

Thermo-Stable Alkaline Protease Purified from a Novel Endophyte *Brevundimonas diminuta* VKB1 Hosted in *Carica papaya* L. – Production Enrichment Approach

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Abstract: Protease is the central component of enzymes in all lives, from microbes to humans. Their limited applications in the medicinal field demand their need for higher production. Our study enlightens protease's sustainable production using various optimization techniques and purification of the protease purified from *Carica papaya* L's endophytes. The results depict that the protease synthesis was enhanced to five-fold concentration after optimization of the production media. The purified protease was a single protein showing high stability towards alkaline pH and thermo-stable. The metal ions' influence indicates that the protease can be catalyzed highly in the presence of copper, magnesium, iron atoms. Results disclose the purified protease further can be taken for pharmacological studies to understand their efficiency as a therapeutic regime.

Keywords: endophyte; protease; Placket-Burman; response surface methodology; purification.

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1. Introduction

Symbiotic growth of microbes inside the host plant without affecting them are termed endophytes. These symbiotic interactions were highly facilitated by the extracellular enzyme produced by these microbes. It was also reported that the enzymes also inhibit many plant pathogens and withstand biotic stress. Hence the capability of endophytes living together with host and synergistic production of these enzymes helps counteract emerging issues. Further, these extracellular enzymes can be taken for various large-scale fermentation studies, as they adapt extremely to various environmental conditions [1,2]. Some examples of bacterial endophytes from *Rhizophoraceae* [3], *Acanthaceae* [4], and various mangrove plants [5] have exhibited significant enzyme activity. Also, it has the capability of producing rare enzymes such as L-Asparaginase [6], heat liable glucoamylase [7], and fibrin degrading protease [8,9].

Carica papaya L. is a fruit that is extensively grown in the region of tropical and subtropical environments [10]. The latex and unripe fruit are rich in protease enzymes like papain, chemo papain, peptidase A and B, lysozymes [11,12]. Though it is a pack of enzyme-rich fruit, researchers restricted it to the limit of purifying the papain, a well-known protease enzyme [13,14]. Also, the production of the inactive form of protease, low purification yield, production based on varieties, wastage of fruits is some of the drawbacks that limit the enzyme production and purification [15,16]. In an alternative method of enzyme purification, microbes residing inside the fruit termed endophytes can be isolated and identified for further studies. Studies reported that strains isolated from *Carica papaya* L. exhibited predominantly *Bacillus*

species followed by other bacterial species such as *Kocuria*, *Acinetobacter*, *Staphylococcus*, and *Enterobacter*. They can produce various extracellular enzymes like amylase, protease, pectinase, cellulase, etc. [11].

Hydrolytic enzymes are the central components found in all animals, plants, humans, and microbes; they cleave peptide bonds of a long polypeptide amino acid chain through the hydrolysis process. The degradative nature derives protein modification based on their selectivity and specificity of the enzyme as they contribute 60% worldwide application in industrial sectors. Microbial source of protease purified in a shorter time with high substrate specificity and catalytic activity [17,18]. Hence many industrial and biotechnological sectors concentrate on the microbial enzyme production that replaces or rejuvenates the host enzyme activity. The active therapeutic proteases have been in research based on their diversity and specificity. Depletion in the body enzyme leads to several pains, injuries, inflammation, and diseases. The underlying cause of many unexplored health issues paved the way for developing novel enzymes [19].

Hence, the study's insight relies on identifying and screening protease-producing endophytic bacteria from the host *Carica papaya* L. Substantial increment of the enzyme production was obtained using statistical optimization techniques Plackett-Burman and response surface methodology designs. Further enzyme purification was carried out under the optimized medium condition, and various biochemical characterization was performed to understand its stability and molecular weight.

2. Materials and Methods

2.1. Sample collection.

The unripe *Carica papaya* L. (Papaya) was collected from the tree near the Vellore Institute of Technology, Vellore.

2.2. Surface sterilization.

Surface sterilization was carried out by a continuous tap water wash followed by rinsing in 70% ethanol for one minute. Further, the sample was soaked in a 0.1 % Sodium Hypochlorite solution containing 1 % tween-20 surfactant for four minutes to remove cell debris and other microbes. Then the sample was rinsed and washed thrice in sterilized double-distilled water. The third time used water was taken as a positive control for analyzing sterilization quality [20].

2.3. Isolation of bacterial samples from the surface-sterilized papaya.

The isolation procedure was carried out by the impregnation method. The surface-sterilized sample was made into small pieces using a sterilized knife. For the impregnation method, the endocarp region was placed in contact with the nutrient agar medium. The plates were incubated for seven days at 37° C. Colonies; specifically, bacterial cultures were identified based on their distinct shape, size, and color and separately pure cultured for further screening [20,21].

2.4. *Qualitative selection of protease producing bacteria.*

The selection of strains based on their enzyme activity was carried out before strain identification using the skim milk agar test. The isolate VKB1 that is exhibiting the maximum zone of degradation was taken for further studies.

Bacterial cultures were inoculated as a single streak on skimmed milk agar plates containing (g/l) peptone, 1; glucose, 1; beef extract, 5; skim milk powder, 8; and agar, 15. The appearance of a zone of degradation was found in plates after following the incubation for two days at 37 °C. Strains showing the degradation of casein by forming a clear zone were taken for fibrin plate assays [22,23].

2.5. *Molecular and morphological characterization of bacteria.*

The screened-out bacteria were chosen for their species identification using morphological, biochemical, and molecular characterization. Genomic DNA of the selected strain was extracted from cells of a 24 h culture. The 16S rDNA sequencing of the selected strain was PCR amplified using universal primers using ABI VERTIC thermocycler. The PCR product was purified with a base pair of approximately 1777 bp were sequenced using the upstream primer 27F and downstream primer 1492R. Samples were performed with PCR analysis using the ABI PRISM[®] BigDye[™] terminator cycle kit. The product resulted were electrophoresed in an ABI3730XL automated DNA sequencing system. Then the obtained nucleotides were subjected to a homology search using the National Centre for Biotechnology (NCBI) BLAST program [24]. The obtained sequence was submitted to GenBank.

2.6. *Factors influencing protease production under submerged fermentation.*

Submerged fermentation for protease production is preferred due to its advantages, such as consistency in enzyme characteristics, effortless downstream approach, and processing conditions. Hence studies have focused on process development and scale-up of the protease purification under optimized physical parameters and nutrient factors [25–28]. The variables of each factor influencing protease synthesis in the bacteria were selected based on the one-factor at a time approach (OFAT). Physical parameters such as pH (4, 6, 7, 8, 9, 12), RPM (50, 100, 150, 200), fermentation period (12, 24, 36, 48, 60, 72, 84, 96), temperature (4, 25, 37, 45, 60 (°C)) was studied. Similarly, nutrient factors such as carbon source (Dextrose, Maltose, Xylose, Sucrose, Starch and Galactose), organic nitrogen source (Yeast Extract (YE), Beef Extract (BE), Casein, Peptone, Urea, and Gelatin) inorganic nitrogen source (Ammonium Chloride, Sodium Carbonate, Sodium di-hydrogen Phosphate, Potassium di-hydrogen Phosphate, and Ammonium Sulphate) was analyzed. All the optimization was carried out in triplicates. The mean value was taken for the estimation of enzyme activity by the following protease assay. With some modifications in the experiment, the casein solution (2.5 mL, 1.2% w/v) was mixed with the crude enzyme dissolved in 2.5 mL of the 0.1 M Tris-HCl buffer (pH 7.8) incubated for 30 min at 37°C. Further, the reaction was stopped by adding 5 mL of trichloroacetic acid (0.11 M) containing sodium acetate (0.22 M) and acetic acid (0.33 M). Precipitation of the remaining protein was centrifuged at 10000 rpm for 10 mins, and the samples were measured at 275 nm against Tris-HCl as blank [29]. Total protein estimation was carried out by the Lowrys method [30].

2.7. Statistical optimization for improvised enzyme yield.

The major drawback of the traditional OFAT approach is difficulty analyzing the optimal conditions resulting from the interaction among different enzyme synthesis factors. Other disadvantages are time-consuming and expensive in screening out the variables out of substantial experimental factors. Hence, to eliminate these difficulties, experimental factorial design and response methodology can be used for medium optimization [2,31,32].

The experimental factorial design, such as Plackett-Burman (PB) design, helps select significant factors from many fermentation variables. Further, the results from this design are used in the collection of variables in further optimization. Response surface methodology (RSM) guides in designing experiments, building models, evaluating the effects of different factors, and searching the optimal conditions upon the interaction of various factors for the desired enzyme activity [33–35].

2.7.1. Significant variable analysis using Plackett-Burman design approach.

Plackett-Burman's (PB) factorial design was used to screen the significant factors that are affecting the production of a protease by the strain. A total of six factors was selected from the OFAT approach for the experiment that includes maltose as a carbon source, yeast extract as a nitrogen source, sodium di-hydrogen phosphate as an inorganic nitrogen source, 72 h of the fermentation period, pH, and rate of agitation (Table 1). Based on the PB design, every selected factor was analyzed at two levels: lower level (−1) and higher level (+1). The list of factors and levels were given in Table 1.

Table 1. Factors selected for Plackett-Burman design.

Factors	Units	Coded levels	
		Low	High
Maltose (A)	% (w/v)	0.5	1
Yeast Extract (B)	% (w/v)	0.1	1
NaH ₂ PO ₄ (C)	% (w/v)	0.05	0.25
pH (D)		4	9
Fermentation period	H	72	96
Agitation	rpm	100	150

The experimental design is based on the first-order polynomial model:

$$Y = \beta_0 + \sum \beta_i X_i \tag{1}$$

where Y is the response activity (total enzyme activity in U/mL), β_0 termed model intercept, and β_i is the linear coefficient, and X_i is the level of the independent variable. Though the model does not explain the interaction between factors, it evaluates the significant factors that influence the response. All the design experiments were performed in triplicates, and their average enzyme activity was taken as a response (Y). All the calculation of statistical analysis was performed using Analysis of Variance (ANOVA) and the significant factors ($p < 0.05$) for the protease production were optimized using response surface methodology (RSM). All the experiments were carried out using statistical software, Design-Expert 10 [36].

2.7.2. Optimization using response surface methodology: central composite design approach.

The response surface methodology (RSM) was performed to analyze the effects of selected variables on the protease yield. Three independent variables (yeast extract, pH, and fermentation period) were selected for the central composite design (CCD) of RSM to estimate the relative significance of the factors even in their complex interactions (Table 2). Selected main factors were studied at five different levels ($-\alpha$, -1 , 0 , $+1$, and $+\alpha$) in a set of twenty experiments that includes eight factorial, six axial, and six center points.

Table 2. Individual variables and their coded values for central composite design.

Factors	Units	Coded levels				
		$-\alpha$	-1	0	$+1$	$+\alpha$
Yeast Extract	% (w/v)	-0.206807	0.1	0.55	1	1.30681
pH		2.29552	4	6.5	9	10.7045
Fermentation period	h	63.8185	72	84	96	104.182

The experimental design data were evaluated as a second-order polynomial regression equation comprising both individual and combinatorial effects of each variable.

$$Y = a_0 + \sum_{i=1}^3 a_i C_i + \sum_{i=1}^3 a_{ii} C_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 a_{ij} C_{ij} \tag{2}$$

where Y is the experimental and predicted response, a_0 is the offset term, a_i , a_{ii} and a_{ij} are the coefficients of linear terms, square terms, and the interactive terms, respectively and C_i 's were A, B, and C, C_{ij} 's are AB, BC, and CA. All the experiments were performed in duplicates, and the mean proteolytic activity (U/mL) was taken as a response (Y).

ANOVA was done to estimate the model. The Values of “Prob > F” (< 0.05) indicated that the model factors were significant. The fitted polynomial equation was given as 3D surface plots to view the relationship between the responses and each factor's levels in the design. The combinatorial effect of each optimized parameter that gave maximum enzyme activity (Y) was tested experimentally to confirm the model's validity [37,38].

2.8. Separation and purification of protease.

Based on the optimized parameters, the production medium was modified, and the culture was mass-produced. After incubation, the medium with culture was centrifuged, and the cell-free supernatant was collected. The crude enzyme was subjected to ammonium sulfate precipitation with 60 % saturation. The enzyme salt mixture was left overnight at 4° C, and then the protein precipitate was separated by centrifugation at 10000 rpm for 10 min. The pellet was collected and dissolved in 2 mL of 0.02 M Tris-HCl (pH 7.8), desalted with a dialysis bag with the same buffer at 4° C overnight. The obtained dialysis fraction was concentrated by lyophilization. The lyophilized sample was resuspended in 0.02 M of Tris-HCl buffer (pH 7.8). The resuspended sample was loaded onto a DEAE-Cellulose column, and the protein samples were eluted by sequential extraction with the same buffer with a gradient increase in NaCl concentration (0 M to 1 M). All the samples from each fraction were measured with the protease activity were pooled together. The pooled-out samples were dialyzed again with Tris-HCl buffer (pH 7.8), then lyophilized and resuspended in the same buffer for gel filtration chromatography. Lyophilized and the concentrated sample was applied to the Sephadex G100 column pre-equilibrated and eluted (at a flow rate of 30 mL/h) with 0.5 M of Tris HCl

containing 0.15 M NaCl. Active fractions were collected and pooled together after protease analysis and protein concentration determination. The pooled fractions were lyophilized and stored at $-20\text{ }^{\circ}\text{C}$ for further characterization [24,39,40].

2.9. HPLC analysis.

The lyophilized purified sample obtained from gel filtration chromatography was dissolved in 20 mM of Tris-HCl buffer (pH 7.8) and analyzed for purity using High-Performance liquid chromatography equipped with C18 column, UV Detector-2487 (Waters HPLCPump-1525, Milford, MA, USA). The mobile phase was water and acetonitrile in the ratio 1:1. The purity was authenticated at 280 nm [41].

2.10. SDS-PAGE.

The purified sample was subjected to analyze the mass and purity by SDS-PAGE (12% [w/v]). The purified proteins were loaded onto gels after being heated in an SDS sample buffer. The molecular weight was determined using a wide range of protein markers (Genei, Bangalore). The protein bands were visualized by performing a silver stain method [27,42].

2.11. Biochemical characterization of the purified enzyme.

The effect of pH on the extracellular protease was studied with varying pH ranging from 3 to 12 and various buffers (20 mM): sodium acetate buffer (pH 3-6); Tris HCl buffer (6.5-8.8); Phosphate buffer (9-12). The enzyme activity was estimated by performing the protease assay after incubating the purified enzyme in all the buffer pH ranging from 3 to 12 for four hours at room temperature. Similarly, the effect of temperature was also studied by incubating the purified protein at a different temperature ranging (4, 25, 37, 45, and $60\text{ }^{\circ}\text{C}$) for four hours for the stability analysis, and the enzyme assay was performed. Various inhibitors: PMSF, DTPA, EDTA, and metal ions: MgSO_4 , MnSO_4 , CuSO_4 , ZnSO_4 , FeSO_4 , and CaCl_2 at a concentration of 5 mM was mixed with one μg of the purified enzyme and incubated for two hours and their enzyme activity was evaluated. The evaluated enzyme activity was reported as the percentage residual activity [40,43,44]

3. Results and Discussion

3.1. Isolation and screening of endophytes for protease activity.

The study revealed that the unripe papaya was enriched with various beneficial endophytic bacteria.

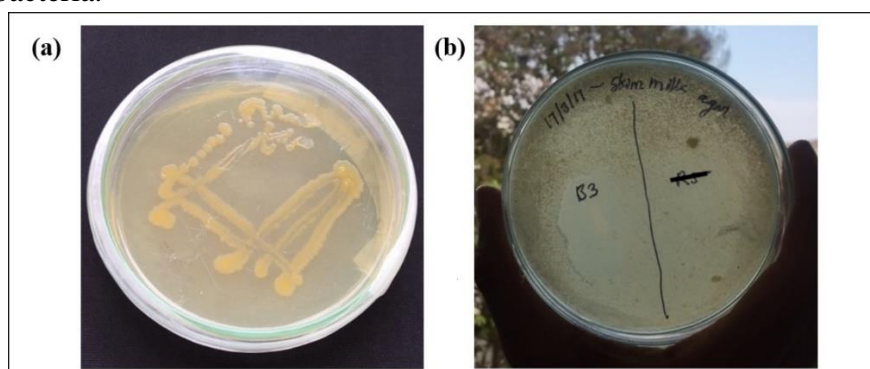


Figure 1. Endophytic bacterium purified and screened for its protease production. (a) Streak culture of the pure cultured endophytic bacterium VKB1; (b) zone of degradation in skim milk agar to screen protease activity.

Around fifteen colonies were isolated from the endocarp region. The control plates without bacterial colonies define that the isolated colonies are endophytes. All the isolates were screened for protease activity qualitatively using the skim milk agar test. Out of all the strains, VKB1 (Figure 1 (a)) has exhibited good extracellular protease activity by degrading the casein in the skim milk agar plate (Figure 1 (b)).

3.2. Strain identification.

The morphological characterization using gram staining concluded that the selected strain VKB1 is a gram-negative bacterium. Further biochemical characterization depicts that the bacteria can hydrolyze casein and gelatin. It also illustrates that the VKB1 strain can produce other enzymes such as catalase, oxidase, etc. The strain VKB1 was further confirmed as *Brevundimonas diminuta* based on its 16s rRNA sequence and named *Brevundimonas diminuta* VKB1. The 1239 bp 16s rRNA sequence of *Brevundimonas diminuta* VKB1 was submitted in the GenBank database and assigned with an accession number MN744337.1 (Figure 2). The detailed evolutionary relationship between the strain VKB1 and other closely related *Brevundimonas* species families.

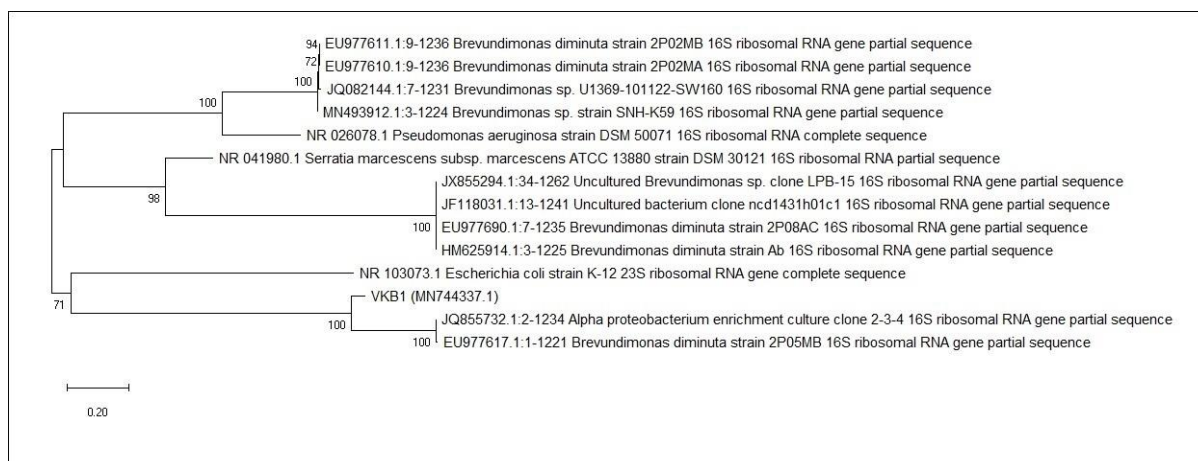


Figure 2. Phylogenetic tree constructed based on the 16s rRNA sequence showing that the strain VKB1 is closely related to Alpha-proteobacterium and *Brevundimonas diminuta* strain. *Escherichia coli* strain (K-12, NR103073.1) and *Serratia marcescens* strain (DSM 30121, NR041980.1) were used as an outgroup.

3.3. Preliminary screening of parameters using OFAT.

Understanding the protease's kinetics and catalytic mechanisms from a new microbe is crucial due to their distinct nature in genetics, metabolic, and enzyme production pathways[45]. It is necessary to understand the influence of physical and nutrient parameters for the fermentation and production of an enzyme [27,36]. A set of OFAT approach experiments was performed to establish a cost-effective and cheap formulation of media for protease production. The extracellular protease synthesis from a bacterium doesn't depend only on the carbon and nitrogen sources but other physical factors such as Incubation time, temperature, pH, and agitation. Studies reported that the alkaline pH and optimal temperature between 30 to 70 °C influence the production of protease at a higher concentration [28,46]. The synthesis of protease by the endophyte VKB1 was highly influenced by the nutrient factors' maltose, yeast extract, and sodium dihydrogen phosphate (Figure 3) that has grown at 37 °C for 72 h under an alkaline condition of pH 8. Hence these parameters were chosen for further optimization in PB design for protease production.

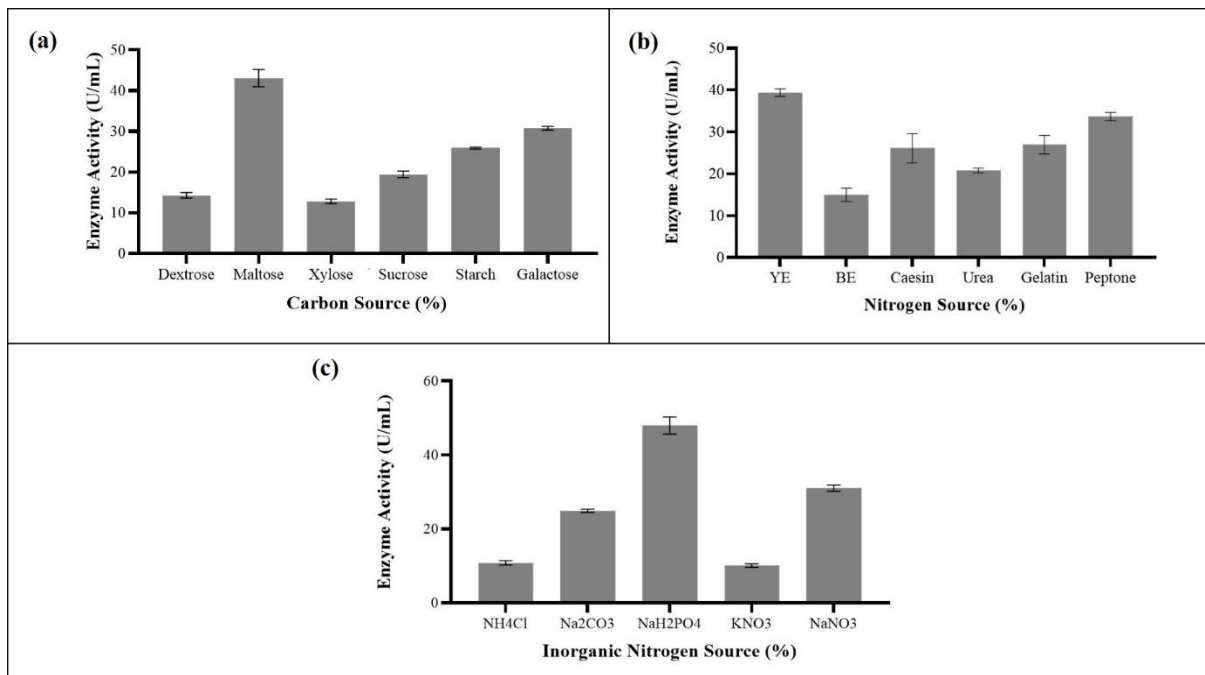


Figure 3. Effects of different carbon and nitrogen sources at an optimum condition (37 °C and pH 7 with 100 rpm).

3.4. Selection of significant factors using the PB design.

Totally six factors were analyzed for their influence on the extracellular protease synthesis using PB design. The PB design matrix for evaluating effective variables in the enzyme production and their response (Y) is given in Table 3.

Table 3. Design model and response (Y) for the Plackett-Burman design.

Run	Maltose	Yeast Extract	NaH ₂ PO ₄	pH	Fermentation Period	Agitation	Enzyme Activity (U/mL)
1	1	1	0.05	4	72	100	333.52
2	0.5	0.1	0.25	9	72	150	261.38
3	0.5	1	0.05	9	96	150	820.31
4	1	0.1	0.05	4	96	150	361.67
5	1	0.1	0.25	9	96	100	505.18
6	0.5	1	0.05	9	96	100	688.62
7	1	1	0.25	4	96	150	688.62
8	1	0.1	0.05	9	72	150	401.66
9	0.5	0.1	0.05	4	72	100	260.34
10	0.5	1	0.25	4	72	150	291.95
11	1	1	0.25	9	72	100	542.94
12	0.5	0.1	0.25	4	96	100	134.18

The fitness of the model and their significant effect were calculated using the student's t-test ANOVA. Factors that are showing a low p-value ($p < 0.05$) are the significant variables for the enzyme production, and they were selected for subsequent optimization studies. Hence significant variables like Yeast extract (B), pH (D) and Fermentation period (F) shows p-value of 0.0099, 0.0265 and 0.0461 respectively comparing maltose (A) (0.3188), NaH₂PO₄ (C) (0.2599) and agitation (F) (0.3188). The main effect of the medium components, the ANOVA, regression coefficient, F values, a p-value of the variables are shown in Table 4.

Table 4. ANOVA for selected Plackett-Burman design.

Source	Sum of Squares	df	Mean Square	F Value	Prob > F	significant
Model	4085.51	3	1361.84	7.73	0.0095	significant
B-Yeast Extract	1990.48	1	1990.48	11.30	0.0099	
D-pH	1298.13	1	1298.13	7.37	0.0265	

Source	Sum of Squares	df	Mean Square	F Value	Prob > F
E-Fermentation Period	796.91	1	796.91	4.52	0.0461
Residual	1409.38	8	176.17		
Cor Total	5494.89	11			

The model F-value 7.73 implies that the models are significant, and there is only a 0.95 % chance that an F-value this large could occur due to noise. The “Predicted R-squared” of 0.4229 is in reasonable agreement with the “adjusted R-squared” of 0.6473. Adequate precision measure the signal-to-noise ratio. A ratio greater than four is desirable. In this model, the adequate ratio is 8.203. Hence the final first-order regression equation derived in terms of coded factors for this experimental model revealed the yield of protease production.

$$\text{Enzyme Activity} = +147.61 + 12.88 * B + 6.24 * D + 8.15 * E$$

From the analysis of protease production under the PB design method, it is revealed that both nutrient and physical factors play a role in the influence of protease production. Significant factors and their positive effect were selected for the CCD analysis to understand their specific effects on enzyme production's combinatorial range.

3.5. Central composite design - response surface methodology.

CCD was used for the optimization and interaction of three variables such as yeast extract, pH, and fermentation period each were studied at five coded levels of (-α, -1, 0, +1, and +α) as shown in Table 2. The models were performed in twenty different runs (Table 5). The maximum enzyme activity of 678.17 U/mL was observed at run 15. The results from the design were calculated using ANOVA. P-value = <0.0001 shows that the model is significant (Table 6). Also, there is only a 4.16% chance that this large F-value could occur due to noise. According to the model and ANOVA experimentation, it was observed that the A, B, C, AB, BC, AC, A², B² and C² are important terms. The second-order polynomial models were used to relate the independent variables with enzyme activity. The calculated coefficient of determination (R²) 0.9998 indicates that the model could describe 91% of the variability. The “lack of fit F value” of 0.718 shows the lack of fit is not significant relative to the pure error determining that the non-significant lack of fit value is good. The signal-to-noise ratio was measured using adequate precision, which should be greater than 4. In this model, the ratio is 193.623 indicates an adequate signal, and therefore, the model is significant for the enzyme bioprocess.

Table 5. Experimental design and response (Y) for central composite design.

Run	Yeast Extract	pH	Fermentation period	Response (Y)			
				Enzyme (U/mL)	Activity	Predicted (U/mL)	Activity
1	0.55	6.5	63.8185	1361.4		1383.41	
2	0.55	6.5	84	1494.09		1493.13	
3	1.30681	6.5	84	1393.25		1377.92	
4	1	4	96	1208.53		1261.8	
5	0.1	9	96	1376.31		1357.27	
6	0.55	6.5	84	1499.09		1493.13	
7	-0.206807	6.5	84	1149.9		1121.13	
8	0.1	4	72	1143.66		1117.8	
9	1	4	72	1340.89		1320.4	
10	0.55	2.29552	84	1084.68		1107.76	
11	0.55	6.5	104.182	1423.51		1457.4	
12	0.55	6.5	84	1490.11		1493.13	
13	0.55	6.5	84	1493.56		1493.13	
14	0.1	4	96	1193.94		1162.15	
15	1	9	72	1478.17		1416.43	
16	0.55	10.7045	84	1319.77		1352.59	

Run	Yeast Extract	pH	Fermentation period	Response (Y)			
				Enzyme (U/mL)	Activity	Predicted (U/mL)	Activity
17	1	9	96	1573.72		1460.05	
18	0.55	6.5	84	1497.23		1493.13	
19	0.1	9	72	1183.49		1210.69	
20	0.55	6.5	84	1494.3		1493.13	

Table 6. ANOVA for response surface methodology.

Source	Sum of Squares	Df	Mean Square	F Value	Prob > F	
Model	4.169E+005	9	46321.04	61.56	< 0.0001	significant
A-Yeast Extract	90735.17	1	90735.17	120.59	< 0.0001	
B-pH	91858.31	1	91858.31	122.08	< 0.0001	
C-Fermentation Period	7070.67	1	7070.67	9.40	0.0119	
AB	9818.91	1	9818.91	13.05	0.0047	
AC	9793.70	1	9793.70	13.02	0.0048	
BC	17154.15	1	17154.15	22.80	0.0008	
A ²	73227.78	1	73227.78	97.32	< 0.0001	
B ²	1.323E+005	1	1.323E+005	175.79	< 0.0001	
C ²	11743.02	1	11743.02	15.61	0.0027	
Residual	7524.34	10	752.43			
Lack of Fit	1.328E+005	5	2.656E+022	2.73	0.756	Not significant
Pure Error	4.85E+07	5	9.71			
Cor Total	5.139E+005	19				

The response (Y) was well correlated with a quadratic second-order polynomial equation. The final equation in terms of coded factors:

Enzyme Activity

$$= +494.0 + 70.35 * A + 146.41 * B - 122.69 * C + 71.04 * AB - 2.63 * A - 76.44 * BC - 133.46 * A^2 - 86.35 * B^2 - 86.32 * C^2$$

The 3D surface plots show the impact of the interaction of two variables for the enzyme production while the third one is kept as a zero-coded level (Figure 4 (a-c)). Among the variables used in CCD, yeast extract and pH significantly affect enzyme production compared with the other variables. The perturbation plot represents that the factors fermentation period, pH, and yeast extract have a vital role in enzyme production. Comparing the previous OFAT (43.7 U/mL) optimization, the enzyme's synthesis has increased fifteen-fold to the enzyme activity 678.17 U/mL in the RSM. Similarly, the validated experimental results conducted with the optimized conditions show that the enzyme production was 678.17 U/mL. It shows a good agreement with that of the predicted value that validates the model design.

3.1. Separation and partial purification of the protease.

The extracellular protease from *Brevundimonas diminuta* VKB1 was partially purified, as shown in Table 7. Specific activity was calculated at each step of purification by the obtained enzyme activity and protein concentration. It was observed that the gel filtration purified enzyme was showing 5.4-fold activity comparing the crude protein content, and the protein recovery was near 53.92%. The protein's molecular weight was determined by SDS-PAGE, which showed protein bands with a mass value of ~25 kDa (Figure 5 (a)).

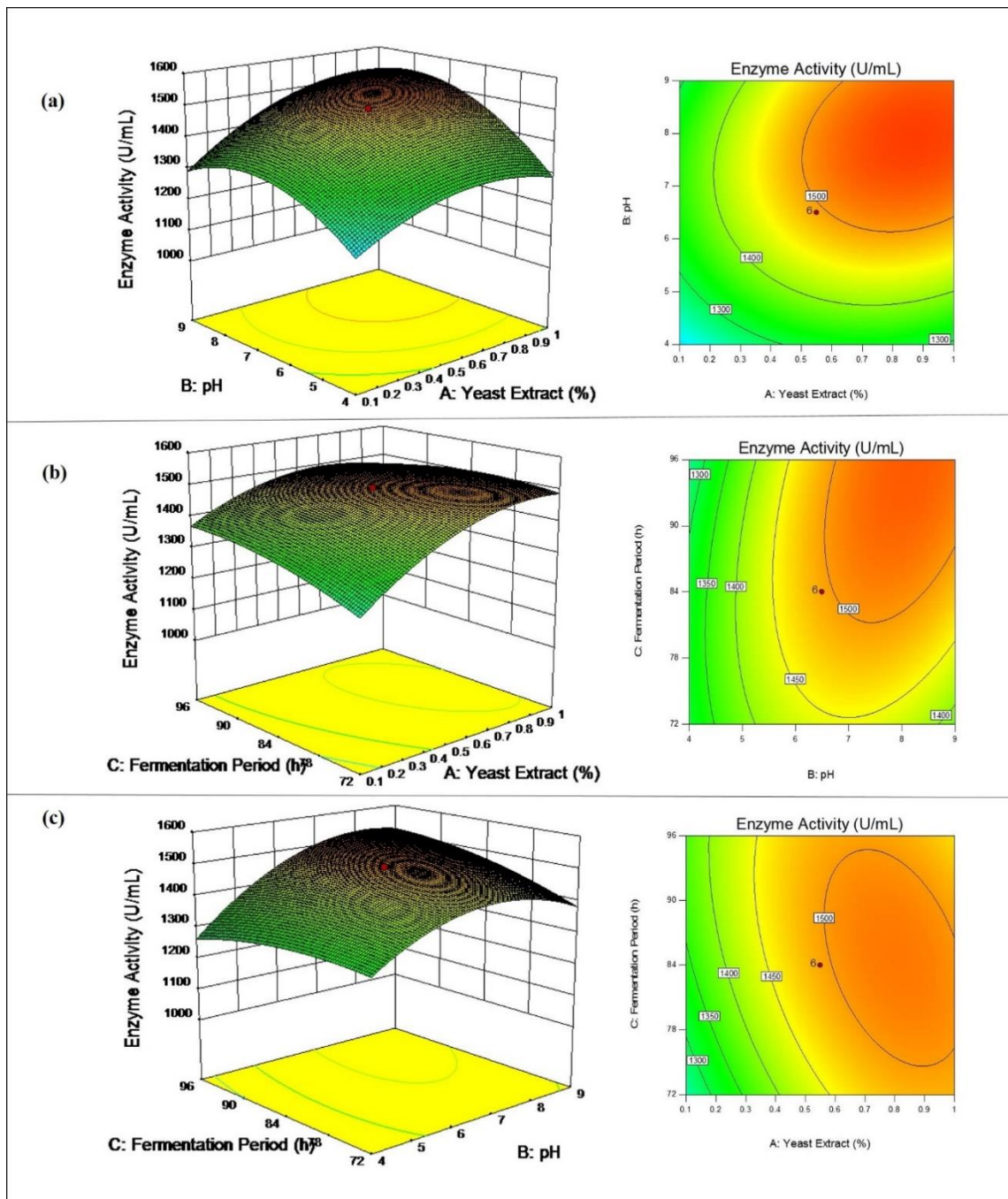


Figure 4. 3D surface representation of the RSM analysis. (a) shows elliptical contour plots indicating significant interactions among yeast extract and pH; (b) shows elliptical plots revealing moderate interaction among fermentation period and yeast extract; (c) also showing elliptical plots with high interactions among fermentation period and pH.

Also, according to the results of HPLC, the partially purified enzyme was showing two peaks. As the protein peaks were merged, it was assumed that they could be a single protein that might get degraded. The efficiency of purified protease in protein degradation using fibrin and casein as substrate was performed, and the zone of degradation was observed (Figure 5 (b) and (c)).

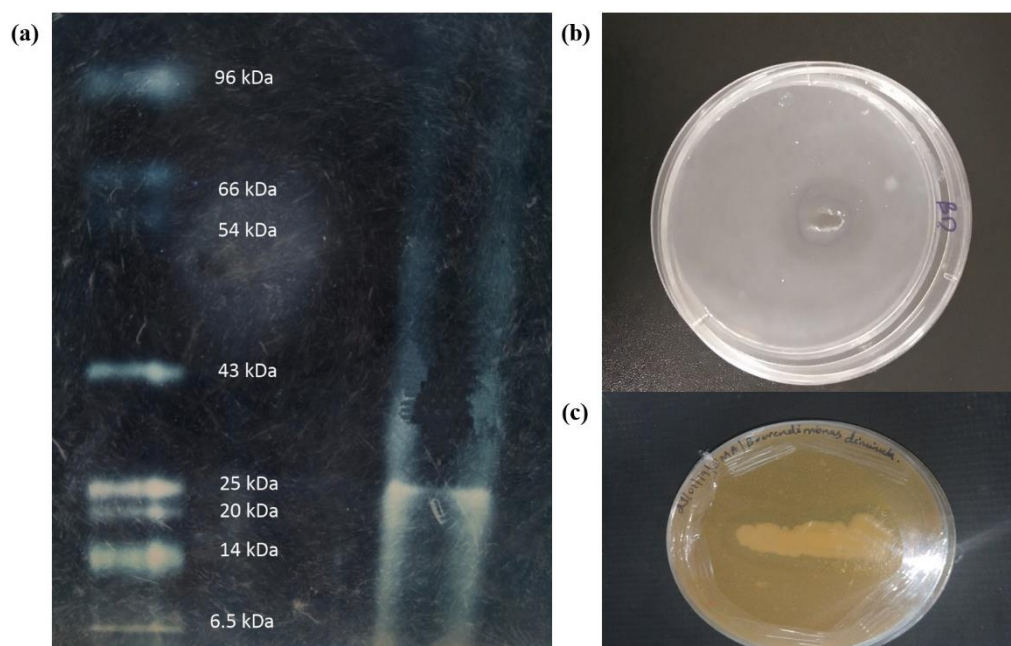


Figure 5. Molecular weight determination and protein degradation. **(a)** SDS-PAGE analysis revealing the approximate molecular weight of the protease purified using marker lane; **(b)** zone of degradation appeared after incubating the substrate fibrin with protease overnight using fibrin plate assay; **(c)** zone of degradation appeared after incubating the substrate casein with protease overnight using skim milk agar plate.

Table 7. Summary of the purification of VKB1 protease from *Brevundimonas* sp.

Purification steps	Total Protein (µg/mL)	Total activity (U/mL)	Specific Activity (U/mg)	Recovery (%)	Purification fold
Crude Extract	956	157.6	164.81	100	1
Ammonium Sulphate Precipitation	728	131.40	180.50	83.37	1.09
Dialysis	443	127.83	288.57	81.11	1.75
Ion-Exchange Chromatography	225	96.9	430.75	61.50	2.61
Sephadex G-100	95	84.96	894.39	53.92	5.4

3.2. Biochemical properties of the purified enzyme.

The effect of pH on the protease activity was determined (Figure 6 (a)), and the results depicted that the enzyme was highly active at pH 7-8. In general, the protease enzymes are active at neutral and alkaline pH conditions with an optimum pH of 8 and 10 [24,40]. The enzyme activity was dropdown below pH 6. Thus, the purified enzyme was assumed to be an alkaline protease. As per temperature, the enzyme showed higher stability till 40 °C and the activity dropdown at 60 °C (Figure 6 (b)).

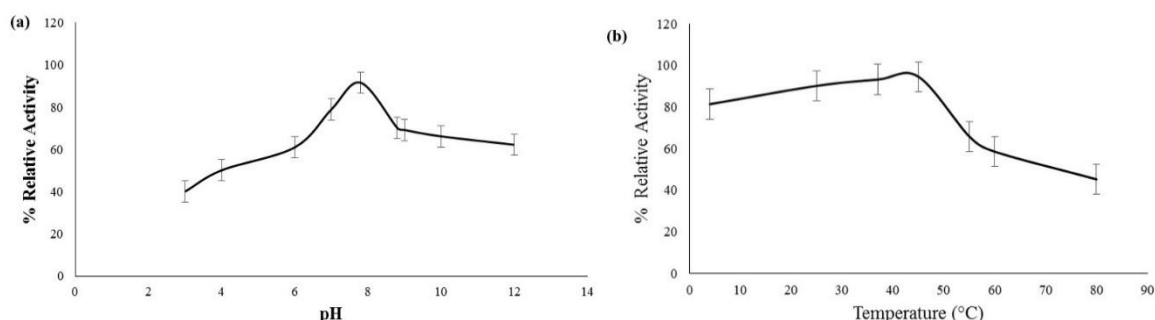


Figure 6. Various analyses performed to understand the purified protease stability. **(a)** effect of pH on enzyme activity; **(b)** effect of temperature on enzyme activity.

The enzyme activity was determined with inhibitors; it was shown that the inhibitors PMSF and DTPA showed less enzyme activity whose relative activity percentage was 80.50% and 75.66% comparing the EDTA and Pepstatin A (95.82% and 85.86%). The results indicate that the enzyme belongs to the serine protease class. Also, the enzyme was showing high relative activity percentage with the metals Cu^+ , Mg^+ , Fe^+ , comparing the other metals Ca^+ and Zn^+ (Table 8). Hence from the biochemical characterization, the enzyme shows excellent stability by confirming their productive enzyme activity comparing the other proteases produced by other bacterium sources [42,47,48].

Table 8. Effect of protein inhibitors and metal ions towards the VKB1 enzyme activity.

Inhibitors (5 mM)	Relative activity (%)
PMSF	80.50
DTPA	75.66
EDTA	95.82
Pepstatin A	85.86
CaCl_2	72.80
MgSO_4	84.68
MnSO_4	88.48
FeSO_4	90.66
CuSO_4	95.90
ZnSO_4	67.74

3.3. Discussion.

This study's significant aspect is the production of thermostable protease from the endophytic strain *Brevundimonas diminuta* VKB1 host in *Carica papaya* L fruit. The industrially-produced large-scale level of protease enzymes is predominantly from the *Bacillus* species. The commercially available Subtilase, Nattokinase, Savinase are produced by the *Bacillus subtilisin*, *Bacillus natto*, and *Bacillus lentus* [49]. Protease from *Brevundimonas diminuta* was not highly reported. Out of fifteen isolates, strain VKB1 showed high extracellular proteolytic activity and selected for further studies. If two bacteria shared 99% homology in their 16s rRNA sequence are found to be in the same genus. The strain VKB1 was identified through 16s rRNA sequencing and share 99% homology with both Alpha proteobacterium and *Brevundimonas diminuta*. However, the *Brevundimonas diminuta* and *Brevundimonas* species located in other clades also showed above 92% similarity defines that the strain belongs to the *Brevundimonas diminuta* genus.

Enhancement of the protease production through medium components was formulated based on classical and statistical optimization techniques. The medium components for protease production differ from microbes to microbes. Hence the conventional OFAT approach was first examined to find out the significant factors from each variable. As a disaccharide, maltose followed by sucrose and galactose influences the high enzyme activity comparing the monosaccharides [29,50,51]. This is because of their readily metabolizing nature and limited availability [27,52]. Yeast extract and sodium dihydrogen phosphate were found to be more efficient in the synthesis of the enzyme. Nitrogen sources are generally utilized for their role in the synthesis of cell components, amino acids, etc. [10,22]. Physical factors influence the growth condition of bacteria to grow in its adaptive condition. Upon optimization, the VKB1 strain utilized maltose, yeast extract, and sodium di-hydrogen phosphate under pH 8 at 37 °C with 100 rpm incubated for 72 h for the enhanced protease production.

The statistical method of optimization helps scrutinize the main and significant factors for their influence and interaction in enzyme production. They are cheaper and time-consuming comparing the OFAT approach. Plackett-Burman Design was followed in this study to initiate

the selection of critically significant factors necessary for the next Response Surface Methodology. Among the six variables from the OFAT approach, the design exhibited yeast extract, pH, and incubation time as significant, influencing the protease production's positive effect. PB design lags the exact quantification of factors needed for the production and does not define the interactive response among variables. Hence RSM was carried out to perform the interaction among variables using CCD. The 3D representation exaggerates the regression analysis of the interacting variables at an optimum level. The 2D contour plots with the elliptical design depict the significant interaction and circular for the non-significant interaction among the variables. The study plots that yeast extract's interaction: pH and pH: incubation time showing the significant influence on protease production comparing the yeast extract: incubation time. The results show that the depletion in yeast extract concentration with increasing fermentation period decreases the enzyme production leading to non-significant results. The purified protease characterization enlightens that it can be serine alkaline protease that withstands a high-temperature environment. The involvement of metal ions in the enzyme activity widely helps understand the enzyme's nature, whether it can be a metalloprotease or not. Further, metal ions highly stabilize the enzyme acting as a cofactor. Hence, copper metal ion plays a major role in influencing enzyme activity, followed by iron and magnesium. Though it is not a metalloprotease, the above trace metal ions are highly reported as a cofactor for various proteins, defining that they can be acting as a cofactor for the purified enzyme. Further studies on understanding the molecular nature and purity of the protease through SDS PAGE and HPLC analysis determines that the protein is not stable under detergents and they degrade so easily because the bands appear to be a single protein of the same molecular weight depicting, they can be a single protein of molecular weight ~25 kDa.

3. Conclusion

The study highlights that the purified protease from endophyte shows thermo-stability at a higher temperature and alkaline protease. The protease's enhanced production from the endophyte *Brevundimonas diminuta* VKB1 hosted in unripe fruit *Carica papaya* L. depicts that the production was increase fifteen-fold at the stage of response surface methodology comparing the OFAT. The influence of the copper and other divalent metal ions acting as a cofactor for the enzyme activity defines that the protease can be an antioxidant enzyme. Further studies on the protease towards their biological activity may throw light on the therapeutic properties.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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