Environmental Science and Pollution Research
https://doi.org/10.1007/s11356-018-2859-4

RESEARCH ARTICLE

Use of statistical experimental methods for optimization of collagenolytic protease production by *Bacillus cereus* strain SUK grown on fish scales

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Received: 17 October 2017 / Accepted: 27 July 2018

Abstract
In this study, novel and cheap sources like fish scales and molasses were used for the production of collagenolytic protease. Statistical optimization of different parameters for the production of collagenolytic protease by *Bacillus cereus* strain SUK has been carried out using response surface methodology (RSM). Three most significant medium components identified by Plackett-Burman (PB) were fish scales, molasses, and incubation time, which were further optimized using central composite design (CCD). The medium having fish scales 9.38 g l^{-1}, molasses 2.42 g l^{-1}, and incubation time of 67.34 h was found to be optimum for maximum collagenolytic protease production. *B. cereus* strain SUK has shown multiple plant growth-promoting traits, whereas degraded fish scale hydrolysates (FSHs) were having antimicrobial as well as plant growth-promoting abilities. The collagenolytic efficiency of this isolate can be exploited in an eco-friendly process of bioconversion of fish waste, representing an alternative way of waste management that could be used to produce various value-added products, such as collagenolytic protease, microbial biomass, amino acids, protein hydrolysates, and collagen peptides.

Keywords Collagenolytic protease · *Bacillus cereus* strain SUK · Response surface methodology · Plant growth-promoting traits · Fish scale hydrolysates

Introduction

In a world with 7.3 billion people, which is expected to grow by another two billion by 2050, human race has the huge challenge of feeding the planet while safeguarding its natural resources for future generations. Fishing industry is one of the established food sectors, which can supply ample amount of food to deliberately growing population (FAO 2014). A huge amount of fish production additionally inclines nearly equal amount of waste as that of final product. During processing of fish, significant amount of waste (20–80% depending upon the level of processing and type of fish) is generated (Ghaly et al. 2013). Immense quantity of waste produced should be properly treated, but a good care cannot be taken for all the waste produced. The numbers of ways are used to dispose the waste, which is polluting our environment in a very rigid manner. Landfilling and incineration are some of the methods which are used many times, but they are not fruitful as they are costly as well as require a good maintenance (Kim and Venkatesan 2014). Moreover, it results in the loss of useful biological resources, such as proteins.

Maintenance of high agriculture productivity is also a prerequisite to cater the demands of growing population (Perez-Montano et al. 2014). Currently, the productivity is diminishing due to the lack of sufficient nutrient availability to plants. Use of plant growth-promoting bacteria (PGPB) is gaining strong impulse among researchers and scientist. PGPB are reported for escalating the yield of various crops (Ahemad and Kibret 2014). Various bacteria, such as the *Bacillus*, *Rhizobium*, and *Pseudomonas*, have been extensively studied for their plant growth-promoting (PGP) factors in vitro as well as under field conditions (Vejan et al. 2016).

Responsible editor: Philippe Garrigues

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Published online: 03 August 2018

Various PGP factors, such as indole acetic acid (IAA), phosphate solubilization, nitrogen fixation, and bio-control attributes like production of hydrolytic enzymes, siderophore, HCN, and antibiotics, have been previously reported for *Bacillus* sp. (Kumar et al. 2011; Kumar et al. 2012; Sunar et al. 2015). Some of the *Bacillus* sp. were reported to produce collagenolytic protease; (Liu et al. 2010; Wu et al. 2010; Suphatharaprateep et al. 2011) a type of hydrolytic enzyme which may act as an effective bio-control agent against phytopathogenic bacteria, fungi, insects, and pests (Shimoi et al. 2010; Ruiu 2013; Yang et al. 2013).

Collagen-rich waste like fish scales, which either considered as an environmental pollutant, can be effectively used in multiple ways (Bhagwat and Dandge 2018). This waste plays an important role in global carbon and nitrogen cycles, and its metabolism is of both environmental and biotechnological interest. It can be used as a simple source for the production of collagenolytic protease, or its degraded products can be used as a good source of biofertilizer. Due to high nitrogen content as well as PGP activity of protein hydrolysates, it could be effectively applied in agriculture. Thus, microbial degradation of fish scales represents an alternative for the development of slow-release nitrogen fertilizers. Collagen peptides, which are reported to have antimicrobial activity against pathogenic microorganisms, are the added benefit of the protein hydrolysate (Najafian and Babji 2012; Ennaas et al. 2015; Ennaas et al. 2016).

So, the present study comprises the following points: (a) isolation of potent fish scale degrading isolate, (b) response surface methodological (RSM) optimization of the collagenolytic protease, (c) evaluation of the PGP attributes of the isolate, and (d) to check the potential of isolate as well as protein hydrolysate for growth promotion of *Vigna radiata*.

Materials and methods

Isolation and selection of the potent collagenolytic protease producing microbial strain

Microorganisms were isolated from soil samples collected at meat and poultry waste disposal site, Kolhapur, (MH) India. Selection of potent collagenolytic protease producing microorganisms was carried out as per our earlier designed protocol (Bhagwat et al. 2015). Briefly, microorganisms were first screened on synthetic casein medium plates, and bacteria showing a zone of hydrolysis were isolated and retained for subsequent screening on gelatin agar plates. The gelatin agar plates with well-grown bacteria were flooded with 35% trichloroacetic acid and organisms showing a zone of gelatin hydrolysis were screened. Further, potent organisms were selected on the basis of the ratio of a zone of hydrolysis to the colony diameter (Table 1).

Collagenolytic protease production

Removal of non-collagenous proteins and minerals from fish scales were carried out by using the protocol of Pati et al. (2010). Processed fish scales were used as the only source of nutrition in the production medium. The potent organisms were inoculated with 1% *v*/*v* in the production medium containing processed fish scales 10 g l^{-1}. The flasks were shaken at 120 rpm on an orbital shaking incubator at 37 °C until the fish scales get degraded completely. After every 12 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 potent collagenolytic protease producer

Potent collagenolytic protease producer was identified by 16S rDNA sequencing. DNA was extracted, and 16S rDNA gene was amplified by polymerase chain reaction using the forward primer (5′ AGHGTBTGHTCMTGNCTCAS 3′) and reverse primer (5′ TRCGGYTMCCTTGTWHCGACTH 3′). The PCR reactions were performed in a final volume of 100 μl having approximately 20 ng of DNA, 10× PCR buffer, 3 U of Taq polymerase enzyme, 400 ng of each primer, and 2.5 mM of each dNTPs. The DNA amplification conditions were as follows: initial denaturation at 95 °C for 5 min, 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min 30 s, and the final extension step at 72 °C for 7 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. Neighbor-joining method was used to infer the evolutionary history (Saitou and Nei 1987). The optimal tree with the sum of branch length = 82.22 was obtained. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Maximum composite likelihood method was used to compute the evolutionary distances (Tamura et al. 2004), which were in the units of the number of base substitutions per site. 1st + 2nd + 3rd + non-coding codon positions were included, and all positions containing gaps and missing data were eliminated from the dataset. The evolutionary analysis was conducted in MEGA6 (Tamura et al. 2013).

Media optimization and experimental design

Screening of significant factors affecting collagenolytic protease production

The most significant factors affecting the collagenolytic protease production by the strain under study were screened by using Plackett-Burman's factorial design (PB design). Seven

Table 1 Fish scale degradation optimization studies of the potent isolates

Sr. no.	Strain	Zone of hydrolysis in mm	Colony diameter in mm	Ratio	Degradation time (hours)	Highest enzyme produced (U/ml)
1	EC	6	1	6	96	3.21 ± 0.17
2	EV	5	1	5	120	3.43 ± 0.14
3	FL	8	1	8	72	5.03 ± 0.22
4	FQ	9	1.5	6	84	4.22 ± 0.14
5	GD	5	1	5	120	3.74 ± 0.15
6	HA	6	1	6	108	3.89 ± 0.16

Each point in case of enzyme activity represents the mean ($n = 3$) ± SD

factors were selected for this study and 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 (Table 2). Total seven variables were evaluated in 13 trials under this investigation with varying the levels of each factor (Table 3). The response was recorded by averaging the experimental values of collagenolytic activity carried out in triplicate. The first order equation can model the data properly (Eq. (1)):

$$Y = \beta_0 + \sum \beta_i X_i \quad (1)$$

where Y is the predicted response of collagenolytic protease production, β_0 is the intercept, and βi is the linear coefficient. Most significant variables affecting collagenolytic protease yield were determined by regression analysis, and those variables were optimized in further experiments (Tables 4 and 5).

RSM for statistical optimization of collagenolytic protease production

To study the interactive effects of significant variables on the collagenolytic protease yield, RSM was used. The results generated by PB experiment used to identify the significant variables affecting collagenolytic protease yield in RSM optimization studies. Central composite design (CCD) was used for the RSM experiment. The significant independent variables were evaluated at five levels (− 1.68, − 1, 0, 1, 1.68) as showed in Table 5. A total of 20 experiments were formulated with 6 central points selecting the ranges of respective variables

Table 2 Variables and levels for Plackett-Burman experiment

Code value	Variables	− 1	0	1
A	Fish scales (g l^{-1})	5	10	15
B	Molasses (g l^{-1})	2	4	6
C	Inoculum (ml l^{-1})	5	10	15
D	pH	6	7	8
E	Incubation time (hours)	48	60	72
F	Stirring (rpm)	100	120	140
G	Temperature (°C)	35	37	39

(Table 6). Multiple regression analysis was carried for a second order polynomial equation (Eq. (2)):

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 E + \beta_{12} AB + \beta_{13} AE + \beta_{23} BE + \beta_1{}^2 A^2 + \beta_2{}^2 B^2 + \beta_3{}^2 E^2 \quad (2)$$

where Y is the predicted response of collagenolytic protease production; β_0 is the intercept; A, B, and E are the independently coded variables; β_1, β_2, and β_3 were the linear coefficients; and $\beta_1{}^2$, $\beta_2{}^2$, and $\beta_3{}^2$ are the quadratic coefficients. β_{12}, β_{13}, and β_{23} are the interactive coefficients. Statistical software package "Design Expert" software version 10.0 was used to develop and analyze the design (Stat-Ease Inc., Minneapolis, USA). Response surface graphs were obtained using multiple regression analysis, which illustrates the effect of variables individually as well as in combination.

Enzyme assay

Collagenolytic activity was measured as per our earlier protocol (Bhagwat et al. 2016). The reaction started with the addition of 0.1-ml enzyme in 0.6-ml 100-mM Tris-HCl buffer, pH 7.5, containing 10-mM $CaCl_2$ and 0.3 ml of 0.2% (*w*/*v*) gelatin. The reaction mixture was incubated at 37 °C for 30 min and reaction was stopped by the addition of 0.5 ml of 10% trichloroacetic acid. Ninhydrin method was used to measure the amount of released free amino acid 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.

Amino acid and protein determination of optimized medium degraded by *Bacillus cereus* strain SUK

The response surface methodologically optimized medium was then degraded by *B. cereus* strain SUK. The amount of released free amino acid was measured by the ninhydrin method using standard leucine curves (Moore and Stein 1954), and the protein content was determined by Lowry method using bovine serum albumin (BSA) as a standard protein (Lowry et al. 1951).

Table 3 Plackett-Burman design for seven 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/ml)		
	A	B	C	D	E	F	G	Observed	Predicted
1	1	1	−1	1	1	1	−1	4.78	5.40
2	−1	1	1	−1	1	1	1	6.95	7.07
3	1	−1	1	1	−1	1	1	5.21	5.16
4	−1	1	−1	1	1	−1	1	7.28	7.07
5	−1	−1	1	−1	1	1	−1	8.59	8.64
6	−1	−1	−1	1	−1	1	1	6.90	6.83
7	1	−1	−1	−1	1	−1	1	7.38	6.98
8	1	1	−1	−1	−1	1	−1	3.03	3.59
9	1	1	1	−1	−1	−1	1	4.14	8.81
10	−1	1	1	1	−1	−1	−1	5.79	5.26
11	1	−1	1	1	1	−1	−1	7.15	6.98
12	−1	−1	−1	−1	−1	−1	−1	6.18	6.83
13	0	0	0	0	0	0	0	10.19	10.19

Antimicrobial activity assessment of the fish scale hydrolysate

After complete degradation of scales, the culture broth was centrifuged at 8000 rpm for 20 min at 4 °C. The resulted cell-free supernatants, containing FSHs (fish scale hydrolysates), were freeze-dried to obtain powder and further used to determine its antibacterial potential. Antibacterial activities of FSHs were tested against two Gram-positive bacteria: *Staphylococcus aureus* (NCIM 2654) and *Enterococcus faecalis* (NCIM 2403) and two Gram-negative bacteria: *Escherichia coli* (NCIM 2832) and *Pseudomonas aeruginosa* (NCIM 5032). Log-phase bacterial cultures containing 10^8 CFU ml^{-1} of each bacterial strain grown in nutrient broth were swabbed on the surface of Mueller-Hinton agar plates. The FSHs were dissolved in distilled water at various concentrations ranging from 25 to 200 mg/ml and sterilized by filtration through a 0.22-μm Nylon membrane filter. Then bores were made using a sterile borer and were loaded with 100 μl of various concentrations of FSHs to determine their antimicrobial efficacy.

Table 4 Factors and levels in central composite design (CCD)

Factor	Level				
	−1.6817	−1	0	1	1.6817
A (g l^{-1})	5.00	7.03	10.00	12.97	15
B (g l^{-1})	1.00	1.80	3.00	4.20	5
E (hours)	36	46	60	74	84

A: fish scales, B: molasses, and E: incubation time

In vitro screening of *B. cereus* strain SUK for its plant growth-promoting activities

B. cereus strain SUK was then tested for the various PGP traits like phosphate solubilization (Pikovskaya 1948), IAA production (Gordon and Weber 1951), siderophore production (Schwyn and Neilands 1987), ammonia production (Marques et al. 2010), hydrogen cyanide production (King et al. 1954), and exopolysaccharide production (Nicolaus et al. 1999).

Plant growth promotion study

The ability of studied organism along with fish scale lysate to promote growth of *V. radiata* was evaluated by pot trials. The seeds of *V. radiata* were surface-sterilized with 75% (*v*/*v*) ethanol for 10 min and were washed several times with distilled water. Briefly, 10 seeds were sown in each pot containing sterilized soil. Each pot was supplemented separately with water only (control; S1), sterilized degraded fish scales (S2), and degraded fish scales with the bacterial inoculum (having 10^8 cells/ml) (S3) in 10-ml amount. Plants were watered with water at 48-h interval, and after 2 weeks, plants were uprooted and analyzed for root, shoot length, and chlorophyll contents (Arnon 1949).

Statistical analysis

Results obtained were mean of three or more determinants. Microsoft Office data analysis tool pack was used to carry out one way ANOVA. The difference of $P \leq 0.05$ was considered as significant.

Table 5 Experimental design and results of central composite design (CCD). The observed values are average of triplicate determinations

Trials	A	B	E	Collagenolytic protease activity (U/ml)	
				Observed	Predicted
1	− 1	− 1	− 1	9.42	9.26
2	1	− 1	− 1	9.90	9.82
3	− 1	1	− 1	7.00	6.56
4	1	1	− 1	6.90	6.77
5	− 1	− 1	1	15.36	15.03
6	1	− 1	1	13.81	13.80
7	− 1	1	1	12.31	11.94
8	1	1	1	10.67	10.38
9	− 1.68	0	0	12.27	12.82
10	1.68	0	0	11.88	11.97
11	0	− 1.68	0	13.57	13.69
12	0	1.68	0	8.01	8.53
13	0	0	− 1.68	4.63	4.90
14	0	0	1.68	12.41	12.78
15	0	0	0	16.52	16.35
16	0	0	0	15.99	16.35
17	0	0	0	16.37	16.35
18	0	0	0	16.71	16.35
19	0	0	0	16.28	16.35
20	0	0	0	16.32	16.35

Result and discussion

Isolation and identification of collagenolytic protease producer and medium optimization for collagenolytic protease production

Screening for collagenolytic protease producing microbial strain

One hundred and thirty-five microorganisms were isolated on synthetic casein medium showing a zone of hydrolysis. Out of which 44 potent isolates were obtained by subsequent secondary screening on gelatin agar. On the basis of the ratio of a zone of hydrolysis to the colony diameter, significantly potent microorganisms were selected for further investigation (Table 1).

Six potent microorganisms showing the ratio of a zone of hydrolysis to the colony diameter five and more were grown in production medium and then subjected to quantitative tube assay for the selection of most potent microorganism. Demineralized fish scales were the only source of nutrition provided for the growth of microorganisms and enzyme production. All the six isolates completely degraded the fish scales over the period of time. The sequence in which the degradation of fish scales was done by the isolates was FL > FQ > EC > HA > EV > GD. Out of six strains, strain FL showed the highest production of an enzyme at 60 h of incubation and maximum degradation of fish scales within 72 h of incubation (Fig. 1). Hence, strain FL was further used to optimize the production of the collagenolytic protease with the help of response surface methodology.

Table 6 Statistical analysis of the model by Plackett-Burman design

Source	Sum of squares	d.f.	Mean square	*F* value	*P* value *P* > *F*
Model	25.57	3	8.52	34.29	< 0.0001*
A	8.33	1	8.33	33.53	0.0004*
B	7.39	1	7.40	29.75	0.0006*
E	9.84	1	9.84	39.61	0.0002*
Residual	1.99	8	0.25		
Corrected total	42.85	12			

*Significant *P* values at $P \leq 0.05$

Identification of the potent collagenolytic protease producing microbial strain

Identification of bacteria and its phylogenetic analysis was carried out by ribotyping, which is a widely accepted method (Saitou and Nei 1987). 16S rDNA sequencing of the potent isolate was carried out, and the results were submitted to GenBank (accession no. KX831892). The phylogenetic analysis demonstrated 99% sequence similarity of the isolate with *B. cereus*; so, it was named as *B. cereus* strain SUK. Using MEGA6 software, the phylogenetic tree of the sequence was constructed by neighbor-joining method (Fig. 2); it showed the detailed evolutionary relationship between the strain under study and other closely related *Bacillus* species.

Media optimization studies

Plackett-Burman design for screening of significant factors

Total seven variables viz., fish scales (*A*), molasses (*B*) inoculum, (*C*), pH (*D*), incubation time (*E*), stirring (*F*), and temperature (*G*) were selected to study their effects on collagenolytic protease production by using PB design (Table 2). Table 3 represents the yield of collagenolytic protease production for the constructed experimental design.

The statistical analysis using PB design (Table 6) indicated that fish scales (*A*), molasses (*B*), and incubation time (*E*) significantly affected collagenolytic protease production with *P* values less than the significance level, whereas the remaining components inoculum (*C*), pH (*D*), stirring (*F*), and temperature (*G*) were found to be insignificant with *P* values above 0.05. Using regression analysis, first-order polynomial was derived (Eq. (3)), representing collagenolytic protease production as a function of the independent variables.

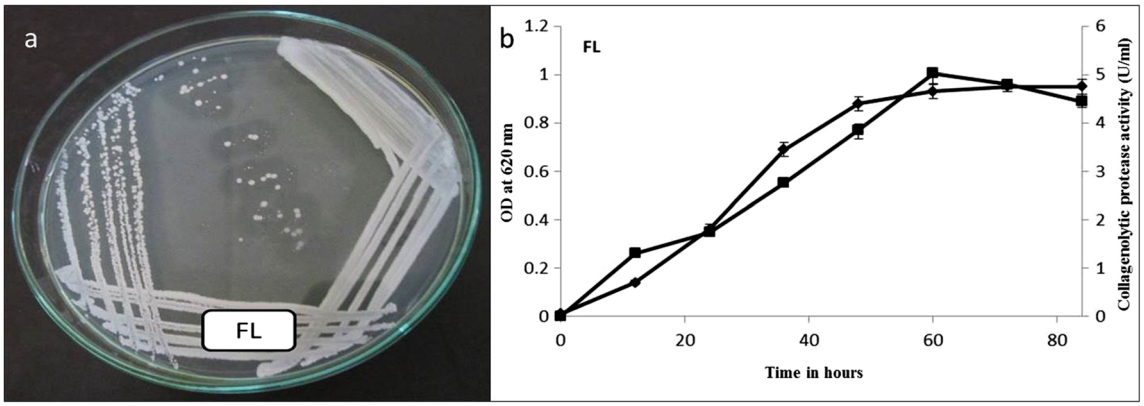

Fig. 1 **a** Gelatin plate assay. **b** Growth curve (♦); collagenolytic protease activity (■) of isolate carried out by standard assay method in 100-mM Tris-HCl buffer, incubated at 37 °C for 30 min. Each point represents the mean ($n = 3$) ± SD

Collagenolytic protease

$$= 6.431438 - 0.83333\ A - 0.78502\ B + 0.905797\ E \quad (3)$$

The model F value of 4.44 implies that the model is significant. The values of at $P < 0.05$ indicate that the model terms are significant. Using first-order equation, evaluation of the relationship between significant variables is not possible; so, in further investigation, second-order model in RSM experiment was used.

Medium optimization by RSM

CCD was used for further optimization of significant variables obtained from PB design. The high and low levels of the significant components are shown in Table 4. Table 5 indicates design of experiment and the yield of collagenolytic protease production for 20 experiments carried out using CCD analysis. The level of collagenolytic protease production as a function of the different variables, such as fish scales, molasses, and incubation time, was provided by the regression equation obtained using ANOVA. The quadratic regression of model was given as:

$$\text{Collagenolytic protease} = 16.34561 - 0.25276\ A - 1.53311\ B + 2.34445\ E - 0.08454\ AB - 0.44686\ AE - 0.09662\ BE - 1.39754\ A^2 - 1.85016\ B^2 - 2.65291\ E^2 \quad (4)$$

Equation (4) is the highly significant model ($P = 0.0001$), which is demonstrated by the ANOVA of quadratic regression (Table 7). The model F value of 173.19 suggests 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

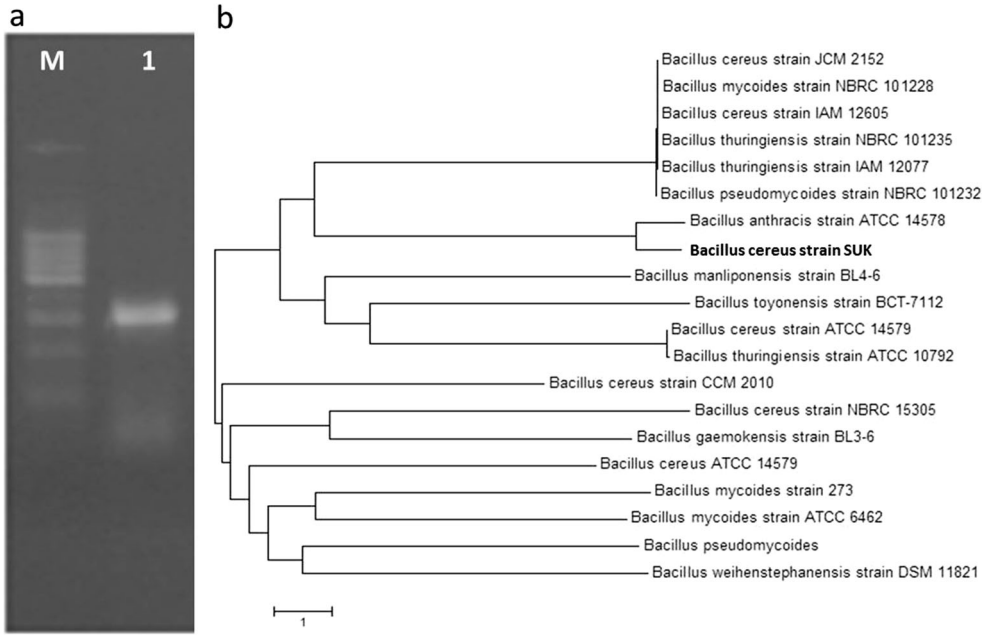

Fig. 2 **a** 1.5-kb PCR amplified product with DNA marker. **b** Phylogenetic tree of the *Bacillus cereus* strain SUK and other closely related *Bacillus* species based on 16S rRNA sequences. The tree was generated by using the neighbor-joining method. Bar = 1 substitutions per site

Table 7 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	262.24	9	29.14	173.19	< 0.0001*
A	0.87	1	0.87	5.19	0.0459*
B	32.10	1	32.10	190.80	< 0.0001*
E	75.06	1	75.06	446.17	< 0.0001*
AB	0.057	1	0.057	0.339	0.5728
AE	1.60	1	1.60	9.50	0.0116*
BE	0.075	1	0.075	0.443	0.5203
A^2	28.15	1	28.15	167.30	< 0.0001*
B^2	49.33	1	49.33	293.22	< 0.0001*
E^2	101.43	1	101.43	602.86	< 0.0001*
Residual	1.68	10	0.17		
Lack of Fit	1.39	5	0.28		
Pure Error	0.30	5	0.059	4.68	0.05771
Corrected Total	263.92	19			

*Significant *P* values at $P \leq 0.05$

is a measure of the total of reduction in the variability of response acquired by using the repressor variables in the model (Bas and Boyac 2007). The goodness of fit of the model was checked by using the determination coefficient (R^2), and it was adequate in this study, $R^2 = 0.9936$. The "Pred R^2" of 0.9585 is in reasonable agreement with the "Adj R^2" of 0.9879. The lack-of-fit value for this quadratic regression was not significant (0.0577) relative to the pure error. Hence, the model equation was acceptable 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 (Anderson and Whitcomb 2005). The "Adeq Precision" ratio of 39.466 indicates an adequate signal. Hence, this model can be used to direct the design space.

Interaction of variables

The significant or insignificant interactions between the corresponding variables were determined considering shapes of the contour plots, circular, or elliptical. Elliptical nature of the contour plots represents the significant interactions between corresponding variables, whereas insignificant interactions among the corresponding variables were shown by circular contour plots (Muralidhar et al. 2001; Jhample et al. 2015; Bhagwat et al. 2015). 3D response surface and 2D contour plots are usually the graphical representations of a regression equation. Using these 3D response surface plots and their respective 2D contour plots, it is very facile and convenient to decipher interactions between two variables and additionally to find their optimum levels. The obtained 2D contour plots can be used to predict values of collagenolytic protease production for different concentrations of the variables.

The 2D contour plots and their respective 3D response surface plots of collagenolytic protease production are shown in Fig. 3. Each figure represents the effect of two variables keeping other factor at zero level. The response surface curves and contour plots represented in Fig. 3a_1 and b_1 shows the interaction between molasses and fish scales. A positive interaction between molasses and fish scales was revealed by elliptical shape of the contour plot. Fish scales are rich in collagen; it acts as an inducer for collagenolytic protease enzyme. Enzyme production was considerably lesser when fish scales were used at 7 g l^{-1} in the production medium; production increases with simultaneous increase in the concentrations of fish scales from 7 to 10 g l^{-1}, but it was linearly decreased when concentrations increased from 10 to 13 g l^{-1}. Similar findings have been previously reported where enhanced protease production by *Bacillus* species was observed with respect to gelatin (Patel et al. 2005; Suphatharaprateep et al. 2011). Molasses is a rich source of sucrose as well as various micro-nutrients, which were equally important for the enhanced production of the enzyme; but in the case of molasses, contrast behavior was observed as compared to fish scales. Increased collagenolytic protease production was observed with lower concentrations of molasses viz., from 1.8 to 3.0 g l^{-1}, whereas concentration over 3.0 gl^{-1} of molasses drastically decreased collagenolytic protease production. These results were corroborated by previous reports where alkaline protease production by *Bacillus alcalophilus* LW8 was enhanced at the lower concentration of molasses (Rathod and Pathak 2014).

Apart from carbon and nitrogen sources, micro-nutrients and physical factors also influence the extracellular protease production by micro-organisms. Previous studies proved that the micro-nutrients (Bernal et al. 2006; Fakhfakh-Zouari et al.

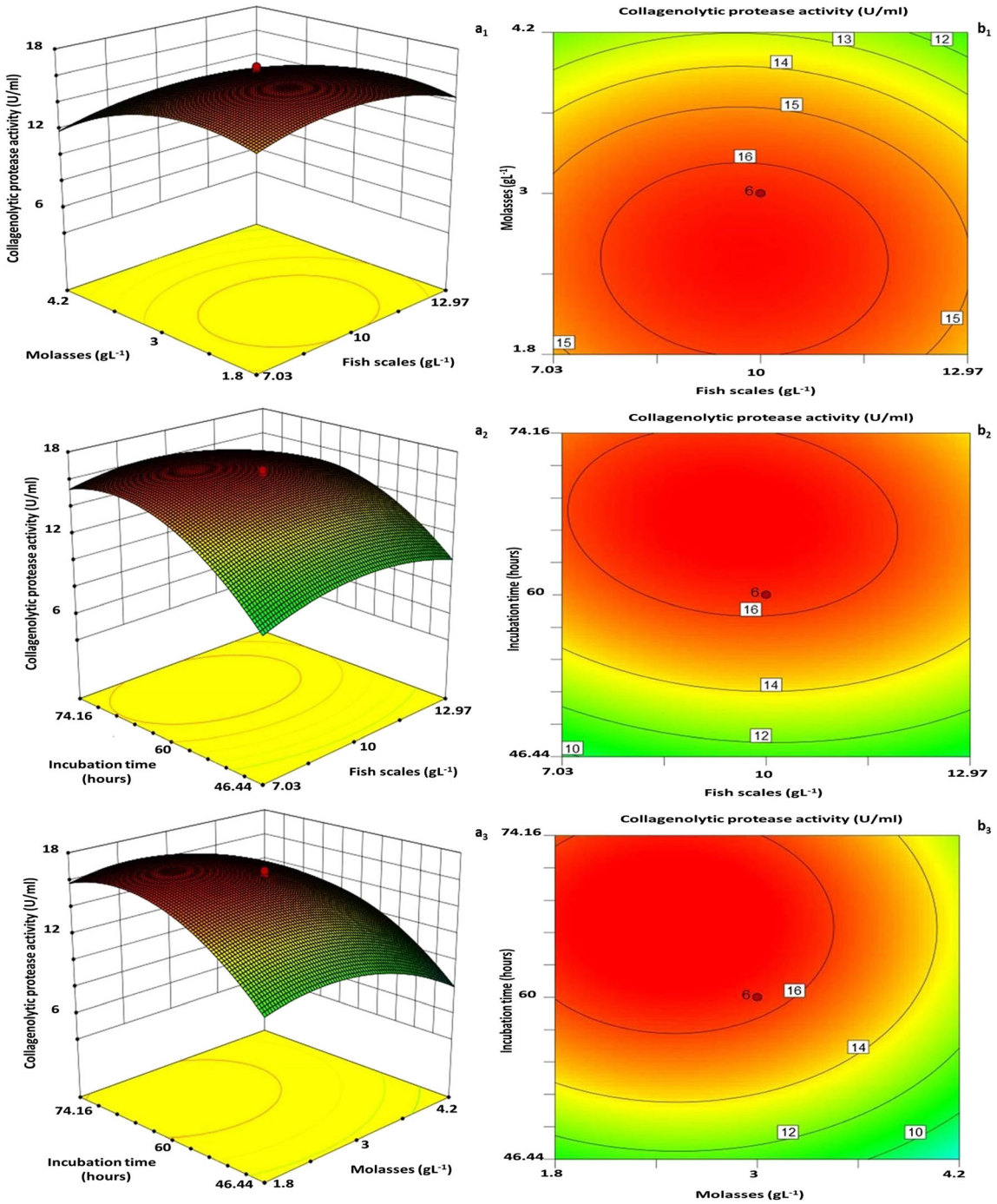

Fig. 3 3D response surface plots (**a**) and 2D contour plots (**b**) of collagenolytic protease production by optimization of significant variables. The interaction between ($\mathbf{a}_1$, $\mathbf{b}_1$) molasses and fish scales, ($\mathbf{a}_2$, $\mathbf{b}_2$) incubation time and fish scales, and ($\mathbf{a}_3$, $\mathbf{b}_3$) incubation time and molasses and its effect on collagenolytic protease production

2010; Bhagwat et al. 2015), and physical factors like incubation time (Razak et al. 1994; Oberoi et al. 2001; Puri et al. 2002) can also affect the yield of extracellular protease. Figure 3a_2, b_2 and a_3, b_3 represents the interactions in between incubation time and fish scales and incubation time and molasses, respectively. Both of them showed elliptical contour plots representing highly significant interaction with each other. Interaction of incubation time with both the substrates revealed that its lower levels showed lesser production of collagenolytic protease yield, while enhanced collagenolytic protease production was observed at higher incubation time. These results were supported by previous studies (Razak et al. 1994; Oberoi et al. 2001; Puri et al. 2002).

Validation of the experimental model

The processed parameters set to achieve maximum collagenolytic protease production which included the optimum concentration of fish scales 9.38 g l^{-1}, molasses 2.42 g l^{-1}, and incubation time of 67.34 h maximum collagenolytic protease production obtained experimentally were found to be 17.82 ± 0.34 U/ml. This was very clearly in close agreement with the model prediction value of 17.21 U/ml. The model can be validated due to the close correlation between the experimental and predicted values.

Amino acid and protein determination of optimized medium degraded by *B. cereus* strain SUK

With the help of response surface methodology, medium was optimized which contains 9.38-g l^{-1} fish scales and 2.42-g l^{-1} molasses; it was inoculated with the active culture of *B. cereus* strain SUK at 1% *v/v*. The medium was incubated at 37 °C, and samples were periodically withdrawn to check the contents of amino acids as well as proteins. Proteins were found to be highest at 54 h of incubation, while amino acid concentration was predominant at 60 h of incubation (Fig. 4). The maximum concentration of fish scales was found to be degraded at 60 h of incubation period, which was better as compared to previously unoptimized medium.

Antimicrobial activity assessment of the fish scale hydrolysate

The antibacterial activities of FSHs were evaluated against two Gram-positive (*S. aureus* and *E. faecalis*) and two Gram-negative (*E. coli* and *P. aeruginosa*) bacteria. As can be seen in Table 8, FSHs exhibited activity against the both Gram-positive bacteria and against *E. coli*; it clearly indicates that Gram-negative bacteria are more resistant than Gram-positive bacteria, which is in line with several works reported before (Lambert et al. 2001; Jemil et al. 2014). In general, antibacterial properties of FSHs could be either due to peptides produced during hydrolysis of proteins or by the collagenolytic protease produced by *B. cereus* strain SUK. As per the literature, various reports reported the antimicrobial potential of the collagen peptides (Najafian and Babji 2012; Ennaas et al. 2015; Ennaas et al. 2016) as well as various proteases (Eshamah et al. 2014); hence, it can be concluded that the antibacterial spectrum of FSHs was may be the synergistic action of collagen peptides and collagenolytic protease.

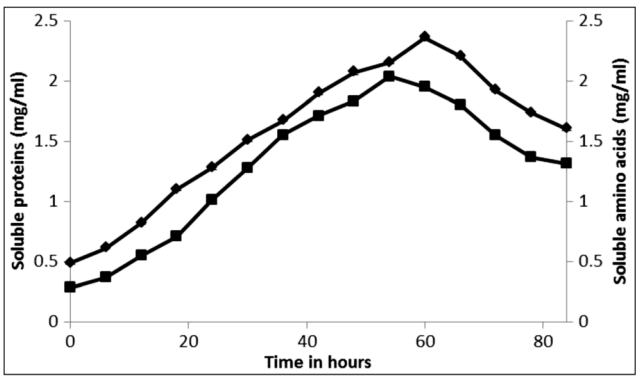

Fig. 4 Amino acids (♦) and protein content (■) solubilized by *Bacillus cereus* strain SUK. Each point represents the mean ($n = 3$) ± SD

Table 8 Antibacterial activity of FSHs at different concentrations

Microorganisms	FSHs concentration (mg/ml)			
	200	100	50	25
Staphylococcus aureus (NCIM 2654)	+	–	–	–
Enterococcus faecalis (NCIM 2403)	+	+	+	–
Escherichia coli (NCIM 2832)	+	+	–	–
Pseudomonas aeruginosa (NCIM 5032)	–	–	–	–

(+) presence of growth inhibition; (−) absence of growth inhibition

In vitro screening of *B. cereus* strain SUK for its plant growth-promoting activities

Isolated *B. cereus* strain SUK was positive for various PGP traits. It showed clear zone on Pikovskayas agar plate and yellowish orange halo zone around colony on CAS (chrome azurol S) agar plate indicating phosphate solubilization and siderophore synthesis. Development of brown to yellow coloration in peptone water after addition of Nesslers reagent confirms ammonia production. Strain SUK showed HCN production, which is indicated by change in the color of filter paper (soaked in 1% picric acid and moistened with few drops of 10% $NaCO_3$) (Lorck 1948) from yellow to brown placed on Kings agar plate. The organism also carries IAA production ability which is confirmed by development of pink color in supernatant after addition of salkowskys reagent, whereas *B. cereus* strain SUK was not able to produce exopolysaccharides. Genus *Bacillus* is well known for its PGP abilities, and previously, *Bacillus* has been reported for its variety of PGP traits (Joo et al. 2004; Kumar et al. 2012; Sunar et al. 2015; Bhange et al. 2016).

Table 9 Plant growth promotion study

	S1	S2	S3
Root length (cm)	1.47 ± 0.15	3.17 ± 0.21	3.5 ± 0.3
Shoot length (cm)	12.33 ± 0.35	13.66 ± 0.21	14.5 ± 0.3
Chlorophyll content (mg ml^{-1} g^{-1} FW)	1.64 ± 0.017	1.83 ± 0.023	2.25 ± 0.036

Each point represents the mean ($n = 3$) ± SD

Plant growth promotion study

Fish waste offers tremendous potential to mitigate disadvantages of chemical fertilizers. But, the direct use of fish waste in the soil is problematic as fishmeal attracts field mice, which dig tunnels under the seed beds (Nishio 1996). So, practically, if fish waste is directly applied in the fields, then it may affect the crop productivity extensively. To overcome this problem, fish scales were degraded by *B. cereus* strain SUK having multiple PGP traits.

Effect of supplementation of degraded fish scales on the growth of *V. radiata* was studied after 14 days of incubation. Table 9 clearly indicates the increased growth of *V. radiata* in the presence of fish scale-degraded metabolites and *B. cereus* strain SUK. The results showed that in the presence of degraded metabolites along with bacteria (S3) superiorly promoted the growth of *V. radiata*. Degraded end products of fish scales (S2) also have a beneficial effect on the growth of *V. radiata*. It was observed that the presence of degraded metabolites without and with bacteria considerably increases the root length, shoot length, and chlorophyll content. Similar results were observed where keratinous waste was degraded and utilized for plant growth promotion (Paul et al. 2013; Bhange et al. 2016), whereas collagenous waste was enzymatically solubilized and exploited for plant growth promotion (Bhagwat and Dandge 2016).

Conclusion

In an investigation into making more effective use of underutilized resources, fish scales, which otherwise cause serious environmental pollution, are used as a novel and inexpensive substrates for the production of collagenolytic protease. In the present study, response surface methodological optimization of collagenolytic protease production from *B. cereus* strain SUK was carried out using cheap and natural substrates like fish scales and molasses. The degraded FSHs containing collagen peptides and collagenolytic protease have antibacterial potential, which can restrict the growth of plant pathogens. Isolated collagenolytic producer *B. cereus* strain SUK has various PGP abilities. Further, the degraded products along with *B. cereus* strain SUK showed excellent plant growth promotion. Hence, metabolites released by fish scales degradation along with *Bacillus* sp. SUK could be successfully employed as an economic source of nitrogen fertilizers as well as PGPB for plants. This study could be useful as it facilitates the effective utilization of fish waste resulting into the production of various value-added products in an eco-friendly way.

Funding information Prashant K. Bhagwat is thankful to UGC for awarding BSR Meritorious Fellowship for Doctoral Research. Corresponding author wish to thank UGC-MRP with sanction grant No. F. No. 41-1282/2012 (SR).

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