4H_2PO_4$ and KH_2PO_4 were found to be statistically significant in affecting enzyme production. The magnitude of coefficient of each variable indicated the intensity of its effect on the studied response. The greater the magnitude, higher the significance of the variable. Thus, KH_2PO_4 had the highest influence followed by $NH_4H_2PO_4$ and casein.

Table 4 — Range of variables used for response surface methodology

Variables	Levels		
	-1	0	+1
Casein	0.1	0.55	1
$NH_4H_2PO_4$	0.1	0.88	1.5
KH_2PO_4	0.05	0.28	0.5

Table 5 — Analysis of variance (ANOVA) for face centered central composite design

Source	Sum of square	*df*	Mean square	*F* value	*P* value	
Model	28170000	9	3130000	22.85	< 0.0001	Significant
A	4641000	1	4641000	33.88	0.0002	
B	564000	1	564000	4.12	0.0699	
C	1438000	1	1438000	10.5	0.0089	
A_2	6669000	1	6669000	48.91	< 0.0001	
B_2	1362000	1	1362000	9.94	0.0103	
C_2	1650000	1	1650000	12.05	0.006	
AB	10730000	1	10730000	78.36	< 0.0001	
AC	1045000	1	1045000	7,.63	0.02	
BC	2664000	1	2664000	19.45	0.0013	

Second Step Optimization (Face Centered Central Composite Design)

The significant factors selected by Plackett-Burman design were further optimized by central composite design. The central composite design matrix of the variables (casein A, $NH_4H_2PO_4$: B, and KH2PO_4: C) along with the experimental (n = 3) and predicted values and the actual level of factors is given in Table 3 and 4, respectively. The experimental data were statistically analyzed by the analysis of variance (ANOVA) and the results are shown in Table 5. The

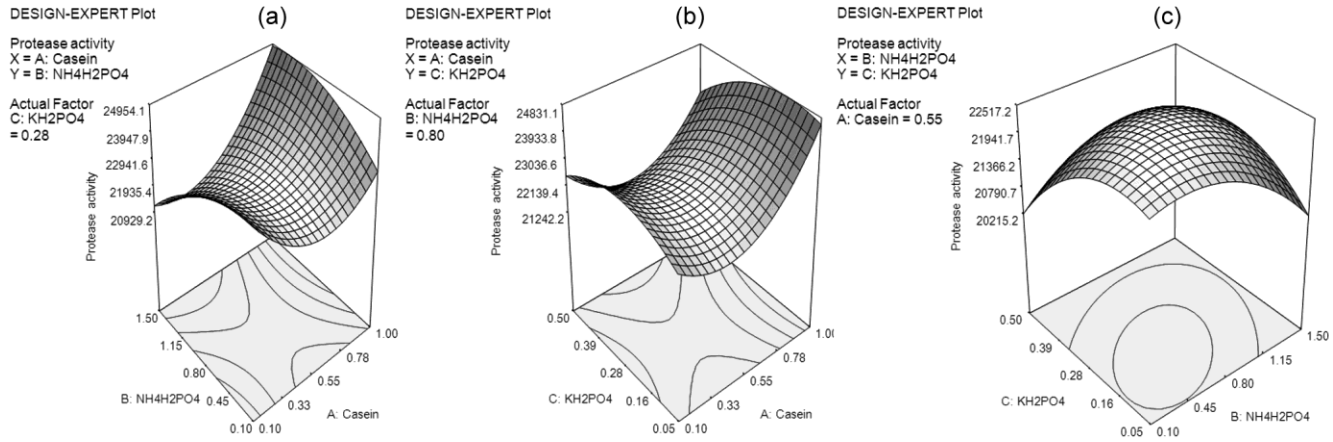

Fig. 1 — Three dimensional graphs showing the effects of (a) $NH_4H_2PO_4$ and casein, (b) KH_2PO_4 and casein & (c) KH_2PO_4 and $NH_4H_2PO_4$ on protease production by *Pseudomonas aeruginosa* MCCB 123.

ANOVA of the quadratic regression model indicated that the model was highly significant ($p < 0.0001$) as indicated by the F-value of 22.85. The 'lack of fit value' was insignificant and the goodness of fit of the model was checked by regression coefficient (R^2). The R^2 value should be between 0 and 1. The closer the R^2 value to 1, the stronger the model and better it predicts the response[19]. R^2 had a value of 0.9536, indicating that the model could explain up to 95.36 % of the variability of the response and the model did not explain 4.64% of the total variation. The value of R^2 (0.9536) indicated a good agreement between the experimental and predicted values of protease production. Adequate precision measures such as signal to noise ratio and a ratio greater than 4 is desirable[20]. The signal to noise ratio (adequate precision) for the model was higher than 4 (18.32) indicating a good fit. The predicted R^2 value 0.9033 is in reasonable agreement with adjusted R^2 value 0.9119. The RSM gave following regression equation for protease activity as a function of casein (A), $NH_4H_2PO_4$ (B) and KH_2PO_4 (C). Final equation in terms of coded factors is:

$$\text{Protease activity} = Y = + 22412.35 + 681.25A - 237.49B - 379.19C + 1560.80A^2 - 703.82B^2 - 774.60C^2 + 1158.34AB - 361.45AC + 577.08BC \quad \ldots (1)$$

Linear coefficients such as A (F ratio 33.88), C (F ratio 10.50) and all quadratic coefficients, A^2 (F ratio 48.91), B^2 (F ratio 9.95), C^2 (F ratio 12.05) were also significant for protease activity suggesting that casein (A), $NH_4H_2PO_4$ (B) and KH_2PO_4 (C) had a significant effect on elastase production by *P. aeruginosa* MCCB 123. However, the linear coefficient B was found to be insignificant (F ratio 4.12). The interaction coefficients

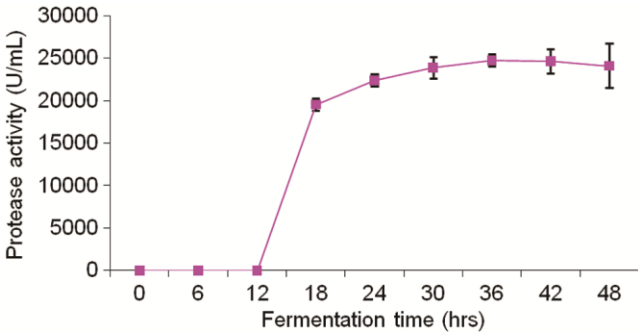

Fig. 2 — Time course of protease production in a 5-L fermentor.

such as AB (F ratio 78.36), AC (F ratio 7.63) and BC (F ratio 19.45) were also found to be significant model terms.

The response surface plots of significant interaction of varying concentrations of casein and $NH_4H_2PO_4$, casein and KH_2PO_4 and $NH_4H_2PO_4$ and KH_2PO_4 on enzyme production when all the other parameters were kept at their optimum level is given in (Fig. 1 a-c). Based on the regression equation (equation 1), the optimum concentration of the optimized components for maximum enzyme production were casein, 0.97 g $l^{-1;}$ $NH_4H_2PO_4$, 1.28 g l^{-1} and KH_2PO_4, 0.31 g l^{-1}. The interaction effects between casein and $NH_4H_2PO_4$ (AB) and $NH_4H_2PO_4$ and KH_2PO_4 (BC) were found to be most significant with F values of 78.36 and 19.45, respectively and coefficient estimates of 1158.34 and 577.08, respectively.

Model Validation Experiments

The model validation was carried out in shake flasks under optimum conditions predicted by the model. The experimental values for protease activity (24479.2 U mL^{-1}) was closer to the predicted value (24623.9 U mL^{-1}), thus validating the model.

Time Course of Enzyme Production in 5 L Fermenter

The time course of protease production by *P. aeruginosa* MCCB 123 in the optimized medium is shown in Figure 2. Enzyme production reached a maximum at 36th hour and thereafter protease activity was getting decreased. At the 36th hour, the enzyme production reached maximum with 24754.17 U mL^{-1} protease activity which was 1.83 fold higher than that in the unoptimized medium (13481.30 U mL^{-1}).

Conclusion

For the successful industrial application of an enzyme, bioprocess optimization, purification and characterization are important prerequisites. In the present study, LasB protease (elastase) was purified from the culture supernatant of *P. aeruginosa* MCCB 123. The culture conditions were optimized to maximize the enzyme production by response surface methodology which resulted in 1.82 fold increment in the protease activity. The very high protease activity of the enzyme and its persistence in the presence of calcium and magnesium ions makes it an ideal choice for detergent for hard water washes. The 5.5 fold higher activity in comparison to commercial protease suggests its commercial viability in many industrial applications. Elastases find potential application in the leather industry and as a therapeutic agent. Development of a successful bioprocess technology suggests the viability of this enzyme in industrial sector. This is the first report on the bioprocess methodology of elastase production in *P. aeruginosa*. So far, proteases from *Bacillus* sp. have been used for various industrial applications. *Pseudomonas* species also produce extracellular proteases similar to those produced by *Bacillus* species.

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