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EXPERIMENTAL ARTICLES

Statistical Medium Optimization for the Production of Collagenolytic Protease by *Pseudomonas* sp. SUK Using Response Surface Methodology[1]

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Abstract—*Pseudomonas* sp. SUK producing an extracellular collagenolytic protease was isolated from soil samples from meat and poultry industrial area based in Kolhapur, India. Response surface methodology was employed for the optimization of different nutritional parameters influencing production of collagenolytic protease by newly isolated *Pseudomonas* sp. SUK in submerged fermentation. Initial screening of production parameters was performed using Plackett-Burman design and the variables with statistically significant effects on collagenolytic protease production were identified as gelatin, peptone, and K_2HPO_4. Further, optimization by response surface methodology (RSM) using Central Composite Design showed optimum production of collagenolytic protease with 12.05 g L^{-1} of gelatin, 12.26 g L^{-1} of peptone and 1.29 g L^{-1} of K_2HPO_4. Collagenolytic protease production obtained experimentally has very close agreement with the model prediction value and the model was proven to be adequate. The statistical optimization by response surface methodology upsurges collagenolytic protease yield by 2.9 fold, hence the experimental design is effective towards process optimization. Moreover, ammonium sulphate precipitated, partially purified enzyme has shown to cleave collagen from bovine achilles tendon, which was observed by phase contrast microscopy, and SDS-PAGE. Hence, extracellular collagenolytic protease of *Pseudomonas* sp. SUK could have considerable potential for industrial as well as medical applications.

Keywords: *Pseudomonas* sp. SUK, collagenolytic protease, optimization, response surface methodology

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Proteases are the specialized class of enzymes, which can hydrolyze the peptide bond of proteins. Proteases are one of the largest groups of industrial enzymes, with increasing market demand due to their noteworthy applications in various industries as well as in basic research [1]. Collagen is the fibrous, structural protein; most abundant in mammalian body, which can be cleaved only by collagenases [2]. Proteases with collagenolytic activity has enormous industrial applications; potential restorative applications include wound healing [3], treatment of sciatica in herniated intervertebral discs [4], preparation of intact mammalian cells [5], treatment of retained placenta [6], and preclinical therapeutic studies on various types of destructive fibrosis, such as liver cirrhosis [7], Dupuytren's contracture [8] and Peyronie's disease [9].

Production of collagenolytic proteases by bacteria is well documented from long time. Microorganisms like *Clostridium, Porphyromonas*, and *Vibrio* has found to be potential candidates for the production of collagenolytic protease [10–12]. But pathogenic and anaerobic nature of these microorganisms moreover, their toxin producing ability limits the application of their collagenases. Hence, the possibilities of outbreak of such microorganisms and increasing cost of enzyme production due to their such kind of nature directed researcher's thirst to find the alternate sources of microorganisms which are non-pathogenic or less pathogenic and would be able to produce this enzyme in cost effective manner.

With respect to increasing biotechnological applications, collagenolytic enzymes are supposed to be produced in sufficient amounts, leading the concept of medium optimization for improvement of the yield. Media optimization by classical method is very laborious, time consuming and fails to include the interactive effects of the variables under study. Use of Response Surface Methodology (RSM) can overcome the limitations of classical medium optimization technique. RSM is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes in which a response of interest is influenced by several variables and the objective is to optimize this response [13].

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Literature data showed that significant increase in product formation can be achieved by employing RSM [14–16]. Also some of the reports mentioned that use of more than one protein source is responsible for increase in protease yield [17, 18]. Hence, the present study involves the isolation of potent collagenolytic protease producing microorganism and optimization of medium with combination of two protein sources for the enhanced production of the collagenolytic protease using RSM.

MATERIALS AND METHODS

Isolation and selection of the potent collagenolytic protease producing microbial strain. Microorganisms were isolated from meat and poultry industry soil samples collected at Kolhapur, (MH) India. Microorganisms were first screened on synthetic casein medium plates containing 7 g L^{-1} of K_2HPO_4, 3 g L^{-1} of KH_2PO_4, 0.1 g L^{-1} of $MgSO_4 \cdot 7H_2O$, 23 g L^{-1} of agar, 10 g L^{-1} Casein and pH 7.2–7.5; and plates were incubated at 37°C for 24 h. Bacteria showing zone of hydrolysis on synthetic casein medium were isolated and retained for subsequent screening on gelatin agar plates containing 4 g L^{-1} of peptone, 1 g L^{-1} of yeast extract, 12 g L^{-1} gelatin, 20 g L^{-1} of agar and pH 7.2–7.5. Bacteria that grew well on gelatin agar plates were subjected to 35% trichloroacetic acid and organisms showing zone of gelatin hydrolysis were screened. Further, most potent organism was selected on the basis of ratio of zone of hydrolysis to the colony diameter.

The most potent organism was inoculated with 1% v/v in the fermentation medium which was designed by referring previous scientific literature [19, 20]. The fermentation medium contains gelatin 10 g L^{-1}, yeast extract 1 g L^{-1}, K_2HPO_4 1 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.2 g L^{-1}, $CaCl_2 \cdot 2H_2O$ 0.01 g L^{-1}, $MnCl_2 \cdot 4H_2O$ 0.01 g L^{-1}, $ZnSO_4 \cdot 7H_2O$ 0.01 g L^{-1}, $FeSO_4 \cdot 7H_2O$ 0.01 g L^{-1} and pH 7.0. The flasks were shaken at 120 rpm on an orbital shaking incubator at 37°C. After 36 h of incubation the culture broth was centrifuged at 4°C and 8000 rpm for 20 min and supernatants were analyzed for enzyme activity.

Identification and phylogenetic analysis of the microorganism. Potent collagenolytic protease producer was identified by 16S rDNA sequencing [21]. DNA was extracted and 16S rDNA gene was amplified by polymerase chain reaction using the primers 27F (5'-AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3'). The PCR reactions were performed in a final volume of 50 μL having approximately 20 ng of DNA, 1× PCR buffer, 3U of *Taq* polymerase enzyme, 100 ng of each primer, 250 μm of each dNTPs. The DNA amplification conditions were: initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 30 s, 55°C for 30 s 72°C for 2 min, and final extension step at 72°C for 10 min. The purified PCR product was sequenced and analyzed by using BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and resultant homologous sequences of other species were used for phylogenetic analysis. The evolutionary history was inferred using the Neighbor-Joining method [22]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [23] and were in the units of the number of base substitutions per site. Codon positions included were of 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analysis was conducted in MEGA6 [24].

Media Optimization and Experimental Design

Screening of significant factors affecting collagenolytic protease production. Preliminary screening of factors affecting collagenolytic protease production were comprised of the selection of best carbon sources (glucose, fructose, lactose, sucrose, starch and cellulose) and additional nitrogen (proteose peptone, malt extract, tryptone, peptone, casein, beef extract and gelatin) in the fermentation medium by using one factor at a time strategy. It was followed by Plackett-Burman's factorial design (PB design) which was used for screening the most significant factors affecting the collagenolytic protease production by the strain under study. Nine factors were selected for this study by considering classical optimization results. Each factor was studied at three levels: low level (−1), high level (+1) and a center point for the evaluation of linear and curvature effects of the variables. Collagenolytic activity was carried out in triplicate and the average of these experimental values was recorded as response. The factors under investigation and levels of each factor used in the experimental design are illustrated in Table 1; total 9 variables were evaluated in 13 trials (Table 2). The first order equation can model the data properly (Eq. 1):

$$Y = \beta_o + \Sigma\beta_i X_i. \quad (1)$$

Where Y is the predicted response of collagenolytic protease production, β_o is the intercept, β_i is the linear coefficient. Regression analysis determined the most significant variables affecting collagenolytic protease yield, and were evaluated in further optimization experiments.

RSM for statistical optimization of collagenolytic protease production. RSM was used to estimate the main effects of significant variables on the collagenolytic protease yield. Significant variables determined by PB experiment were used for further optimization studies. Central composite design (CCD) was used for the RSM experiment. The independent variables were studied at five levels (−1.68, −1, 0, 1, 1.68). The ranges of respective variables selected for the

Table 1. Variables and levels for Plackett-Burman experiment

Code value	Variables, g L^{-1}	−1	0	1
A	Gelatin	5	10	15
B	Peptone	5	10	15
C	K_2HPO_4	0.5	1	1.5
D	Yeast extract	0.5	1	1.5
E	$MgSO_4$	0.1	0.2	0.3
F	$CaCl_2$	0.01	0.02	0.03
G	$MnCl_2$	0.01	0.02	0.03
H	$ZnSO_4$	0.01	0.02	0.03
I	$FeSO_4$	0.01	0.02	0.03

experiment (Table 3) and a total of 20 experiments were formulated with 6 central points (Table 4). A second order polynomial equation was fitted to the data by carrying out multiple regression analysis (Eq. 2):

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_1^2 A^2 + \beta_2^2 B^2 + \beta_3^2 C^2. \quad (2)$$

Where Y is the predicted response of collagenolytic protease production, β_o is the intercept, A, B, and C are independent coded variables, β_1, β_2 and β_3 were linear coefficients, β_1^2, β_2^2, and β_3^2 are quadratic coefficients. β_{12}, β_{13}, and β_{23} are interactive coefficients. The design was developed and analyzed by a statistical software package Design Expert software version 8.0 (Stat-Ease Inc., Minneapolis, United States). With multiple regression analysis, response surface graphs were obtained by which effect of variables can be studied individually as well as in combination.

Enzyme assay. Collagenolytic activity was measured as described by Warinda et al. with few modifications [19]. The reaction mixture contained 0.3 mL of 0.2% (w/v) gelatin in water, 0.6 mL 100 mM Tris-HCl buffer, pH 7.5, containing 10 mM $CaCl_2$ and 0.1 mL enzyme. The reaction mixture was incubated at 37°C for 30 min and stopped by the addition of 0.5 mL of 10% trichloroacetic acid. The amount of released free amino acid was measured by the ninhydrin method using standard leucine curves. One unit activity of collagenolytic protease was expressed as 1 μmol of leucine equivalents released per minute per ml of enzyme.

Partial purification of collagenolytic protease by ammonium sulphate precipitation. *Pseudomonas* sp. SUK was cultivated in optimized medium for 36 h; the culture was centrifuged at 8000 rpm for 20 min to remove the cell biomass. The collagenolytic protease from cell free supernatant was precipitated by adding solid ammonium sulphate to a final concentration of 60% saturation. The precipitate was harvested by centrifugation at 8000 rpm for 20 min, dissolved in 100 mM Tris-HCl buffer (pH 7.5) and dialysed against the same buffer overnight (4°C). The protein content was determined by Lowry method using bovine serum albumin (BSA) as a standard protein [25].

Table 2. Plackett-Burman design for 9 variables with coded values along with the predicted and observed results. The observed values are average of triplicate determinations

Trial	Coded variable level									Collagenolytic protease activity, U	
	A	B	C	D	E	F	G	H	I	observed	predicted
1	1	1	−1	1	1	1	−1	−1	−1	7.09	7.70
2	−1	1	1	−1	1	1	1	−1	−1	4.00	4.91
3	1	−1	1	1	−1	1	1	1	−1	6.08	6.33
4	−1	1	−1	1	1	−1	1	1	1	4.34	3.79
5	−1	−1	1	−1	1	1	−1	1	1	2.31	2.42
6	−1	−1	−1	1	−1	1	1	−1	1	0.91	1.30
7	1	−1	−1	−1	1	−1	1	1	−1	5.88	5.21
8	1	1	−1	−1	−1	1	−1	1	1	7.53	7.70
9	1	1	1	−1	−1	−1	1	−1	1	9.02	8.81
10	−1	1	1	1	−1	−1	−1	1	−1	5.84	4.91
11	1	−1	1	1	1	−1	−1	−1	1	6.46	6.33
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	1.25	1.30
13	0	0	0	0	0	0	0	0	0	7.19	7.19

The degradation of collagen was observed by phase contrast microscopy. The reaction was carried out with the addition of 1 mL of partially purified collagenolytic protease to 25 mg of bovine achilles tendon collagen in 100 mM Tris-HCl buffer, pH 7.5, containing 10 mM $CaCl_2$ at 37°C. Images were captured using an inverted microscope (Lawrence and Mayo) fitted with digital camera (Jenoptic CCD) and were processed using the ProgRes C14 imaging software.

Collagen degradation was also observed by SDS-PAGE analysis. The reaction started with the addition of 0.5 mL of partially purified collagenolytic protease to 5 mg of bovine achilles tendon collagen (dissolved in 0.1 M acetic acid) in 100 mM Tris-HCl buffer, pH 7.5, containing 10 mM $CaCl_2$ at 37°C (21 Lima et al.). The samples were recovered after every hour of incubation and analysed by SDS-PAGE using 4% stacking gel and 8% separating gel, according to the method of Laemmli [26].

RESULTS AND DISCUSSION

Screening for collagenolytic protease producing microbial strain. Fifty four microorganisms were isolated on synthetic casein medium showing zone of hydrolysis; out of which potent fifteen microorganisms were obtained by subsequent secondary screening on gelatin agar. On the basis of ratio of zone of hydrolysis to the colony diameter significantly potent organism was selected for further investigation (data not shown).

Identification of the potent collagenolytic protease producing microbial strain. Ribotyping is a widely accepted technique for the identification of bacteria and its phylogenetic analysis [22]. So identification of microorganism was carried out with partial 16S rDNA sequencing and the results were submitted to GenBank (Accession no. KP403955). The 16S rDNA based phylogenetic analysis demonstrated 99% sequence similarity of isolated organism with genus *Pseudomonas*. The phylogenetic tree of the sequence was constructed using MEGA6 software by neighbor-joining method (Fig. 1); which showed the detailed evolutionary relationship between the strain under study and other closely related *Pseudomonas* species.

Clostridium histolyticum is extremely pathogenic in nature and even though it is currently used for collagenolytic protease production [8]. Although *Pseudomonas* is reported as an opportunistic pathogen; its pathogenicity is negligible when compared with *Clostridium histolyticum*. The activity of collagenolytic protease from isolated strain was observed at neutral pH range, which opens an avenue for its use in human system where normal physiological blood pH range is 7.35–7.45 [27]. Therefore this could be a pragmatic solution for various medical applications.

Media optimization. All of the tested carbon sources in production medium at 1% w/v level, drastically decreased the enzyme production suggesting negative effect of all carbohydrates on collagenolytic protease production (Fig. 2). Glucose (1.78 U/mL) and fructose (1.49 U/mL) that are monosaccharides, provided least production of respective enzyme, and may be due to the phenomenal characteristic of catabolite repression by these simple carbohydrates. Inability of some of the *Pseudomonas* species [28] to degrade lactose (4.34 U/mL) and sucrose (3.57 U/mL) resulted comparatively higher enzyme activity than that of monosaccharides, which is although less as that of control (4.82 U/mL). Similar reports were also reported where use of carbohydrates in the production medium lowered the protease production [29, 30]. So,

Table 3. Factors and levels in Central Composite Design (CCD)

Factor	Level, g L^{-1}				
	−1.6817	−1	0	1	1.6817
A	2.5	6.05	11.25	16.45	20
B	2.5	6.05	11.25	16.45	20
C	0.25	0.605	1.125	1.645	2

Table 4. Experimental design and results of Central Composite Design (CCD)

Trials	A	B	C	Collagenolytic protease activity, U	
				observed	predicted
1	−1	−1	−1	3.81	4.07
2	1	−1	−1	6.46	6.08
3	−1	1	−1	6.51	6.14
4	1	1	−1	4.68	4.60
5	−1	−1	1	2.31	2.62
6	1	−1	1	6.46	7.07
7	−1	1	1	7.14	7.76
8	1	1	1	8.69	8.66
9	−1.68	0	0	4.87	4.50
10	1.68	0	0	6.90	6.94
11	0	−1.68	0	2.84	2.49
12	0	1.68	0	5.55	5.57
13	0	0	−1.68	7.62	8.08
14	0	0	1.68	11.05	10.27
15	0	0	0	13.42	13.45
16	0	0	0	13.32	13.45
17	0	0	0	13.61	13.45
18	0	0	0	13.23	13.45
19	0	0	0	14.14	13.45
20	0	0	0	12.89	13.45

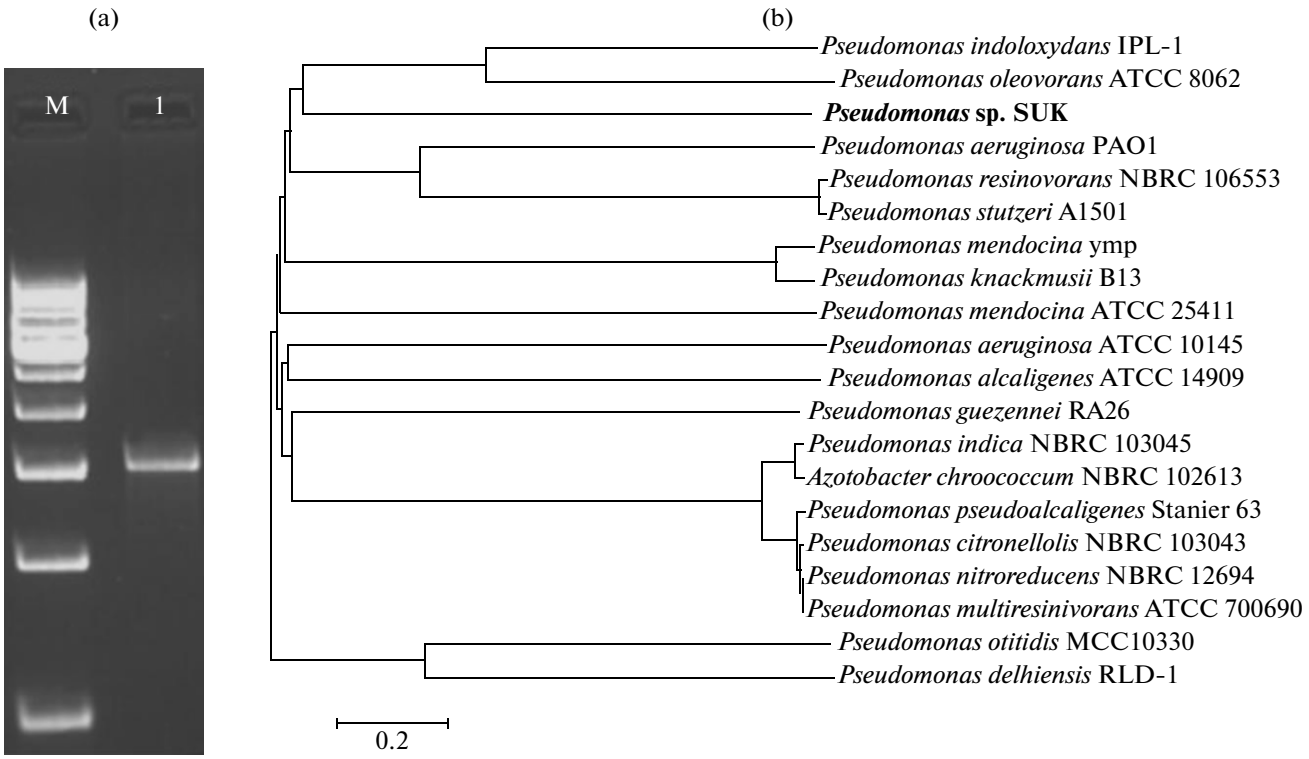

Fig. 1. (a) 1.5 kb PCR amplified product with DNA marker, (b) phylogenetic tree of the *Pseudomonas* sp. SUK and other closely related *Pseudomonas* species based on 16S rRNA sequences. The tree was generated by using the neighbor-joining method. Bar = 0.2 substitutions per site.

carbohydrates are omitted from the production medium.

While in case of various additional nitrogen sources tested at 1% w/v level in the production medium, peptone exhibited conspicuously higher collagenolytic protease production (7.19 U/mL) and the phenomenon is shown in Fig. 3. Peptone which is enzymatic digest of animal proteins proved as a best source of nitrogen, which enhanced the production of collagenolytic protease from 4.82 to 7.19 U/mL. Similar results were reported previously in case of *Bacillus stearothermophilus* F1 and *Pseudomonas aeruginosa* MTCC 7926 entailing enhanced protease production by addition of peptone [29, 30].

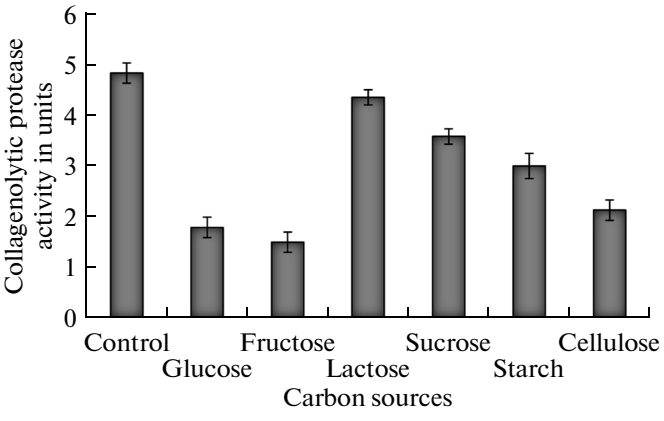

Fig. 2. Effect of carbon source on collagenolytic protease production. Error bars indicate standard deviation.

Plackett-burman design for screening of significant factors. Based on previous experiments, total nine variables were studied for their effect on collagenolytic protease production by using PB design. Table 2 represents the yield of collagenolytic protease production for each experimental design. The Statistical analysis using PB design (Table 5) indicated that gelatin (A), peptone (B), and K_2HPO_4 (C) significantly affected collagenolytic protease production with *p*-values less than the significance level, whereas the remaining components yeast extract (D), $MgSO_4 \cdot 7H_2O$ (E), $CaCl_2 \cdot 2H_2O$ (F), $MnCl_2 \cdot 4H_2O$ (G), $ZnSO_4 \cdot 7H_2O$ (H), $FeSO_4 \cdot 7H_2O$ (I) were found to be insignificant with P-values above 0.05. First order polynomial was derived by using regression analysis (Eq. 3), representing collagenolytic protease production as a function of the independent variables.

$$\text{Collagenolytic protease} = 5.227383 + 1.952498A + 1.243965B + 0.559582C. \quad (3)$$

The model *F*-value of 27.91 implies that the model is significant and there was only 0.01% chance that a model *F*-value this large could occur due to noise. The values of at $P < 0.05$ indicate that the model terms are significant. Evaluation of relationship between significant variables is not possible by first order equation; so further investigation was conducted through a second order model in RSM experiment.

Medium optimization by RSM. Significant variables from PB design were further optimized using central composite design. The high and low levels of the components are shown in Table 3. Table 4 indicate design of experiment and results of 20 experiments carried out using CCD analysis. Regression equation obtained after ANOVA provided the level of collagenolytic protease production as a function of the different variables, such as gelatin, peptone and K_2HPO_4. The quadratic regression of model was given as:

$$\begin{aligned}\text{Collagenolytic protease} &= 13.45061 + 0.727397\text{A} \\ &+ 0.916826\text{B} + 0.652316\text{C} - 0.88768\text{AB} \\ &+ 0.6099\text{AC} + 0.7669\text{BC} - 2.73181\text{A}^2 \\ &- 3.32958\text{B}^2 - 1.51058\text{C}^2.\end{aligned} \quad (4)$$

Equation 4 is highly significant model ($P = 0.0001$) which is demonstrated by the ANOVA of quadratic regression (Table 6). The model *F*-value of 103.22 implies that the model is significant and there was only 0.01% chance that a model *F*-value this large could occur due to noise. The values of at $P < 0.05$ indicate that the model terms are significant. R^2 is a measure of the amount of the reduction in the variability of response obtained by using the repressor variables in the model [13]. By using the determination coefficient (R^2) goodness of fit of the model was checked and it was adequate in this study, $R^2 = 0.9893$. The "Pred R^2" of 0.9352 is in reasonable agreement with the "Adj R^2" of 0.9798. The lack-of-fit value for this quadratic regression was not significant (0.1613) relative to the pure error. Hence the model equation was adequate for the prediction of collagenolytic protease production. "Adeq Precision" with a ratio greater than 4 is regarded as desirable which is a measure of signal to noise ratio [31]. The "Adeq Precision" ratio of 27.481 indicates an adequate signal. Thus, this model can be used to navigate the design space.

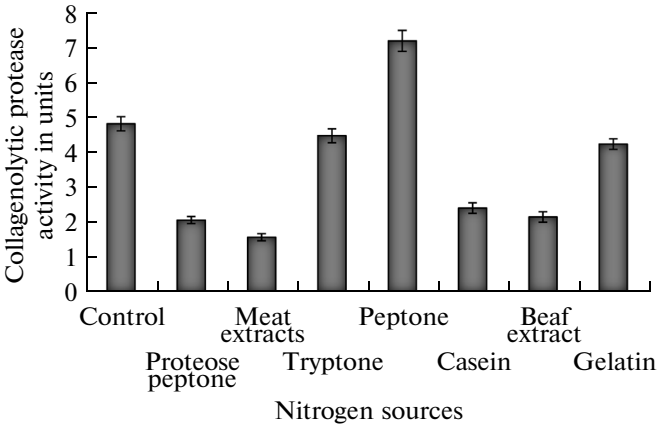

Fig. 3. Effect of nitrogen source on collagenolytic protease production. Error bars indicate standard deviation.

Interaction of variables. Figure 4 shows the 3D response surface plots and their respective 2D contour plots of collagenolytic protease production. Each figure represents the effect of two variables keeping other factor at zero level. 3D response surface plots and 2D

Table 5. Statistical analysis of the model by Plackett-Burman design

Source	Sum of squares	*d.f.*	Mean square	*F*-Value	*p*-Value $P > F$
Model	67.94	3	22.64	58.62	<0.0001*
A	45.63	1	45.63	118.11	<0.0001*
B	18.65	1	18.65	48.28	0.0001*
C	3.65	1	3.65	9.46	0.0152*
Residual	3.09	8	0.38		
Corrected total	74.91	12			

* Significant *p*-values at $P \leq 0.05$.

Table 6. Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of collagenolytic protease production

Source	Sum of squares	*d.f.*	Mean square	*F*-Value	*p*-Value $P > F$
Model	295.79	9	32.86	103.32	<0.0001*
A	7.22	1	7.22	22.71	0.0008*
B	11.47	1	11.47	36.08	0.0001*
C	5.81	1	5.81	18.26	0.0016*
AB	6.30	1	6.30	19.81	0.0012*
AC	2.97	1	2.97	9.35	0.0121*
BC	4.70	1	4.70	14.79	0.0032*
A^2	107.54	1	107.54	338.10	<0.0001*
B^2	159.76	1	159.76	502.26	<0.0001*
C^2	32.88	1	32.88	103.38	<0.0001*
Residual	3.18	10	0.31		
Lack of fit	2.29	5	0.45	2.57	0.16
Pure error	0.88	5	0.17		
Corrected total	298.97	19			

* —Significant *P*-values at $P \leq 0.05$.

contour plots are graphical representations of the regression equation, which is very easy and convenient to understand the interactions between two variables that give insights to find their optimum levels. Shapes of the contour plots, circular or elliptical, emphasize the significant or insignificant interactions between the corresponding variables. Insignificant interactions amongst the corresponding variables were shown by circular contour plots while elliptical contour plots represent the significant interactions [14, 32].

The response surface curves and contour plots represented in figure a_1 and b_1 show the interaction between gelatin and peptone. The elliptical shape of contour plot shows highly significant interaction between gelatin and peptone. Gelatin is partially hydrolysed collagen; it acts as an inducer for collagenolytic protease enzyme. Enzyme production was considerably less when gelatin was used at 6 g L^{-1} in the production medium, production increases with simultaneous increase in the concentrations of gelatin from 6 g L^{-1} to 12 g L^{-1}. Similar findings have been observed in previous report where gelatin has been reported for enhanced protease production by *Bacillus* species [19, 33]. Peptone also exhibited similar behavior as that of gelatin. Increased protease production with peptone as an organic nitrogen source is also reported earlier [34]. Production of enzyme drastically decreases when both gelatin and peptone concentration were used over 15 g L^{-1}.

Substantially carbon and nitrogen are not the only sources, which influence the extracellular protease production by micro-organisms. Previous studies proved that the trace elements can also affect the yield of extracellular protease [35, 36]. Indeed, inline with above studies, our studies showed that, K_2HPO_4 enhance the enzyme production. The Figs. 4 a_2, b_2 and a_3, b_3 represents the interactions in between gelatin-K_2HPO_4 and peptone-K_2HPO_4, respectively. Both of them showed nearly elliptical contour plots representing their positive interactions with each other. Interaction of K_2HPO_4 with both the protein sources reveals that it's lower and higher levels decreased col-

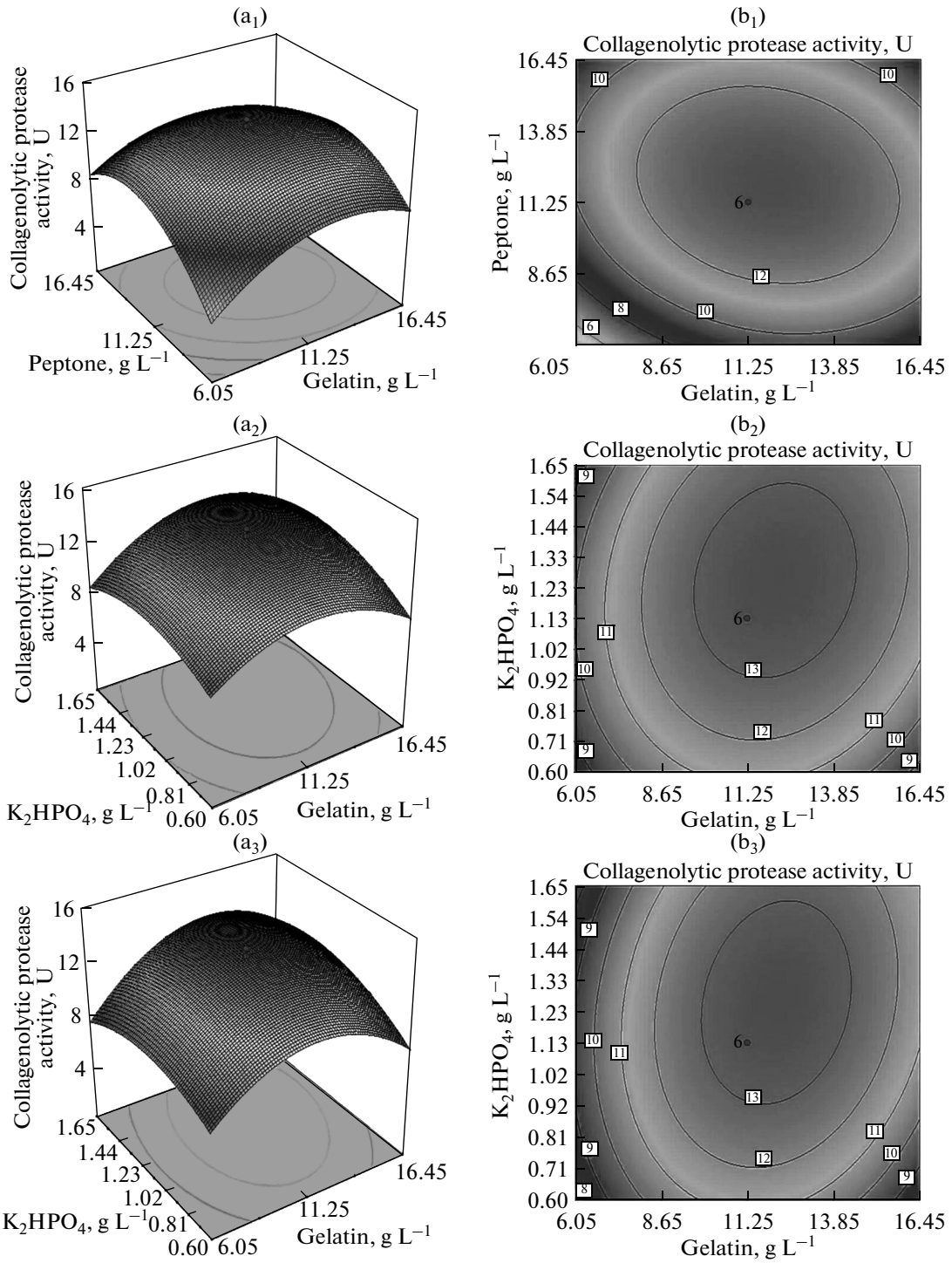

Fig. 4. 3D response surface plots (a) and 2D contour plots (b) of collagenolytic protease production by optimization of variables. The interaction between (a_1, b_1) gelatin and peptone, (a_2, b_2) gelatin and K_2HPO_4, and (a_3, b_3) peptone and K_2HPO_4 and its effect on collagenolytic protease production.

lagenolytic protease yield while middle levels enhanced collagenolytic protease production.

Validation of the experimental model. After processing of model parameters, maximum collagenolytic protease production could be achieved when gelatin, peptone and K_2HPO_4 were set at 12.05, 12.26 and 1.29 g L^{-1}, respectively. Maximum collagenolytic protease production obtained experimentally was

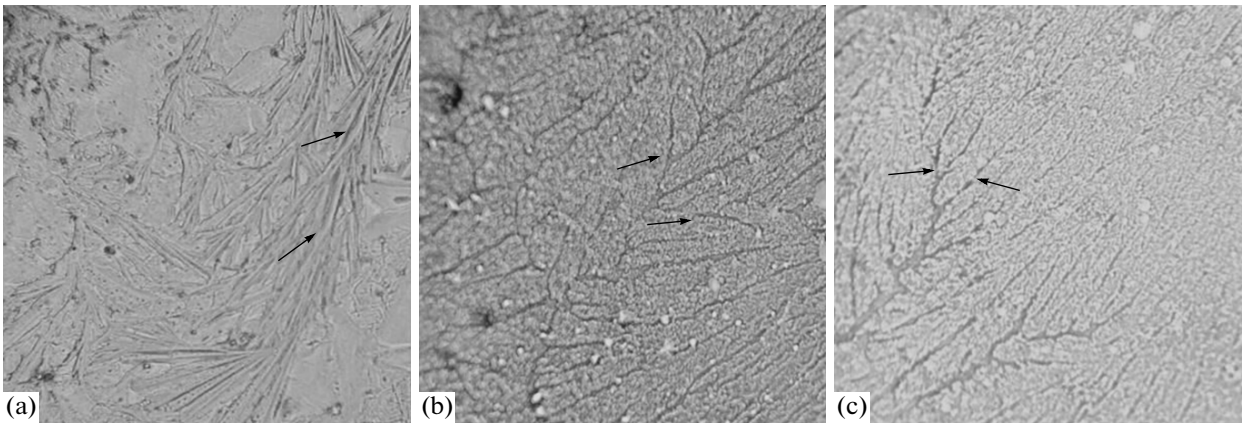

Fig. 5. Progressive degradation of collagen observed under phase contrast microscope. (a) Control (b) 1 h treated sample (c) 2 h treated sample.

13.81 U. This was very close agreement with the model prediction value of 13.65 U. The observed close correlation between the experimental and predicted values validated the model.

Collagenolytic activity of partially purified enzyme. The collagenolytic protease of cell free supernatant showed specific activity of 12.44 U/mg whereas 60% ammonium sulphate precipitated partially purified enzyme showed specific activity of 51.40 U/mg. Partial purification gives yield of 55.93% with 4.13 fold purification. Further, this partially purified enzyme tested on bovine achilles tendon collagen and degraded products were observed by phase contrast microscopy and SDS-PAGE. Figure 5 shows the phase contrast microscopic observation, which clearly states the degradation of collagen. While in case of SDS-PAGE analysis (Fig. 6) sequential dissolution of beta and alpha chains of collagen confirmed the ability of respective enzyme to degrade collagen.

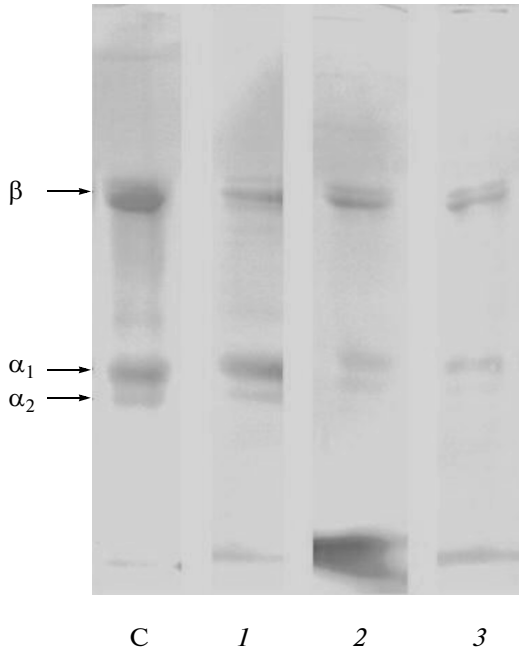

Fig. 6. Progressive degradation of collagen observed by SDS-PAGE. (C) Control (*1*) 1 h treated sample (*2*) 2 h treated sample (*3*) 3 h treated sample.

CONCLUSIONS

Blending of interdisciplinary sciences has always resulted in incredible discoveries. Use of statistical analysis in media optimization has shown its remarkable triumph in increased product formation. CCD based optimization of culture parameters involved in this submerged fermentation process. The whole process is potentially useful for industrial applications as appropriate substrate concentrations can be deduced by this, which can make the whole process more economically feasible.

So far the use of RSM for production of collagenolytic protease was not explored and to the best of our knowledge this is the first report giving detailed medium optimization of collagenolytic protease from a new and promising source *Pseudomonas* sp. SUK. The yield of the enzyme is enhanced up to 2.9 fold in optimized medium as compared with initial production medium. The developed model was validated to be precise and reliable for predicting the production of collagenolytic protease from the studied strain. The next logical step of this research is to purify this collagenolytic protease and its complete characterization for possible industrial exploitation.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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