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Production of Protease Showing Antibacterial Activity by *Bacillus subtilis* VCDA Associated with Tropical Marine Sponge *Callyspongia diffusa*

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Abstract

The marine sponge *Callyspongia diffusa* associated bacterium produced protease enzyme which effectively inhibited the human pathogen *S. aureus* and fish pathogens *viz., V. fluvialis*, *V. anguillarum*, *V. vulnificus* and *E. cloacae*. The species identity of *Bacillus subtilis* VCDA (GenBank Accession No. KJ789102) was confirmed by 16S rRNA gene sequencing. The maximum protease activity was noted at 30°C and at pH 9. Protease production was stimulated in the presence of glucose (1.5%), tryptone (1.5%), NaCl (1.5%) and metal ions Ca^{2+} (1 mM) and Fe^{2+} (10 mM). The 46 K Da *Bacillus subtilis* VCDA protease was partially purified by ammonium sulphate precipitation and Sephadex G-100 column.

Keywords: Marine sponge; *Bacillus subtilis*; Culture dependent methods; Antibacterial activity; Protease

Introduction

The well-balanced association established with many of the marine organisms and the survival mechanisms developed by marine sponges make them ideal candidates for the bioprospecting of bioactive compounds from them. The association or involvement of microorganisms may be suspected when the diverse sponge metabolites are purified from taxonomically diverse species or when the production of metabolite is low from the sponge of interest [1]. One of the most effective methods of discovering natural products is the cultivation of new microbial strains that may represent a novel important chemotype [2,3]. There are several reports and hypothesis that insists that the symbiotic bacteria associated with the sponge are the original producers of bioactive compounds [4,5]. The actual proof for the contribution of microorganisms in secondary metabolism of sponges can be provided if the microbes produce the compound of interest under laboratory conditions [1]. Antimicrobial compounds are produced in conditions like competitions for sharing ecological niche, prevention of phagocytosis, establishment of symbiotic association with invertebrates [6,7]. A myriad of compounds having wide applications have already been discovered from marine sponges; many of them are suspected to be of microbial origin because they resemble the bacterial natural products such as polyketides or nonribosomal peptides [8]. Moreover, being exposed to highly challenging environmental conditions which trigger the bioactive production, these microbes display broad spectrum of activity. The marine microbial symbionts associated with the marine sponge may be the actual source of bioactivity and in this context the studies on the sponge symbiosis in pure culture will be of great advantage for screening bioactive assays. The sponge associated bacteria are acclimatized in nutrient rich microhabitat of their hosts and hence are excellent producers of multiple hydrolytic enzymes which are stable, active at low temperature and specific in action [9]. With this prospective aspect, the bacteria associated with the tropical marine sponge *Callyspongia diffusa, Bacillus subtilis* VCDA which had proven antibacterial activity was screened for its production of bioactive extracellular proteases [10].

Materials and Methods

Collection of sponge and isolation of associated bacteria

Sponge specimens of *Callyspongia diffusa* were collected during post-monsoon (September 2011), monsoon (July 2012) and late post-monsoon (December 2013) seasons from Vizhinjam (8°22'45°N: 76°59'29°E), South-west coast of India at depths ranging from of 6 to7 m at a distance of about 1.5 km from the shore [10]. The sponge specimens found attached to submerged rocks were gently removed and transferred to new polythene covers to minimize external contaminants and transient bacteria. The collected sponge samples were rinsed thrice with sterile filtered sea water to remove adhering debris and mucus on the surface.

The sponge samples immediately after collection were brought to the laboratory under sterile condition. The sponge tissue (1.0 g) was grind in sterile sea water using mortar and pestle in the laminar flow hood and serially diluted using aged sea water. The diluted sample was spread plated on modified culture medium of Zobell Marine Agar 2216 medium (HiMedia).

Determination of antibacterial activity of *B. subtilis* VCDA against selected pathogens

The inhibitory potential of sponge associated bacterial isolate VCDA is already established [10]. The activity of the Cell Free Supernatant (CFS) of the sponge isolate VCDA was tested against the pathogens *viz*., *Klebsiella pneumoniae* MTCC 3384, *Staphylococcus aureus* KU2, *Bacillus pumilus*, *Arthrobacter* sp. and *Micrococcus luteus* MTCC 105 as per described by Rachanamol et al. [10]. The Muller Hinton Agar plates seeded with the pathogens were loaded with CFS of *B. subtilis* VCDA and incubated for 24 to 96 h at 37°C.

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Molecular characterization of the sponge associated bacteria

The marine sponge associated bacterial strain VCDA with potent inhibitory activity and proteolytic activity was characterized using 16S rRNA sequencing. The 16S rRNA gene of the isolate was sequenced (ABI 3100 sequencer and genotyper; Genei) after the DNA isolation and PCR amplification. The sequence obtained was compared to the Gen Bank nucleotide database with BLAST and phylogenetically analyzed using MEGA 5.03 software [11].

Screening of *Bacillus subtilis* VCDA for protease production and determination of protease assay

The overnight culture of the sponge isolate *Bacillus subtilis* VCDA was streaked on Skim Milk Agar (HiMedia) to detect the production of protease. The protease activity was determined by following the methodology described by Mayer et al. [12].

For determination of proteolytic activity, the protease producing strain (1%; v/v inoculum) was inoculated in autoclaved Zobel Marine Broth (Hi-Media) with 1% casein and pH adjusted to 7.0. The culture broth was inoculated for 24 h at 37°C and centrifuged at 15,000 rpm for 15 min. The resulting cell free supernatant was used for assay of protease enzyme. The protease activity was determined in triplicate by measuring the release of trichloro acetic acid soluble peptides from 1% (w/v) casein in 1.25 ml of 1 M Tris buffer (pH 9.0) at room temperature (27 ± 2°C) for 30 min; adding 0.25 ml of cell free supernatant. The 2 ml reaction mixture was terminated by adding 0.5 ml of 5% (w/v) trichloroacetic acid. The mixture was again centrifuged at 5000 rpm for 15 min after incubation for 10 min at 4°C. The supernatant (0.5 ml) was added to 2.5 ml of 0.5 M sodium carbonate and incubated for 20 min. After incubation, 0.5 ml folin phenol reagent was added and the absorbance was read at 660 nm in a UV Spectrophotometer (Shimadzu Corp-04367). One unit of enzyme activity was defined as the amount of enzyme required to release 1 µg of tyrosine/min under standard conditions [13,14].

Effect of various physical parameters on protease production

For determining the effect of various physical parameters *viz.*, Incubation Period, Temperature and agitation on protease production, the producer strain was grown in modified ZMB (ZMB+1% casein) with pH 7.0. For determining the effect of pH, the bacteria were inoculated in to media with varied pH 6.0 to 10.0. The aliquots were drawn every 24 h for determination of growth and protease production from 24 to 120 h of incubation. The producer strains were inoculated in modified ZMB and incubated for 48 h at various temperatures *viz.,* 30, 35, 40, 45 and 50°C for determining the suitable temperature for maximum protease production. To determine the optimum agitation speed required for the maximum protease activity the Zobell Marine Broth (ZMB) (pH8) with bacterial inoculums (24 h) was incubated in a shaker incubator under optimized temperature at different agitation conditions (80, 100 and 120 rpm). In each case, triplicates were maintained and the growth, protein content and protease assay was determined [15].

Effect of various chemical parameters on protease production

The effect of selected carbon and Nitrogen sources, salts and surfactants on Protease Production by *Bacillus subtilis* VCDA was studied. The different concentrations *viz.*, 0.1, 0.5, 1.0, 1.5 and 2% w/v each of glucose, sucrose, fructose, were added to the modified Zobell Marine Broth (mZMB); inoculated with 24 h old producer culture and incubated at optimized temperature for 48 h and the protease content determined. The organic nitrogen sources *viz.*, tryptone and beef extract (0.1, 0.5, 1.0 and 1.5% w/v) and inorganic nitrogen sources *viz.*, $NaNO_3$, NH_4, NO_3, KNO_3 and $(NH_4)_2SO_4$ (0.1, 0.5, 1.0 and 1.5% w/v) were added to the ZMB to determine their effect on protease production and protease assay performed. The effect of salts on the growth and protease production by the sponge isolate was determined by supplementing different concentrations *viz.*, 1.5, 2.0 and 2.5%; w/v (0.25 M to 0.42 M) of NaCl to the ZMB and determination of protease activity.

The influence of various metal ions on protease production was determined by culturing *B. subtilis* VCDA in 10 ml media supplemented with 1 ml divalent cations (10 mM and 20 mM) $CaCl_2$, $FeSO_4$, $MgCl_2$, $MnSO_4$ and $MnCl_2$ and incubated under optimized temperature and pH.

The effect of surfactants like Sodium Dodecyl Sulphate (SDS), Tween 80 and EDTA on protease production was determined by supplementing various concentrations (0.5, 1.0, 5, 10, 15 and 20 mM) of surfactants to the culture broth.

Partial purification of protease

The protease enzyme produced under the optimized culture conditions by the marine sponge symbiont *B. subtilis* VCDA was subjected to partial purification using Ammonium sulphate precipitation (20-60%). The resultant precipitate after centrifugation at 10,000 rpm for 30 min at 4°C was solubilised with minimum volume of 0.05 M Tris buffer (pH 8.0). Fractions with high enzyme activity were dialysed in 1000 da cut off tubing (HiMedia) at 4°C overnight. The dialyzed enzyme sample was loaded on to the Sephadex G-100 column (Sigma) and equilibrated 0.5 M Tris buffer (pH 8.0). The collection tubes with protein content were also analysed for protease activity and tubes with high protease activity were pooled. The molecular weight of the enzyme was determined by comparing with standard protein markers using the method described by Laemmli (1970) using a mini slab gel apparatus (Biorad laboratories). Protein hydrolyzing enzyme separated on SDS PAGE was confirmed by Zymography [16].

Results

Isolation of sponge associated bacteria and determination of antibacterial activity

The tissue homogenate from the marine tropical sponge *Callyspongia diffusa* collected off south-west coast of India was subjected to analysis of the microbial load. A predominant Gram positive rod shaped isolate VCDA inhibited several pathogens [10]. The bacteria was tested for its' antibacterial potency against pathogens *viz.*, *Klebsiella pneumoniae* MTCC 3384, *Staphylococcus aureus* KU2, *Bacillus pumilus, Arthrobacter* sp. and *Micrococcus luteus* MTCC105. The sponge isolate effectively inhibited the pathogen *S. aureus* with 16 mm zone of inhibition (Table 1). The inhibitory activity of *B. subtilis* was also observed against fish pathogens [10].

Molecular characterization of VCDA

The bioactive protease producing sponge isolate VCDA was

Test Pathogen	Zone diameter (mm)
K. pneumonia MTCC B3384	10
M. luteus MTCC B105	12
S. aureus KU2	16
Bacillus pumilus	10
Arthrobacter sp.	11

Table 1: Antibacterial activity of tropical sponge isolate VCDA against selected pathogenic bacteria.

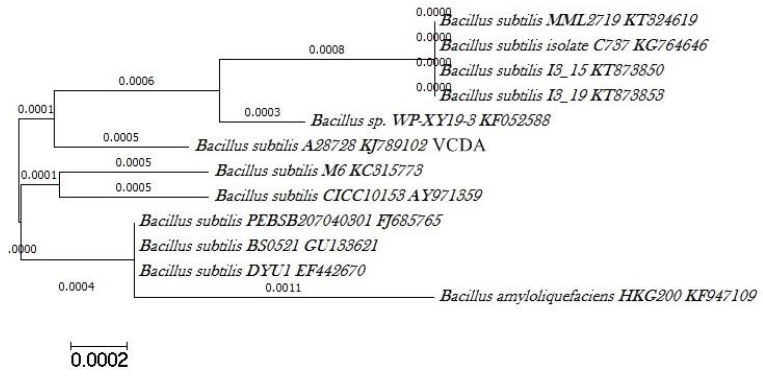

Figure 1: Phylogenetic tree of sponge associated bacteria *B.subtilis* VCDA.

identified as *B. subtilis* VCDA by 16S rDNA sequencing. The base pair was aligned and phylogenetic tree was constructed using Neighbour joining method by Mega 6.0 software. The sequence alignment of *Bacillus subtilis* VCDA using BLAST showed 100% similarity with *Bacillus subtilis* strain M6 KC315773 (Figure 1). The gene sequence was submitted in the GenBank with the accession no. KJ789102.

Determination of protease production by *Callyspongia diffusa* associated bacterial isolate

The protease production was screened in 0.1% skim milk agar medium in which the isolate VCDA exhibited vivid zone of clearance (20 mm). The cell density of the strain *B. subtilis* VCDA maximized at 48 h of incubation at which the optimum protease production (54 U/ml) was noted. The protease activity gradually increased from 46 U/ml to 50 U/ml with respect to the bacterial growth from 24-36 h which declined after 48 h. (Figure 2).

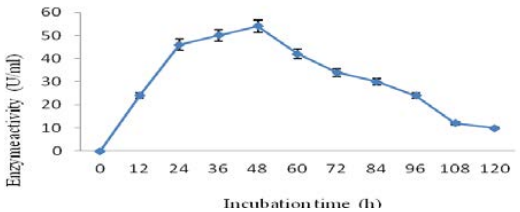

Figure 2: Effect of incubation time on protease production by *B. subtilis* VCDA.

Effect of culture conditions for protease production

The effect of temperature on protease production was determined by observing the protease activity at different temperatures (30°C to 50°C). The maximum enzyme activity was 48 U/ml with 50 µg/ml of protein at 40°C. Protease production increased from 30°C to 40°C (Figure 3). Further increase in temperature decreased the protease activity and it is observed that at 50°C the enzyme activity was 38 U/ml.

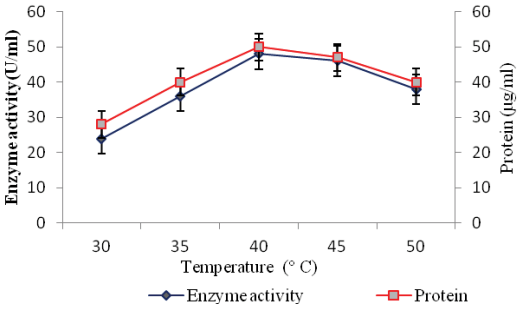

Figure 3: Effect of temperature on protease production.

Effect of pH

Protease production varied with varied range of pH from 7 to 10. At initial pH 7 the protease activity 29 U/ml with 34 µg/ml of protein. The enzyme activity gradually increased from pH 7 and maximum enzyme activity was observed at pH 9 with 54 U/ml corresponding to a protein content of 58 µg/ml. The protease yield decreased at higher pH 10 with 48 U/ml (Figure 4).

Effect of agitation speed

The marine sponge isolate *B. subtilis* VCDA showed maximum protease production at agitation speed of 100 rpm when the bacteria inoculated culture broth was incubated at 48 h at optimized temperature of 40°C and pH of 9. The enzyme activity was 68 U/ml. Increased agitation (120 rpm) resulted in decline in the enzyme activity.

Effect of carbon sources on protease production

Among the carbon sources tested, glucose at 1.5% w/v concentration was found most effective in increasing the production of the enzyme protease by the sponge isolate. The protein content was 85 µg/ml with protease activity of 62 U/ml when glucose was supplemented (Figure 4a). Addition of the sugar fructose yielded protein of 18.65 µg/ml at 1.5% w/v concentration with activity of 14 U/ml (Figure 4b). Sucrose influences the production of protease with protease activity (14.8 U/ml) at 0.5% w/v concentration (Figure 4c). The results confirm glucose as the best source for the production of protease by *B. subtilis*.

Effect of nitrogen source

The nitrogen sources such as Tryptone and Beef extract were tested for increase in production of protease by *B. subtilis* VCDA. The activity of protease gradually increased with the increase in concentration of Tryptone, with enzyme activity of 40 U/ml and protein content of 44.5 µg/ml. The organic nitrogen source, beef extract at higher concentration (1.0%) enhanced the protease production to 26 U/ml with the protease activity of 32 µg/ml. The higher concentration of 1.5% reduced the enzyme activity to 24 U/ml.

In case of inorganic nitrogen sources $NaNO_3$, NH_4, NO_3 and KNO_3 the protease production was low compared to organic nitrogen. Maximum enzyme activity recorded at 1.0% concentration for KNO_3,

i.e., 12 U/ml had further decreased to 9 U/ml at 1.5%. The $NaNO_3$ and NH_4SO_4 influenced the enzyme activity at 1.0% but the effect of inorganic nitrogen sources gradually reduced the enzyme activity. The protease activity and its protein content are given in the Table 2.

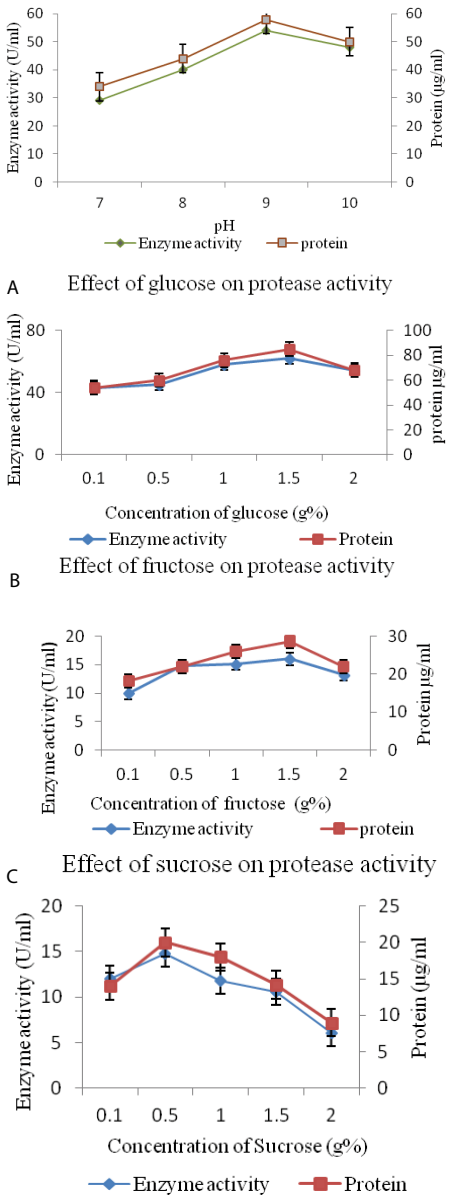

Figure 4: Effect of pH on protease production. (a) to (c): Effect of carbon sources on protease production of *B. subtilis* VCDA.

Effect of NaCl on enzyme activity

The supplementation of 1.5% (0.25 M) NaCl increased the protease production (38 U/ml) (Figure 5). Beyond this level, the enzyme activity decreased and at 2.0%, the observed enzyme activity was 32 U/ml.

Effect of metal ions on enzyme activity

Among various metal ions Ca^{2+} and Fe^{2+} influenced the production of enzyme and a substantial increase in protease production was observed in the presence of Ca^{2+}. The supplementation of media with 1 mM and10 mM of $CaCl_2$ showed higher activity with 42 U/ml and 34 U/ml and Fe^{2+} at 10 mM also showed increase in protease activity with 35 U/ml (Table 3). The other metal ions Mn^{2+} and Cu^{2+} played no significant effect on the production of enzyme.

Effect of surfactants

The different concentrations of surfactants such as Tween 80 and SDS did not favour the enzyme activity compared to the control. SDS and Tween 80 at 0.5 mM had maximum enzyme activity of 10.0 U/ml and further increase in concentration reduced the enzyme activity (Table 4).

Purification of protease enzyme by ammonium sulphate precipitation and gel filtration chromatography

The bioactive protease enzyme from *B. subtilis* VCDA from 1 L culture supernatant in optimized medium was partially purified by ammonium sulphate precipitation at various concentrations (20-60 g%). Among the various fractions obtained, the fractions F3 (55 g%) showed highest enzyme activity (740 µg/ml of total protein). The dialyzed precipitate of this fraction with 3 mg of protein was loaded in the Sephadex G-100 column. The eluted contents (2 ml) in the

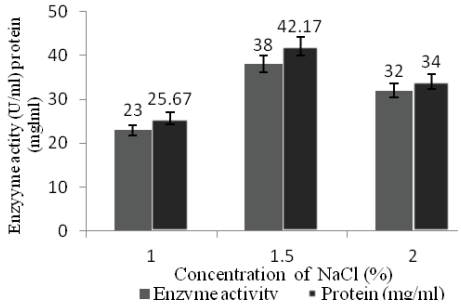

Figure 5: Effect of NaCl on protease production by *B. subtilis* VCDA.

Nitrogen Sources	0.1% w/v		0.5% w/v		1.0% w/v		1.5% w/v	
	Enzyme activity (U/ml)	Protein (µg/ml)	Enzyme activity (U/ml)	Protein (µg/ml)	Enzyme activity (U/ml)	Protein (µg/ml)	Enzyme activity (U/ml)	Protein (µg/ml)
Tryptone	22	33.4	28	36.88	31	39.44	40	44.5
Beef extract	10	13.23	15	18	26	32	24	28.2
$NaNO_3$	24	22.5	30	29.68	22	19.35	16	19.01
NH_4NO_3	14	20.65	29	27.65	31	37.44	26	31.2
KNO_3	8	28.35	10	23.44	12	24.44	9	21.47
$(NH_4)_2SO_4$	10	16.44	16	17.66	12	16.99	8	11.9
Urea	0.9	0.32	8	19.88	6	17.54	12	16.5

Table 2: Effect of nitrogen sources on protease production.

Metal ion conc.	Enzyme activity (U/ml)	Protein (µg/ml)
$FeSO_4$ (1 mM)	24	36
$FeSO_4$ (10 mM)	35	47
$CuSO_4$ (1 mM)	20	24
$CuSO_4$(10 mM)	12	26.98
$MnSO_4$ (1 mM)	18	10.25
$MnSO_4$ (10 mM)	14	23.2
$MgSO_4$ (1 mM)	22	26.5
$MgSO_4$ (10 mM)	16	18.4
$CaCl_2$ (1 mM)	42	58.6
$CaCl_2$ (10 mM)	34	46.8
$MnCl_2$ (1 mM)	15	16.25
$MnCl_2$ (10 mM)	10	12.65
Control	32	42.6

Table 3: Effects of metal ions on production of VCDA protease.

Detergents	Enzyme activity (U/ml)					
	0.5 mM	1.0 mM	2.0 mM	5.0 mM	10.0 mM	20.0 mM
Blank	-	-	-	-	-	-
SDS	10	7.0	5	2.0	0.7	-
Tween 20	8	5	2	0.5	0.05	-

Table 4: Effect of detergents on protease production.

collection tubes (1-50) were checked for the protein concentration at 280 nm. The fraction (13-30 pooled together) which showed specific activity of 35.58 units was concentrated by lyophilization.

SDS-PAGE Electrophoresis

The purified enzyme of the strain VCDA was analyzed on 12% SDS-PAGE. The protein band corresponding to 40-50 KDa of molecular protein marker was visualized from the PAGE. The molecular weight of the enzyme protease from *Bacillus subtilis* VCDA was determined as 46 KDa. The Zymogram activity staining revealed a clear zone of proteolytic activity against the purified sample at the corresponding positions in SDS-PAGE (Figure 6).

Discussion

The bacteria *Bacillus subtilis* VCDA was isolated from the sponge *Callyspongia diffusa* using modified culture medium by the culture-dependent method. The occurrence of Bacillus species in marine environment and its association with marine benthos were reported earlier by Hentschel et al. [6], in which a novel Bacillus species associated with Mediterranean sponge *Alphysina aerophoba* was isolated. The tropical sponge *Callyspongia diffusa* observed to be associated with phylogenetically heterotrophic Bacillus and their association with the marine sponge and its productive source was reported by Fenical and Jensen [2]. Satheesh et al. isolated *B. cereus* and *B. subtilis* from tropical sponge *Sigmadocia* sp. collected from Kanyakumari coast [17]. Two potential biosurfactants producing strains such as *Bacillus subtilis* and *Bacillus amyloliquefaciens* were reported as sponge associated bacteria from *C. diffusa* collected from Kanyakumari coast reported that *B. subtilis* associated with marine sponge can produce anticholinesterase compounds [18,19]. The *Bacillus subtilis* VCDA isolated from *C. diffusa* possessed inhibitory activity against *E. coli* (10 mm), *S. aureus* (16 mm), *Proteus vulgaris* (9 mm), *Vibrio fluvialis* (11 mm) which correlate with the reports of *B. cereus* $SBSO_2$ isolated from sponge *Hyatella cribriformis* that showed activity 12 mm for *S. aureus*, 11 mm for *S. typhii*, 7 mm against *P. mirabilis* and *Vibrio cholerae* (10 mm). Similar potent bioactive producing *Bacillus* sp. were isolated earlier from sponges [20,21].

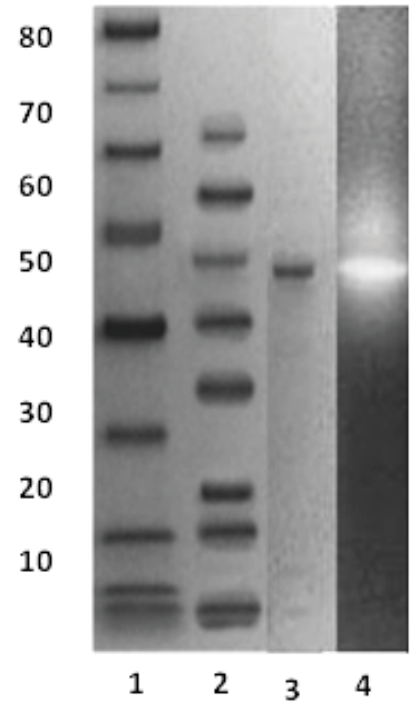

Figure 6: SDS-PAGE analysis of B. subtilis VCDA.
Lane 1: marker, Lane 2: ammonium sulphate precipitated F3 fraction, Lane 3: purified enzyme and Lane 4: zymogram

Bacillus subtilis VCDA isolated from *C. diffusa* produced bioactive protease which was optimized at pH 9 with activity of 54 U/ml. Similar reports were made in case of *B. amyloliquefaciens* MA20 and for *B. cereus* [22,23]. The *Bacillus subtilis* VCDA isolated from *C. diffusa* showed an optimum 48 U/ml activity at temperature 40°C Anand et al., isolated *Halobacterium* sp. JS1, from solar saltern that showed optimum protease production after 96 h of incubation, at 40°C, pH 7.0 with 20% salinity [24]. Mustapha et al. reported about a marine bacteria isolated from east coast of Malaysia which possessed proteolytic activity with optimum growing condition at temperature 35°C and pH 5 [25]. The protease production by the marine sponge isolate *B. subtilis* VCDA was also affected by changes in the salt concentration in which an enhanced activity of 38 U/ml was detected at 1.5% concentration of NaCl and a similar report was observed in case *Bacillus* sp. APCMST-RST with maximum activity at 1.5% NaCl and an reduced protease activity was noted with incearse in salt concentration [26].

Beg and Gupta reported carbon and nitrogen sources, inorganic salts to be important variables that affect bacterial growth and products of microbes [27]. Among carbon source, glucose was observed to have high influence as it produced 62 U/ml protease activities at 1.5% concentration with 85 mg/ml of protein. The *Bacillus licheniformis* N-2 isolated from soil also showed a maximum protease production as carbon source at 1% w/v [28]. The supplementation of glucose to the media proved beneficial for protease production in case of *Bacillus* sp. [29]. From the results obtained in the present study and as per the earlier reports, the media amended with carbon sources, promote the growth and enzyme activity of *B. subtilis* VCDA. The effect of various concentration of tryptone on enzyme activity was observed to be having similar effect on protease activity and maximum enzyme activity of 44 U/ml was observed in the presence of 1.5% tryptone.

The protease activity was 26 U/ml when supplemented with 1.0% beef extract.

Supplementation of NH_4Cl, NH_4NO_3 and KNO_3 resulted in low protein content with lesser enzyme activity. The repression in enzyme activity due to ammonium might be attributed to the fast release of ammonia from inorganic nitrogen sources and it hinders the uptake of ammonia in the medium by bacteria [30]. The surfactants like SDS and Tween 20 also inhibited the enzyme activity indicating the serine nature of protease The effect of surfactants helps to know its effect of denaturing the glycoproteins and enzyme activity of *B. subtilis* VTCC-DVN-12-01was inhibited when added with 0.5 to 1% (w/v) SDS [31]. *Bacillus* sp. NPST-AK15 with enzyme activity of 1000 U/ml was observed in the presence of Ca^{2+} [32].

The crude enzyme of *B. subtilis* VCDA was purified by ammonium sulphate precipitation yielded partially purified protein at 55g% and elution was done through column chromatography. The Sephadex G-100 elution of protease from *B. subtilis* VCDA showed 10.62 fold recovery of crude enzyme. According to the data of Vijaraghavan et al. revealed 9.47 purification fold of protease enzyme could be obtained from *B. subtilis* isolated from cow dung using Sepadex G-75 [33].

Conclusion

In the present study, the molecular mass of partially purified protease from *B. subtilis* VCDA has been determined as 42 kDa. Yang et al. [34] isolated a 44 KDa protease from *B. subtilis* with highest activity of 20.2 U/ml and marine bacillus with 37 KDa as determined by Padmapriya et al. [35]. The protease enzyme with 28.5 KDa was isolated by Vijayaraghavan et al. from *B. subtilis* VV strain [33]. A marine *Streptomyces albidoflavus* isolated by Mohamed et al. was found to produce alkaline protease which on purification by 80% ammonium sulphate precipitation and size exclusion chromatography yielded 20 KDa protein in 12% SDS [36]. The *Bacillus subtilis* VCDA on purification yielded protease with 42 KDa in 12% SDS. According to Bull et al. [37] and Kin [38], the marine enzymes are relatively more stable than enzymes from plants or animals and that had boosted marine microbial enzyme technology in recent years. The potential of marine sponge associated bacteria *Bacillus subtilis* VCDA with ability to synthesis bioactive marine enzyme protease will be a promising source of marine enzymes with unsuspected application potentials in near future.

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