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ORIGINAL ARTICLE

Agro-industrial Waste Utilization, Medium Optimization, and Immobilization of Economically Feasible Halo-Alkaline Protease Produced by *Nocardiopsis dassonvillei* Strain VCS-4

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Abstract

The oceanic actinobacteria have strong potential to secrete novel enzymes with unique properties useful for biotechnological applications. The *Nocardiopsis dassonvillei* strain VCS-4, associated with seaweed *Caulerpa scalpeliformis*, was a halo-alkaline protease producer. Further investigation focuses on medium optimization and the use of agro-industrial waste for economically feasible, high-yield protease production. A total of 12 experimental runs were designed using Minitab-20 software and Placket-Burman design. Among the 7 physicochemical parameters analyzed, incubation time and gelatin were detected as significant factors responsible for higher protease production. Incubation time and gelatin were further analyzed using OVATs. Optimal protease production was achieved with 2% gelatin, 0.1% yeast extract, 0.1% bacteriological peptone, 7% NaCl, pH 8, 5% inoculum, and a 7-day incubation period, resulting in a maximum protease activity (Pmax) of 363.97 U/mL, generation time of 11.9 h, specific growth rate of 0.161 g/mL/h, and protease productivity (Qp) of 61.65 U/mL/h. Moreover, utilizing groundnut cake as an agro-industrial waste led to enhanced production parameters: Pmax of 408.42 U/mL, generation time of 9.74 h, specific growth rate of 0.361 g/mL/h, and Qp of 68.07 U/mL/h. The immobilization of crude protease was achieved using Seralite SRC 120 as a support matrix resulting in 470.38 U/g immobilization, 88.20% immobilization yield, and 28.90% recovery activity. Characterization of both crude and immobilized proteases revealed optimal activity at pH 10 and 70 °C. Immobilization enhanced the shelf-life, reusability, and stability of VCS-4 protease under extreme conditions.

Keywords Actinobacteria · Halo-alkaline protease · Immobilization · Medium optimization · Placket-Burman design · Agro-industrial waste

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Introduction

Microorganisms are pivotal in industries due to their vast biochemical diversity, rapid growth, and adaptability to genetic modification, which enhances enzyme quality and yield. [1]. Protease is one of the most important industrial enzymes. It has a catalytic function that hydrolyzes peptide bonds in proteins. Nowadays, the importance of protease has increased drastically due to its massive usage in research, food supplements for weight reduction, dairy products (to avoid coagulation of milk), detergent formulation, contact lens cleaning, and silk degumming [2, 3]. Protease accounts for 60% of the enzyme market worldwide [4]. The protease market is projected to experience substantial growth, with a CAGR of 7.8% anticipated during the forecast period. In 2023, the market was valued at US$2.1 billion. (www.futuremarketinsights.com). Due to rapid urbanization, the demand for protease with improved characteristics has increased as it plays a significant role in pharmaceutical, clinical, and industrial processes [4, 5]. At the production scale, acidic proteases were mainly extracted from fungi, and neutral and alkaline proteases were extracted from *Bacillus*, *Halobacillus*, and *Pseudomonas* genera [5–8]. However, proteases from actinobacteria are the least explored [9, 10]. According to earlier reports, protease-producing actinobacterial genera included *Streptomyces* [11], *Nocardiopsis* [9, 12], and *Nocardia* [10].

The microorganisms from marine environments have a strong potential to secrete novel enzymes and metabolites with unique properties useful for biotechnological applications [13, 14]. The microorganisms form symbiotic associations with marine flora. The host-associated microorganisms could grow under stressful conditions and encompass remarkable gene clusters for secondary metabolites production with different structural scaffolds having unique characteristic features that protect hosts from predators [15–17]. Till today, diverse floral and faunal species are reported from the coastal areas of Gujarat, India. However, the focus on the microbial resources of coastal Gujarat especially from the Saurashtra region of India is limited [1, 17–21]. Therefore, the Veraval coast from the Saurashtra region was selected in this study to investigate protease-producing actinobacteria associated with seaweed.

The booming global enzyme market demands finding cost-effective alternatives to synthetic media for enzyme production [22, 23]. Therefore, the present research investigates the production of protease using readily available agro-industrial waste from Gujarat, India. Crude protease can also contribute to organic waste management by treating sewage water [6, 24]. However, commercially producing enzymes with extended shelf life, higher yield, and improved purification remains a challenge at the industrial scale. Enzymes are often difficult to recover from reaction systems and lack reusability, and their stability under harsh conditions (like high salinity, temperature, pH, or exposure to metals, solvents, and surfactants) is crucial [25, 26]. Fortunately, medium optimization, utilizing cheaper substrates, and immobilization techniques offer solutions [27, 28]. Immobilized enzymes offer several advantages over free enzymes, such as increased stability, reusability, easier control over enzyme reactions, a final product free of enzymes, and a higher ratio of substrate to enzyme [29]. Therefore, this study investigates halo-alkaline protease production by *Nocardiopsis dassonvillei* strain VCS-4. Key variables such as nutrient source, pH, salt concentration, and incubation time were optimized using the Plackett–Burman design to achieve a higher protease yield. Additionally, agro-industrial wastes were used as a cheaper substrate to make protease production more economical. Finally, the obtained crude protease was immobilized on cation resin (Seralite SRC 120) and anion resin (Seralite SRA 400) to enhance its shelf life, reusability, and stability under high pH and temperature.

Materials and Methods

Isolation and Identification

The protease-producing actinobacterial strain VCS-4 associated with seaweed *Caulerpa scalpeliformis* was isolated by serial dilution and plating technique previously reported by Majithiya and Gohel [21]. Seaweed *Caulerpa scalpeliformis* was collected from the intertidal zone of the Veraval coastal region, Gujarat, India (20°55′1 ″ N and 70°20′32″ E). Genomic DNA was extracted from actinobacterial strain VCS-4 using a previously described method for strain identification [20]. The amplification of the 16S rRNA gene was carried out by using 27F and 1492R universal primer sets. A total of 30 µL of reaction mixture was used for gradient PCR that contained 8 µL genomic DNA, 1 µL each primer, 15 µL PCR reaction master mix, and 5 µL molecular-grade water. Eppendorf nexus master cycler was used for 16S rDNA gene amplification using the following PCR conditions: initial denaturation (94 °C for 5 min), 35 cycles of denaturation (94 °C for 50 s), annealing (56, 58, and 60 °C for 1 min) extension (72 °C for 1 min), and final extension (72 °C for 10 min). Then, the 1500 bp amplified PCR product was confirmed by agarose gel electrophorese using 1.5% agarose gel. The amplified 16S rRNA gene was sequenced using Sanger's sequencing technique. The phylogenetic analysis of the 16S rRNA gene sequence was carried out using the BLAST tool from NCBI to identify 100 nearest homologies. The phylogenetic tree was constructed using the MEGA 11 software. Briefly, 16S rRNA gene sequence alignment was carried out by the CLUSTER W algorithm followed by a phylogenetic tree construction using the maximum likelihood method.

Microscopic Characterization of Nocardiopsis dassonvillei Strain VCS-4

The *Nocardiopsis dassonvillei* strain VCS-4 was grown on actinomycete isolation agar (AIA), ISP-5 agar, and nutrient agar media. Consequently, the plates were incubated at 28 ± 2 °C for 7 days. The colony morphology and growth pattern of *Nocardiopsis dassonvillei* strain VCS-4 were monitored by the naked eye under 32 × magnification using a Carl Zeiss microscope (GmbH 37081 Gottingen, Germany). Further, the detailed growth pattern of *Nocardiopsis dassonvillei* strain VCS-4 was studied using scanning electron microscopy and in situ cultivation. Briefly, *Nocardiopsis dassonvillei* strain VCS-4 spores were inoculated on a thin layer of ISP-5 agar which was placed beforehand on a sterile glass slide. A sterile coverslip was then overlaid to enable direct microscopic observation. The cultures were incubated at 28 ± 2 °C and examined periodically for the formation of aerial mycelia, spore-bearing hyphae, and spore chains using direct microscopic examination of the culture surface.

Physicochemical Characterization of Nocardiopsis dassonvillei Strain VCS-4

Biochemical tests including methyl red, Voges-Proskauer, citrate utilization, catalase, oxidase, triple sugar iron, phenylalanine, ammonia production, and indole production were tested as described previously by Gohel et al. [18]. Further, the effect of NaCl, pH and temperature on the growth of *Nocardiopsis dassonvillei* strain VCS-4 was checked by inoculating the strain in zobel marine broth supplemented with 0–20% NaCl (w/v), pH 6 to 12 (adjusted with 0.5 M NaOH). Later, the broth was incubated at 28 °C, 37 °C, 50 °C, and 60 °C for 7 days in a rotatory incubator shaker at 120 rpm (Innova 42, Germany).

Nocardiopsis dassonvillei strain VCS-4 was grown on gelatin agar, starch agar, cellulose agar, pectin agar and asparagine agar to detect the secretion of protease, amylase, cellulase, pectinase, and L-asparaginase. Furthermore, sugar fermentation ability of *Nocardiopsis dassonvillei* strain VCS-4 was checked using 15 different sugars (20 mg) including arabinose, cellobiose, dextrose, fructose, glucose, galactose, lactose, mannose, maltose, mannitol, raffinose, rhamnose, sucrose, trehalose, and xylose (HiMedia Laboratories Pvt. Ltd, India).

Effect of % NaCl on Growth and Protease Secretion

Detection of alkaline protease on gelatin agar was carried out by sport inoculation method. Briefly, actinobacterial spores were sport inoculated on gelatin agar (g/L: gelatin, 30; bacteriological peptone, 5; yeast extract, 5; agar, 30 and pH 10 adjusted with sterile 0.5 M NaOH) supplemented with 0 to 12% NaCl (w/v). After incubation at 28 °C for 7 days, the plate was flooded with Frazier's reagent (g/L: $HgCl_2$, 150 g; concentrated HCl, 200 mL) to detect the zone of hydrolysis due to protease secretion. The clear zone around the actinobacterial colony indicated the production of extracellular protease. Colony diameter and zone of clearance were measured to assess the relative enzyme secretion as a function of colony size. The ratio of the zone of clearance to the colony diameter was calculated. The data in triplicate were subjected to statistical analysis, and the standard deviation was calculated.

Medium Optimization for the Production of Protease

Inoculum Preparation

A loopful of actinobacterial spores was inoculated into a 100 mL zobel marine broth prepared in a 250-mL Erlenmeyer flask followed by incubation at 28 °C in a rotary incubator shaker at 120 rpm. After 40 h of growth, 5% (v/v) of seed culture ($A_{660\ \mathrm{nm}} = 1$) was added as inoculum to the production medium (Table 1).

Screening of Significant Factors Responsible for the Production of Protease Through Plackett–Burman Design

A total of seven physicochemical parameters (gelatin, yeast extract, bacteriological peptone, NaCl, pH, inoculum size, and incubation time) were checked for the production of protease. A total of 12 experimental runs were carried out for the screening of significant factors responsible for the production of protease (Table 2).

The experiment was designed using Minitab-20 software. The experiment was performed three times for statistical validation. The analysis of variance (ANOVA), pareto chart, residual plot, main effect plot, and contour plot were analyzed.

Screening of Significant Factors Using OVATs Approach

The non-significant factors obtained through the Plackett–Burman design were considered constant such as yeast extract, 0.1%; bacteriological peptone, 0.1%; NaCl, 7%; pH, 8; and inoculum, 5% (v/v). The gelatin was considered a significant factor that affected protease

Table 1 Phenotypic and genotypic characterization of *Nocardiopsis dassonvillei* strain VCS-4

Morphology on:		Biochemical test:	
AIA	White chalky colony, black reverse side pigmentation	Methyl red	–
ISP-2		VP test	–
ISP-5			
Nutrient agar		Nitrate reduction	+
Microscopic observation:	Gram positive long filamentous growth	Citrate utilization	+
Growth at:		Catalase	+
Temperature range (°C)	28–50	Oxidase test	+
Optimum Temperature (°C)	28	TSI	–
pH range	6–12	Phenylalanine	–
Optimum pH	10	Ammonia production	–
NaCl range (%)	0–20	Indole production	–
Optimum NaCl (%)	8	Sugar utilization:	
Hydrolysis of:		Arabinose	+
Starch	–	Cellobiose	–
Gelatin	+	Dextrose	+
Cellulose	–	Fructose	+
Pectin	–	Glucose	+
L-asparagine	–	Galactose	+
Genotypic characteristics:		Lactose	+
16S rRNA gene length	1480 bp	Mannose	+
G + C content (%)	57.09%	Maltose	+
		Mannitol	+
		Raffinose	+
		Rhamnose	+
		Sucrose	+
		Trehalose	+
		Xylose	+

production. Therefore, various concentrations (1 to 3% w/v) of gelatin were used to check optimum protease production.

Protease Activity

The Anson–Hagihara method [30] with few modifications was used to determine the protease activity using Hammerstein casein as the substrate. The crude enzyme (0.5 mL) was added to 3 mL of 0.6% (w/v) Hammerstein casein as a substrate prepared in 20 mM borax–NaOH buffer (pH 10) and incubated at 60 °C for 10 min. The reaction was stopped by adding a 3.2 mL trichloro acetic acid reaction mixture (0.11 M trichloro acetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid). The Whatman filter paper no. 1 was used to remove the precipitates. The release of tyrosine was measured at 280 nm using a SPECORD® 200 plus UV–visible spectrophotometer (Analytikjena, Germany). One unit of protease activity was defined as the amount of enzyme that liberates 1 µg of tyrosine per minute under standard assay conditions.

Table 2 Plackett–Berman design matrix for the production of protease by *Nocardiopsis dassonvillei* strain VCS-4 with experimental and predicted responses

Run order	Gelatin (g/100 mL)	Yeast extract (g/100 mL)	Bacteriological peptone (g/100 mL)	NaCl (g/100 mL)	pH	Inoculum (mL/100 mL)	Incubation time (days)	Tested protease activity (U/mL)	Predicted protease activity (U/mL)
1	1	0.1	1	7	10	5	9	264.41	234.78
2	1	1	0.1	0	8	15	9	154.96	203.63
3	1	0.1	0.1	0	8	5	3	358.18	278.93
4	3	1	1	0	10	15	3	0.00	0.00
5	3	1	0.1	7	10	5	9	0.063	0.00
6	3	1	0.1	7	8	5	3	0.021	0.96
7	3	0.1	0.1	0	10	15	9	89.06	89.15
8	3	0.1	1	7	8	15	3	0.861	48.68
9	3	0.1	1	0	8	5	9	45.51	75.99
10	1	1	1	7	8	15	9	209.98	161.31
11	1	1	1	0	10	5	3	31.14	109.54
12	1	0.1	0.1	7	10	15	3	219.30	249.78

Protease Production Using Agro-industrial Waste

The low-production cost fermentation was carried out using various agro-industrial wastes including skimmed milk powder, wheat straw, millet straw, sorghum straw, groundnut shells, groundnut cake, soya bean meal, and gram flour. The gelatin was used as a control. The fermentation medium was supplemented with 3% agro-industrial waste as a substrate containing 03% NaCl, and pH 9 was set using 20 mM glycine–NaOH buffer. The sterilized production flasks were inoculated with 5% seed culture and incubated at 28 ± 2 °C for 10 days in stationary conditions. The culture supernatant was used as a crude enzyme.

Growth Kinetics

The growth and enzyme kinetics were calculated in terms of maximum cell mass (Xmax), specific growth rate (dx/dt), maximum volumetric enzyme production (Pmax), average enzyme production rate (Qp), and generation time (g). The experiment was carried out by inoculating 5% seed culture in optimized medium and agro-industrial waste. The growth patterns of actinobacteria were observed and measured by harvesting culture aliquots every 24 h. The protease activity of the sample was checked at a preferable time interval of 10 days to monitor growth and protease production. The growth of actinobacteria was monitored at 660 nm by SPECORD® 200 plus UV–visible spectrophotometer (Analytik Jena, Germany) or by measuring cell dry weight collected after centrifugation of broth aliquots at 5000 rpm for 10 min followed by overnight drying at 50 °C.

Immobilization of Crude VCS-4 Protease Using Ionic Binding Method

The cation resin seralite SRC 120 and anion resin seralite SRA 400 were used as a support matrix for immobilization by the ionic binding method. Each resin of 1.0 g was equilibrated with 20 mL of 20 mM borax–NaOH buffer (pH 10) at 28 °C in steering condition for 2 h to activate the support matrix. The cationic resin was then filtered through Whatman filter paper 1 using a Buchner funnel and washed with distilled water until a filtrate of pH 7 was achieved. After filtration, resins were treated with 20 mL of 0.01 N HCl and incubated at 28 °C in steering condition for 1 h followed by washing with distilled water until pH 7 of the filtrate was achieved. For the immobilization, 1.0 mL of crude VCS-4 protease (1845 U/mL) was loaded in each activated support matrix and incubated at 4 °C for 18 h. Following incubation, unbound protease was eliminated through sequential washes with 20 mM phosphate buffer (pH 7) until no detectable protein remained in the wash fractions.

The immobilization efficiency of protease was calculated in terms of immobilized activity, unbound activity, specific activity, immobilization yield, and recovered activity using the following formula.

Immobilization yield (%) = (Loaded enzyme − Unbound enzyme)/Loaded enzyme × 100.

Recovered activity (%) = Immobilized enzyme/(Loaded enzyme − Unbound enzyme) × 100.

Reusability of Immobilized Protease

The reusability of immobilized VCS-4 protease was analyzed by repetitive use of immobilized protease till no protease activity was detected. After each repeat usage, the immobilized protease was recovered by centrifugation at 4 °C (4000 g) for 10 min and stored at

4 °C for 24 h before recycling for another run. The immobilized protease was washed with sterile distilled water to remove the attached substrate particles. The whole process was carried out ten times because after the 10th cycle the shape of the immobilized protease changes and that affects its reusability. The percentage reusability was calculated by considering the initial activity as 100%.

Characteristics of Immobilized Protease

The effect of pH on the activity of immobilized and native protease was determined by preparing 0.6% Hammerstein casein as substrate in 20 mM of each buffer system (pH range 7 to 11). These buffer systems included sodium phosphate (pH 7), tris–HCl (pH 8), glycine–NaOH (pH 9), borax–NaOH (pH 10), and KCl–NaOH (pH 11) buffer. The protease assay was performed as mentioned above.

The effect of temperature on immobilized and native protease was determined by preparing 0.6% Hammerstein casein in 20 mM borax–NaOH buffer (pH 10). The protease assay was performed as mentioned above by incubating enzyme–substrate reaction at a varying temperature ranging from 30 to 90 °C for 10 min.

The effect of NaCl on immobilized and native protease was determined by incubating native and immobilized protease in 1 M and 2 M NaCl at 50 °C, 60 °C, and 70 °C for 2 h. The % residual activity of native and immobilized protease was calculated by considering initial activity as 100%.

Results and Discussion

Isolation and Identification of Actinobacterial Strain

Seaweeds, with their unique properties, diverse applications, and the hidden potential of their associated bacteria, have become a captivating frontier in scientific exploration. The strain VCS-4, isolated from the seaweed *Caulerpa scalpeliformis* using *International Streptomyces Project* 2 (ISP-2) medium, exhibited distinct characteristics. Colonies appeared chalky-white with a raised, irregular filamentous edge and a diameter of 2 mm. *Nocardiopsis dassonvillei* strain VCS-4 displayed similar chalky-white colonial growth with black reverse side pigmentation on various media such as actinomycetes isolation agar (AIA), ISP-2, ISP-5, and nutrient agar (Fig. 1a–c). Previous reports have identified the production of chalky and hard colonial growth, diffusible pigments, and an earthy odor as key characteristics of actinobacteria [3, 55]. The 16S rRNA gene amplification and Sanger sequencing of actinobacterial strain VCS-4 resulted in a 1480 bp gene length having 57.09% G + C content. BLAST analysis of the strain revealed 94.99% similarity with *Nocardiopsis dassonvillei*. The 16S rDNA sequence of *Nocardiopsis dassonvillei* strain VCS-4 was deposited in NCBI GenBank with accession number OM164103. The phylogenetic relationship based on the nearest homology of *Nocardiopsis dassonvillei* strain VCS-4 is shown in Fig. 2.

In Situ *Cultivation of Nocardiopsis dassonvillei Strain VCS-4*

The microscopic examination of *Nocardiopsis dassonvillei* strain VCS-4 confirmed the filamentous nature of this Gram-positive bacterium. *In-situ* cultivation and Gram staining

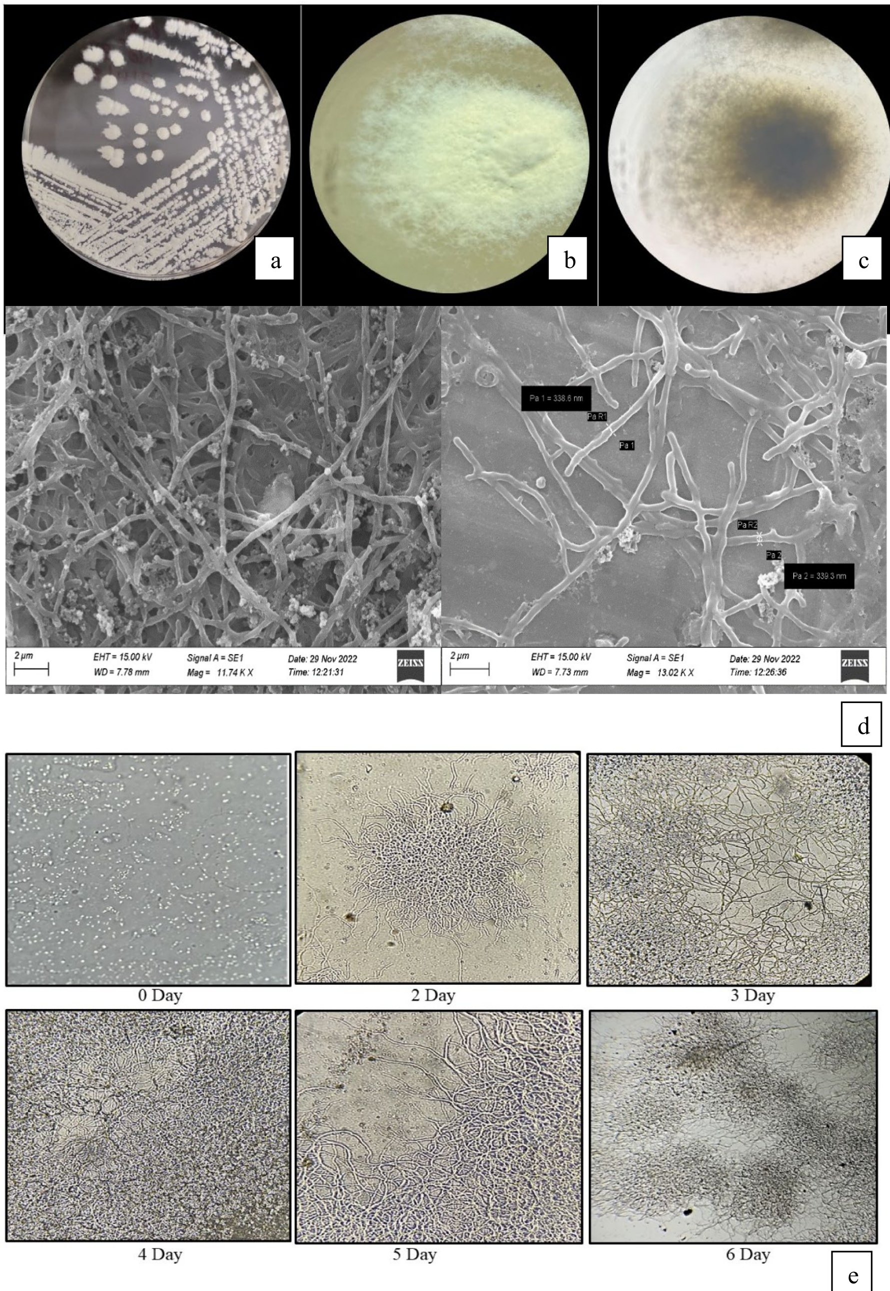


Fig. 1 Morphology of *Nocardiopsis dassonvillei* strain VCS-4. **a** Growth on AIA medium, **b** 32 × magnification of single colony growth on AIA, **c** 32 × magnification of reverse side single colony growth on AIA, **d** filamentous growth under scanning electron microscopy, and **e** in situ cultivation of *Nocardiopsis dassonvillei* strain VCS-4

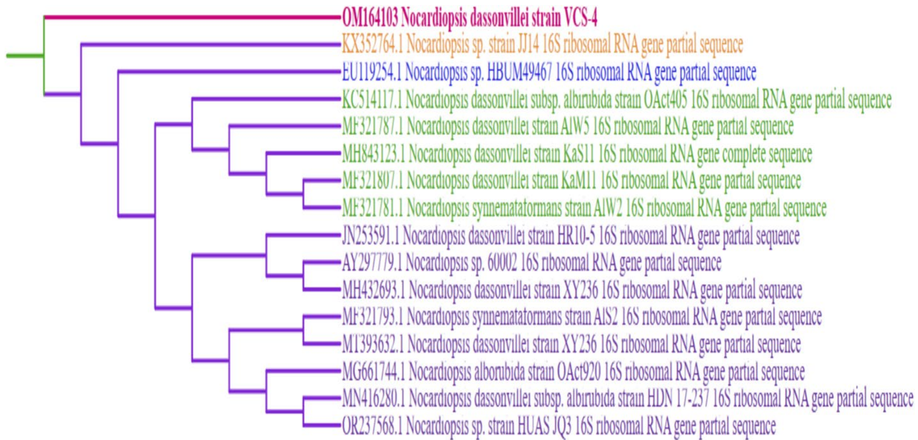


Fig. 2 Phylogenetic analysis of *Nocardiopsis dassonvillei* strain VCS-4 using MEGA 11software

revealed the formation of long, filamentous hyphae after 2 days of incubation, followed by spore formation and colony development after 5 days. Scanning electron microscopy (SEM) analysis (Fig. 1d, e) further corroborated the filamentous structure of the VCS-4 colony, with filaments measuring approximately 338.6 nm in width. According to the earlier report, well-developed vegetative mycelial colonies were reported for *Streptomyces*, *Agromyces*, *Cellulomonas*, and *Cellulosimicrobium* genera [31]. Gohel and Singh [3] reported the growth behavior of *Nocardiopsis xinjiangensis* strain OM-6 by in situ cultivation in starch agar medium (5%, NaCl; pH 9). The *Nocardiopsis xinjiangensis* strain OM-6 displayed curved-shaped mycelia growth followed by fragmentation of mycelia after 4 days of incubation and after 10 days of incubation development of spores were reported. The spores were oval to elongated having smooth surfaces and arranged in short chains of spores [20].

Physicochemical Characterization of Nocardiopsis dassonvillei Strain VCS-4

Microbial identification could be achieved through biochemical tests and sugar utilization assays, which assess their specific metabolic pathways. [32]. The majority of studied actinobacterial species exhibited positive results for nitrate reduction, catalase, and oxidase tests, as reported previously [18]. *Nocardiopsis dassonvillei* strain VCS-4 displayed a similar biochemical profile, demonstrating positive results for these tests along with citrate utilization, as detailed in Table 1. The sugar utilization potential helps actinobacteria to survive in various extreme conditions. Mainly dextrose, sucrose, cellobiose, xylose, arabinose, and galactose were utilized by *Actinoplanes*, *Streptomyces*, and *Nocardiopsis* [33–35]. The *Nocardiopsis dassonvillei* strain VCS-4 could ferment all sugars except cellobiose. The used sugars were arabinose, dextrose, fructose, glucose, galactose, lactose, mannose, maltose, mannitol, raffinose, rhamnose, sucrose, trehalose, and xylose. Earlier, the *Nocardiopsis* genus was reported to utilize raffinose, xylose, maltose, mannitol, fructose, and glucose [36]. *Nocardiopsis dassonvillei* strain VCS-4, isolated in this study, exhibited the remarkable ability to produce protease under haloalkaline conditions (high salt and alkaline pH). This strain demonstrated broad environmental tolerance, growing across a wide range of NaCl concentrations (up to 20% w/v), pH (6 to 12), and temperatures (28

to 50 °C). However, optimal growth was observed at 8% NaCl (w/v), pH 10, and 28 °C. As this example highlights, environmental parameters play a crucial role in microbial growth and secondary metabolite production, even under harsh conditions. The 5% NaCl, pH 9, and moderate temperature were reported to be optimum conditions for the majority of marine bacteria [37, 38]. The bacteria associated with marine hosts produce various enzymes including amylase, protease, keratinase, lipase, L-asparaginase, xylanase, chitinase, cellulase, and dextranase that embrace industrial significance [15, 39]. Therefore, in the present research, *Nocardiopsis dassonvillei* strain VCS-4 was screened for amylase, protease, pectinase, cellulase and L-asparaginase production. Screening results identified *Nocardiopsis dassonvillei* strain VCS-4 as a promising protease producer (Fig. 3a, b).

Effect of %NaCl on Growth and Production of Protease on Gelatin Agar

The *Nocardiopsis dassonvillei* strain VCS-4 was screened for protease secretion on gelatin agar supplemented with 0–12% (w/v) NaCl (w/v) (Fig. 3a, b). The *Nocardiopsis dassonvillei* strain VCS-4 could grow and produce protease in the range of 0–12% NaCl (w/v). The optimum protease secretion with a 3.5 ratio was observed in 6% NaCl (w/v) which is quite enhanced compared to earlier reported proteases from *Nocardiopsis alba* strain OM-5, *Streptomyces lopnurensis* KaM5, and *Bacillus lehensis* JO-26 [7, 9, 19]. The *Nocardiopsis alba* strain OM-5 displayed optimum growth and protease secretion in the presence of 05% NaCl (w/v) [9]. However, protease produced by *Streptomyces lopnurensis* KaM5 was suppressed in more than 4% NaCl (w/v) [19]. Moreover, the recombinant protease from *Bacillus lehensis* JO-26 isolated from a saline desert was reported to produce maximum protease in 2% NaCl (w/v) while 4% NaCl suppressed the growth and protease production [7].

Medium Optimization for the Production of Protease

Screening of Significant Variables by Plackett–Berman Design

Actinobacteria can thrive under extreme environmental conditions and are mainly famous for the production of various bioactive metabolites [40]. Therefore, harnessing enzymes

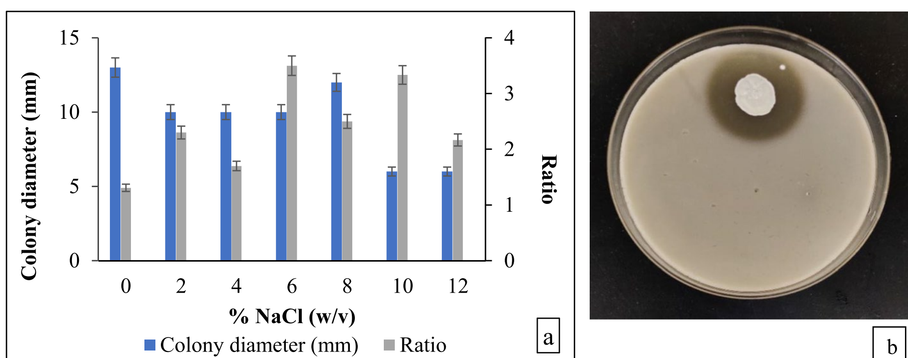


Fig. 3 **a** Effect of % NaCl (w/v) on growth (colony diameter) and protease secretion (ratio: gelatin hydrolysis zone diameter/colony diameter) of *Nocardiopsis dassonvillei* strain VCS-4 on gelatin agar and **b** growth of *Nocardiopsis dassonvillei* strain VCS-4 on gelatin agar plate supplemented with 10% NaCl and pH 10

from marine actinobacteria provides enzymes with enhanced stability toward alkaline pH, and high salt concentrations, which are essential characteristics of enzymes used in detergent and food industries [41, 42]. Optimizing culture medium conditions is crucial for maximizing enzyme production. The composition of the medium directly influences both cell growth rate and the level of enzyme produced by the organism [42]. In the present investigation, a total of seven variables including gelatin, NaCl, pH, yeast extract, bacteriological peptone, inoculum, and incubation time were analyzed to check the production of protease by *Nocardiopsis dassonvillei* strain VCS-4 using the Plackett–Berman design (Table 2 and Fig. 4a). Pareto chart analysis revealed gelatin as the most significant factor influencing protease production (Fig. 4b). This aligns with the Pareto principle, which suggests that a small number of variables often contribute most significantly to an outcome. The maximum protease (358.18 U/mL) activity was observed in run order number 3. The acceptability of the Plackett–Berman design matrix was statically analyzed through a polynomial multiple regression equation to describe and predict the response by ANOVA analysis (Table 3). The level of significance was determined at α value of 0.05. A *p*-value less than 0.05 was considered a significant variable and needed further optimization, whereas a *p*-value more than 0.05 was considered an insignificant variable and could be neglected. The gelatin was a significant variable for the production of protease and therefore selected for further optimization using the OVATs approach, whereas incubation time, yeast extract, bacteriological peptone, inoculum, pH and NaCl were considered constant variables. The value of the constant variable was according to the solution obtained through Plackett–Berman design for optimum protease production with predicted fitted protease activity of 307.30 U/mL (Table 4). Further, the goodness of fit model for protease production was analyzed through residual plot and mean effect plot. The residual plots for protease activity versus order plot resulted in a random distribution of residual that verifies each residual is independent of the other (Fig. 5). The resulting straight-line graph for the normal probability plot of the residual indicated that residuals between variables and response were normally distributed. Further, the skewness of data was explained by histogram analysis

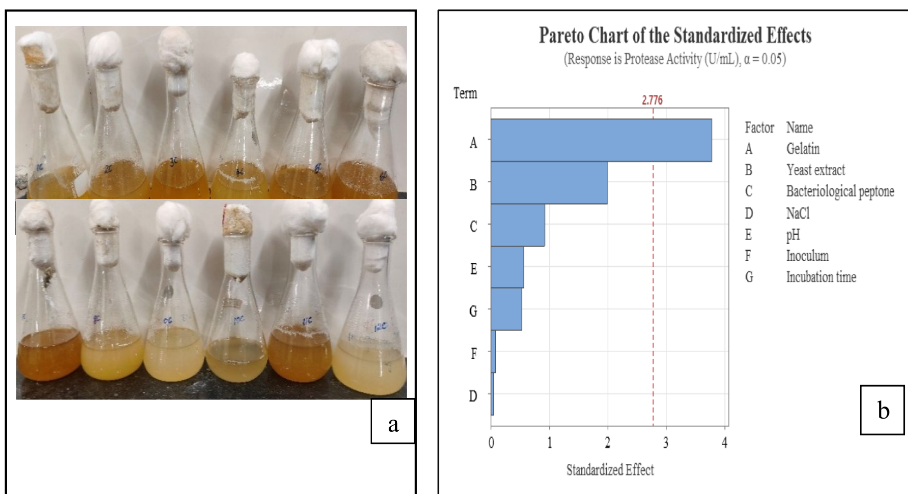


Fig. 4 Protease production by *Nocardiopsis dassonvillei* strain VCS-4 using Plackett–Burman design. **a** Run order-wise growth of *Nocardiopsis dassonvillei* strain VCS-4. **b** Pareto chart for production of protease

Table 3 ANOVA analysis for the production of protease by *Nocardiopsis dassonvillei* strain VCS-4 using Plackett–Berman design

Source	DF	Adj SS	Adj MS	*F*-value	*p*-value
Model	7	139835	19976	2.82	0.167
Linear	7	139835	19976	2.82	0.167
Gelatin	1	101284	101284	14.3	0.019
Yeast extract	1	28145	28145	3.97	0.117
Bacteriological peptone	1	6061	6061	0.86	0.407
NaCl	1	21	21	00	0.959
pH	1	2283	2283	0.32	0.601
Inoculum	1	53	53	0.01	0.935
Incubation time	1	1989	1989	0.28	0.624
Error	4	28334	7083		
Total	11	168169			

Table 4 Response optimization for the production of protease by *Nocardiopsis dassonvillei* strain VCS-4 using Plackett–Berman design

Variable	Setting		
Gelatin	01 g/100 mL		
Yeast extract	0.1 g/100 mL		
Bacteriological peptone	0.1 g/100 mL		
NaCl	7 g/100 mL		
pH	8		
Inoculum	5 mL/100 mL		
Incubation time	9 days		
Response	Fit	95% CI	95% PI
Protease activity (U/mL)	307.30	(116.5, 498.1)	(5.6, 609.0)

(Fig. 5). The response analysis through the main effect plot traces the significance of the individual variable (Fig. 6a). Further, the effect of two variables on protease activity was analyzed by counter plot (Fig. 6b). According to counterplot > 200 U/mL, protease activity was observed with lower concentrations of gelatin and bacteriological peptone. Further, the counterplot of protease activity vs gelatin and incubation time shows optimum protease activity of > 200 U/mL in 1% gelatin and on the 9th day of incubation time, the counterplot of protease activity vs gelatin and yeast extract resulted in > 200 U/mL protease activity in 01.0 to 01.5% gelatin and 0.1 to 0.6% yeast extract, the counterplot of protease activity vs gelatin and NaCl resulted in maximum protease activity in presence of 3 to 7% NaCl and 0.1% gelatin, whereas the counterplot of protease activity vs NaCl and pH resulted in optimum protease activity between 0 to 5% NaCl (w/v) and pH 8 to 9. According to mean effect plot analysis, increasing gelatin, yeast extract and bacteriological peptone concentration suppressed mean protease activity whereas, NaCl, pH and inoculum did not affect protease production. The mean protease activity was maximum with gelatin (01%), bacteriological peptone (0.1%), yeast extract (0.1%), NaCl (7%), pH (8), inoculum (5%), and incubation time (9th day) (Fig. 6a).

Regression equations for calculation of predicted response in Plackett–Berman design are as follows:

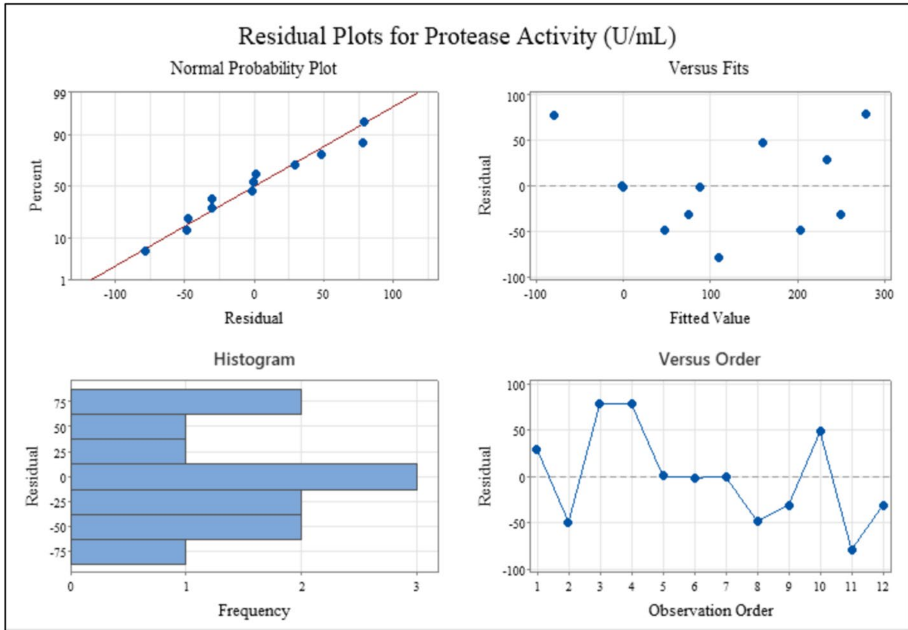


Fig. 5 Residual plot of protease production by *Nocardiopsis dassonvillei* strain VCS-4 using Plackett–Burman design

$$\text{Protease activity (U/mL)} = 486 - 91.9X_1 - 107.6X_2 - 49.9X_3 \\ -0.38X_4 - 13.8X_2 - 0.42X_6 + 4.29X_7$$

where X_1 = gelatin, X_2 = yeast extract, X_3 = bacteriological peptone, X_4 = NaCl, X_5 = pH, X_6 = inoculum, and X_7 = incubation time.

Medium Optimization Through One Variable at a Time (OVATs) Approach

The significant variable obtained through the Plackett—Berman design matrix was gelatin. Therefore, gelatin concentration was further optimized through the OVATs approach (Fig. 7a). A total of 3 different concentrations of gelatin were used to check maximum protease production by using yeast extract, bacteriological peptone, NaCl, pH, and inoculum as constant variables. The effect of gelatin concentration versus incubation time for protease production resulted in maximum protease production with 363.97 U/mL activity on the 7th day of incubation using 2% (w/v) gelatin (Fig. 7a). Hence, the optimized medium for protease production by *Nocardiopsis dassonvillei* strain VCS-4 with 363.97 U/mL tested protease activity and growth absorbance ($A_{660\ nm}$ = 2.322) contained gelatin 2%, yeast extract 0.1%, bacteriological peptone 0.1%, NaCl 7%, pH 8, inoculum 5%, and incubation time 7th day (Fig. 7b). The protease activity of 363.97 U/mL in the optimized medium obtained in the present investigation is much higher compared to earlier reported proteases produced by *Streptomyces*

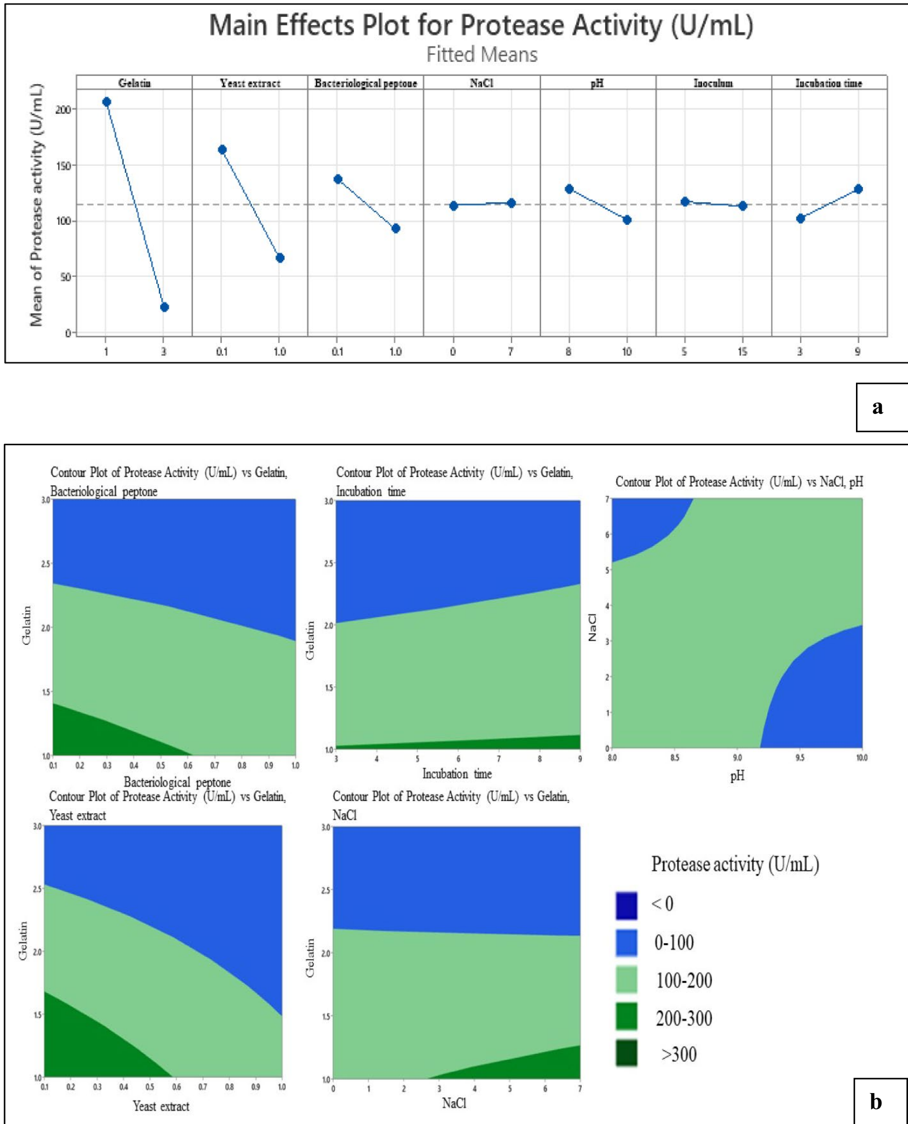


Fig. 6 **a** Main effects plot and **b** counter plot of protease production by *Nocardiopsis dassonvillei* strain VCS-4 using Plackett–Burman design

sp. Al-Dhabi-49 (139.2 ± 2.1U/mL) [11], *Streptomyces lopnurensis* KaM5 (117 U/mL) [19], and *Bacillus subtilis* (143.73 U/mL) [43].

Production of Protease Using Agro-industrial Wastes

The application of proteases in day-to-day life has increased drastically [6]. The wide usage of proteases is in meat tendering, detergent additives, and brewing processes to avoid the

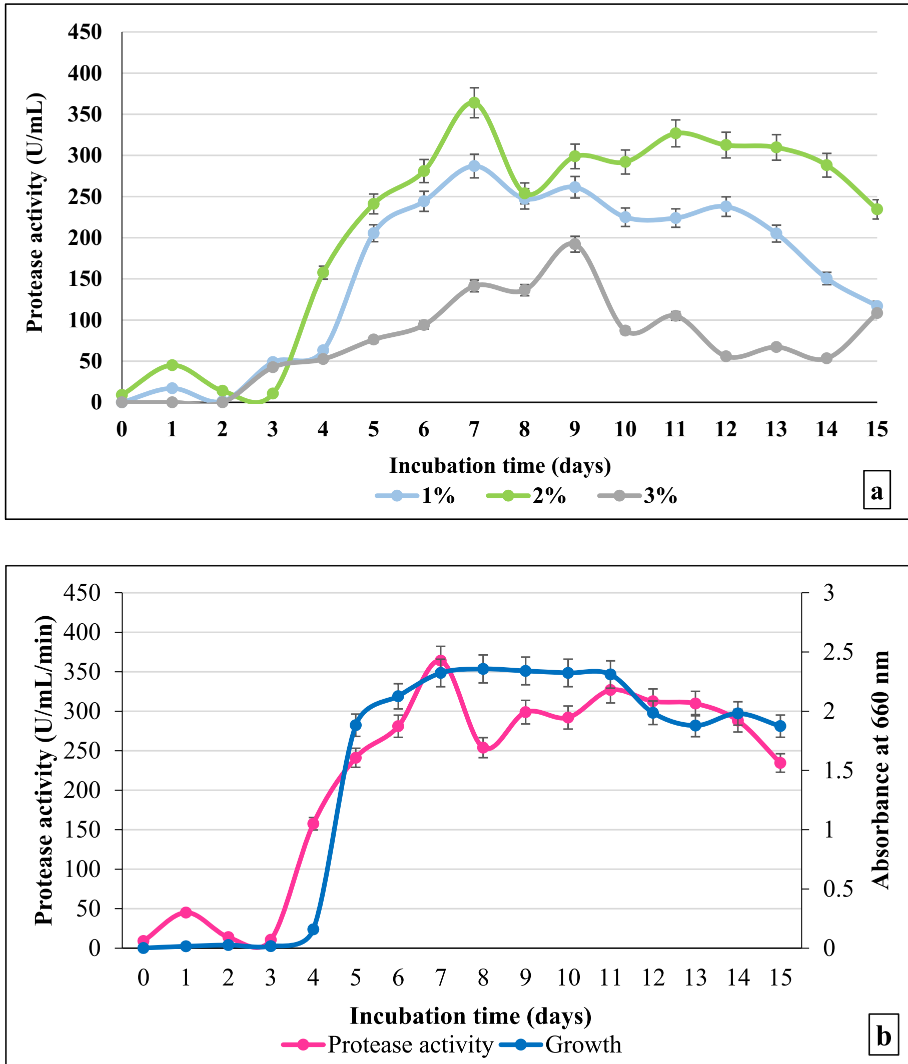


Fig. 7 **a** Effect of % gelatin (w/v) on protease production by *Nocardiopsis dassonvillei* strain VCS-4 using OVATs approach and **b** effect of the optimized medium on protease activity and growth of *Nocardiopsis dassonvillei* strain VCS-4

chill hade of beer [44]. The crude protease also plays a significant role in organic waste degradation and is therefore used for treating sewage water [6, 24]. The rapid growth of the global enzyme market demands the replacement of synthetic media with cheaper substances for the production of enzymes. Therefore, the production of protease was further analyzed using agro-industrial waste to reduce the cost of the production medium. Millets, a diverse group of cereals, are being recognized for their potential to address global nutritional needs, as highlighted by the International Year of Millets 2023. Several millet varieties, including pearl millet, foxtail millet, kodo millet, teff, finger millet, and guinea millet,

are important cereal crops in Asia and Africa (https://www.fao.org/home/en/). The millet, sorghum, groundnut, and wheat are the important crops of the Saurashtra region, Gujarat, India (www.agrifarming.in), and therefore, are used as a substrate for protease production. A total of 7 different agro-industrial wastes were used as substrates. The finely chopped waste of 3% (w/v) was used for the production of protease (Fig. 8a–c) that included wheat straw, millet straw, sorghum straw, groundnut shells, groundnut cake, soybean meal, and gram flour, whereas skimmed milk powder and gelatin were used as control. Groundnut cake emerged as the most effective substrate among all other sources, yielding the highest protease activity (408.42 U/mL) as shown in Fig. 8d. This significantly surpassed the

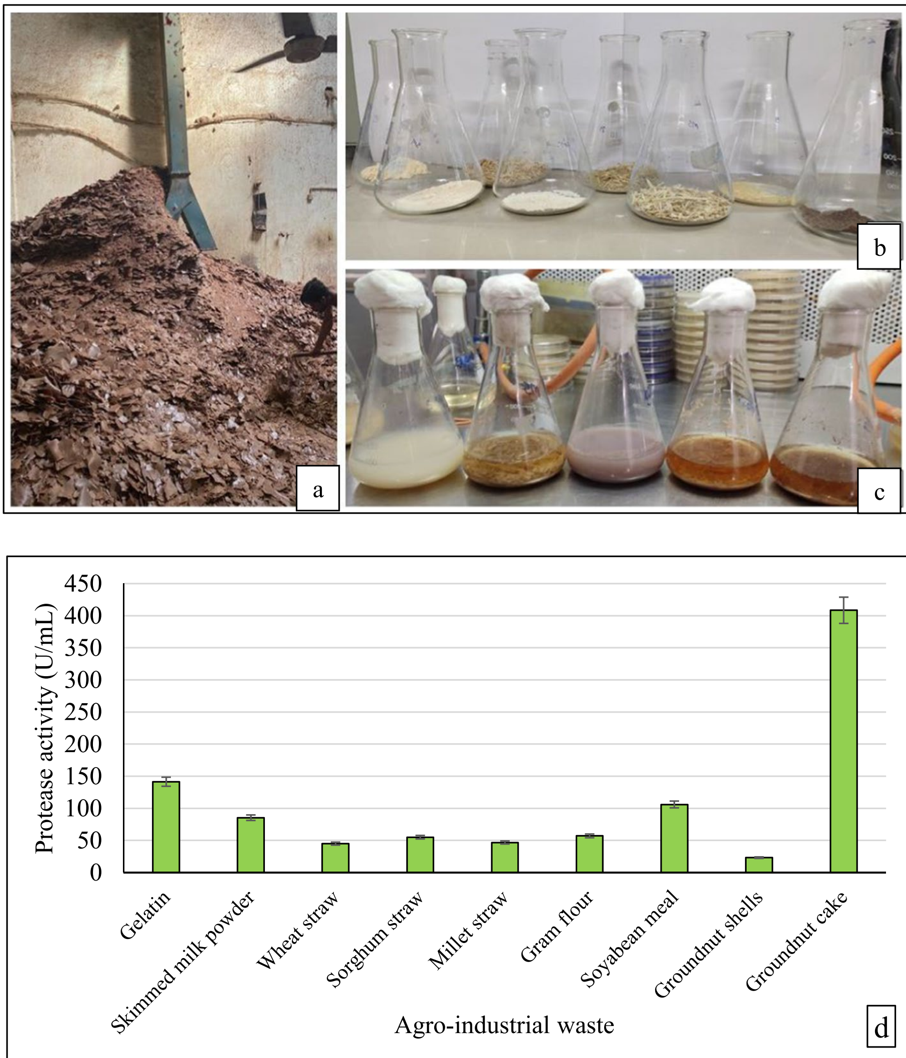


Fig. 8 **a** Groundnut oil factory showing groundnut cake as waste material, **b** finely chopped various agro-industrial waste, and **c** various agro-industrial wastes inoculated with *Nocardiopsis dassonvillei* strain VCS-4 for production of protease

activity observed with substrates such as gelatin (141.41 U/mL), soybean meal (105.98 U/mL), and skimmed milk powder (85.40 U/mL). Conversely, groundnut shells displayed the lowest protease production (23.16 U/mL). The superior performance of groundnut cake can likely be attributed to its rich nutrient profile. Compared to groundnut shells, it boasts a significantly higher protein content (45–50%) [57]. Additionally, it offers a well-balanced composition of other essential nutrients, including carbohydrates (22–30%), fibers (3.8–7.8%), lipids (4–6%), and minerals (4%) [57]. Soybean meal, another protein-rich substrate (32–43%) [58], also supported good protease production, although not as effective as groundnut cake. Skim milk powder and gelatin, while containing protein, seemingly lack the optimal combination of nutrients such as carbon and nitrogen sources for maximum protease production. Till today, less research has been conducted using actinobacteria for enzyme production using agro-industrial waste [45]. Previous studies have explored the potential of agro-industrial waste for L-asparaginase production [46] and vegetable/fruit peels as substrates for amylase and protease production by *Nocardiopsis* sp. [47, 48].

Growth and Protease Production Kinetics

The protease production and growth of *Nocardiopsis dassonvillei* strain VCS-4 were checked using an optimized medium (gelatin, 2%; yeast extract, 0.1%; bacteriological peptone, 0.1%; NaCl, 7%; pH 8; and inoculum, 5%) and agro-industrial waste (groundnut cake; 3%) containing medium. The growth and protease production were measured at a definite incubation time. The growth pattern of *Nocardiopsis dassonvillei* strain VCS-4 was comparable with reported strains of *Nocardiopsis* and *Streptomyces* [47, 49]. The *Nocardiopsis dassonvillei* strain VCS-4 was a slow grower that required 11.9 h and 9.74 h to double the growth in optimized and agro-industrial waste media, respectively (Table 5). Further, the growth of *Nocardiopsis dassonvillei* strain VCS-4 was directly related to enzyme production. The maximum protease production was observed at the stationary phase of the growth cycle. The comparisons of growth kinetics show the highest specific growth rate (dx/dt) of 0.361 g/mL/h in the agro-industrial waste medium. Moreover, the average maximum protease production rate of 68.07 U/mL/h and maximum volumetric protease production (Pmax) of 408.42 U/mL were observed in the agro-industrial waste medium, whereas the optimized medium resulted average maximum protease production rate of 61.65 U/mL/h and maximum volumetric protease activity of 363.97 U/mL. The Pmax of 408.42 U/mL by *Nocardiopsis dassonvillei* strain VCS-4 using groundnut cake was higher compared to

Table 5 Kinetic study of protease production by *Nocardiopsis dassonvillei* strain VCS-4

Sr. no	Parameters	Optimized medium	Agro-industrial waste (groundnut cake)
1	Maximal cell mass: Xmax (g/mL)	0.0023	0.0023
2	Specific growth rate: dx/dt (g/mL/h)	0.161	0.361
3	Maximum volumetric enzyme production: Pmax (U/mL)	363.97	408.42
4	Average enzyme production rate: Qp (U/mL/h)	61.65	68.07
5	Generation time: g (h)	11.9	9.74

earlier reported protease production by *B. subtilis* B22 (334.17 ± 1.8 U/mL at 72 h) using groundnut oil cake [50] and *Bacillus licheniformis* (210.8 U/mL at 96 h) using wheat bran [42].

Immobilization of Crude VCS-4 Protease Using Affinity Binding Method

The distinct size and porosity of cationic and anionic exchangers contribute to their substantial internal and external surface area. The ion exchanger's unique size and porosity facilitate the immobilization of protease through charge-based interactions. Earlier, seralite SRC 120 was used as a catalyst for the synthesis of 3,3′-bis(indolyl)methanes (BIMs) from indoles which could provide a 79% yield even after four repetitive cycles [51]. In the present research, immobilization of protease on seralite SRC 120 by ionic binding method resulted in the highest immobilization activity of 470.38 U/g, unbound activity of 217.76 U/g, immobilization yield of 88.20%, and recovery activity of 28.91%. Comparatively, seralite SRA 400 exhibited the lowest immobilization, with an activity of 360.45 U/g and 992.24 U/g for immobilized and unbound protease, respectively (Table 6). These findings were comparable to those reported for immobilized protease derived from sunflowers [52]. Immobilization using DEAE cellulose D-E52 via ionic binding resulted in a yield of 35.00 ± 3.00%, significantly lower than that achieved with immobilized VCS-4 protease. Further, the sunflower protease was immobilized by physical and covalent binding using chitin, starch, and a combination of chitin + starch. The % yield by covalent binding was 63.50 ± 1.90%, 46.0 ± 2.20%, and 83.20 ± 1.25% on chitin, starch, and a combination of chitin + starch respectively. In comparison, % yield by physical binding was 2.50 ± 0.50% using chitin and 2.8 ± 0.88% using starch as support matrices [52]. Immobilization with a higher reusability rate plays a significant role in the application of protease at the industrial level. Immobilized live cells of *Bacillus circulans* have been reported to produce alkaline protease with activities ranging from 4000 to 5700 U/mL [53]. Subsequently, the live cells in the fermentation medium were reported between 3×10^5 and 9×10^5 CFU/mL with a reusability rate of immobilized cells between 20 and 26 batches. The protease obtained from *Bacillus pumilus* Y-7 was immobilized using polyvinylimidazole/sepiolite (PVI/SEP) hydrogel with 95% immobilization yield and 65% relative reusability activity for 16 repetitive cycles [54]. Immobilized protease on Seralite SRC 120 and SRA 400 exhibited 21.89% and 12.18% residual activity, respectively, after five consecutive cycles (Fig. 9a). The thermotolerant and haloalkaliphilic properties of studied VCS-4 protease render it suitable for application in various detergent and pharmaceutical industries. Moreover, the effect of pH and temperature on native and immobilized protease was investigated and compared with commercial protease from *Streptomyces griseus* (HiMedia, India). The optimum pH and temperature for native and immobilized protease were 10 and 70 °C respectively (Fig. 9b, c), significantly higher than commercial protease (pH 9 and 50 °C). Notably, the

Table 6 Immobilization of VCS-4 protease using ionic binding method

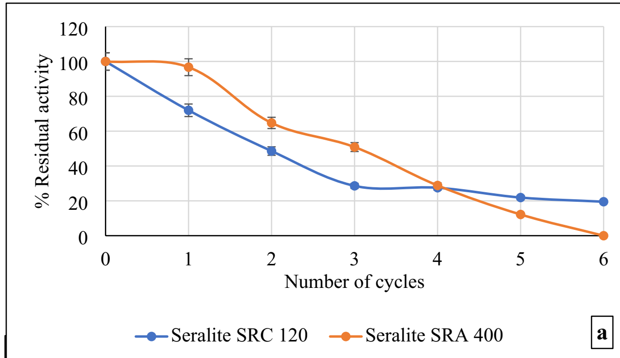
Matrices	Loaded protease (U/g matrix)	Unbound protease (U/g matrix)	Immobilized protease (U/g matrix)	Immobilization yield (%)	Recovery activity (%)
Seralite SRC 120	1845	217.76	470.38	88.20	28.91
Seralite SRA 400	1845	992.24	360.45	46.22	42.27

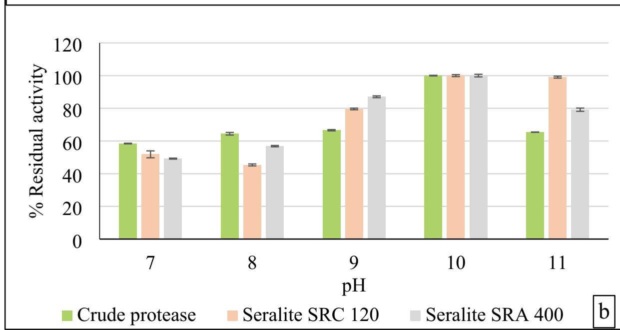
Fig. 9 **a** Reusability rate of immobilized protease, **b** effect of pH on native and immobilized protease, **c** ▶ effect of temperature on native and immobilized protease, and **d** effect of NaCl (1 M and 2 M) and temperature (50 °C, 60 °C, and 70 °C) on native and immobilized protease

immobilized protease on seralite SRC 120 and seralite SRA 400 exhibited enhanced stability at 80 °C, with residual activity of 122.52% and 107.49%, respectively, compared to the native protease (Fig. 9c). Similarly, immobilized protease on seralite SRC 120 and seralite SRA 400 with 99.07% and 79.14% residual activity were more stable at pH 11 compared to native protease having residual activity of 65.43% (Fig. 9b). The protease activity at 60 °C was considered 100%. The native and immobilized VCS-4 protease was active at alkaline pH ranging from 9 to 11. The residual activities were measured by considering the protease activity at pH 10 as 100%. The impact of sodium chloride (NaCl) on protease activity at various temperatures was analyzed. The native (crude) protease exhibited remarkable stability, retaining over 101.54% residual activity even under harsh conditions of 1 M NaCl and 70 °C. This suggests the inherent stability of protease. However, the effect of NaCl concentration varied depending on temperature. At a lower temperature of 50 °C and a higher NaCl concentration of 2 M, the residual activity of the crude protease decreased to 98.54%. Interestingly, immobilization of protease on seralite SRC 120 seemed to improve tolerance under these conditions, with residual activity reaching 115.33% at 2 M NaCl and 50 °C. Furthermore, the influence of temperature on salt tolerance was evident. At a constant high temperature of 70 °C and a higher NaCl concentration of 2 M, the residual activity of the crude protease dropped slightly to 98.57%. Immobilization played a significant role in the stability of protease. Seralite SRA 400 resulted in the highest residual activity (100.07%) at 2 M NaCl and 70 °C, indicating improved tolerance compared to both crude protease and protease immobilized on to seralite SRC 120 (93.84% residual activity). This finding aligns with the notion that high salt tolerance and a high salt requirement for active conformation are often observed in proteases from halophilic microorganisms, suggesting them as a promising source for such enzymes. The observed thermal stability is likely due to higher levels of non-polar amino acids that enhanced hydrophobic interaction. Fascinatingly, this protease also exhibited a "polyextremophilic nature," remaining active/stable in alkaline pH, high temperature, and non-aqueous medium besides activity in the presence of salt [54, 56]. The increasing thermotolerant and haloalkaliphilic nature is a unique feature of immobilized VCS-4 protease compared to earlier reported immobilized protease using polyvinylimidazole/sepiolite (PVI/SEP) hydrogel [54] and DEAE cellulose D-E52 [52].

Conclusion

The *Nocardiopsis dassonvillei* strain VCS-4, isolated from the seaweed *Caulerpa scalpeliformis*, emerged as a promising protease producer. Using a combination of Plackett–Burman design and one-variable-at-a-time (OVATs) optimization, the high average production rate (61.65 U/mL/h) and maximum volumetric activity (363.97 U/mL) were achieved. Various agro-industrial wastes were explored as substrates to minimize production costs. Groundnut cake proved most effective, supporting a maximum protease activity (Pmax) of 408.42 U/mL. Furthermore, the crude protease was immobilized via ionic binding on seralite SRC 120 and seralite SRA 400 carriers to enhance reusability. This resulted in reusability activity of 21.89% and 12.18%, respectively, after five cycles. Both native and immobilized proteases exhibited optimal activity at pH 10 and 70 °C. These findings


% Residual activity
120
100
80
60
40
20
0
0 1 2 3 4 5 6
Number of cycles
Seralite SRC 120
Seralite SRA 400
a


% Residual activity
120
100
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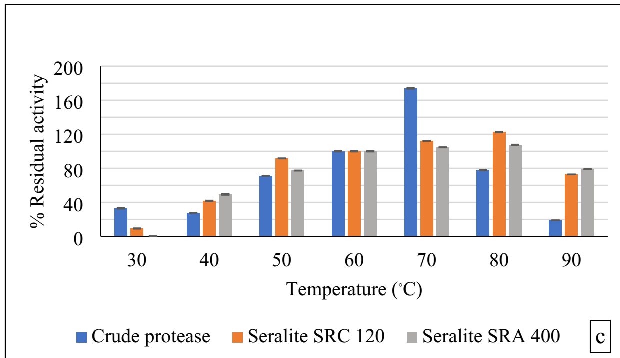
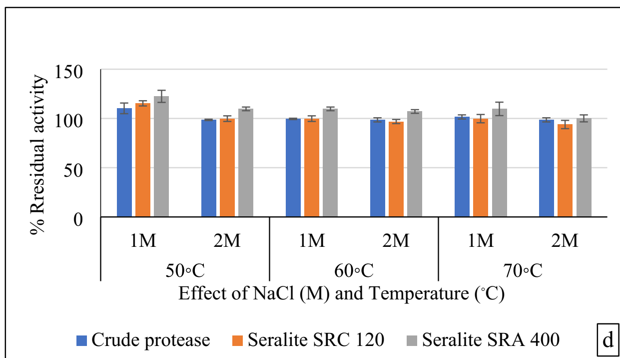
0
7 8 9 10 11
pH
Crude protease
Seralite SRC 120
Seralite SRA 400
b


% Residual activity
200
160
120
80
40
0
30 40 50 60 70 80 90
Temperature (°C)
Crude protease
Seralite SRC 120
Seralite SRA 400
c


% Rresidual activity
150
100
50
0
1M 2M 1M 2M 1M 2M
50◦C 60◦C 70◦C
Effect of NaCl (M) and Temperature (°C)
Crude protease
Seralite SRC 120
Seralite SRA 400
d

highlight the potential of the immobilized VCS-4 protease for various industrial applications due to its economical production, thermotolerance, and haloalkaliphilic nature, making it suitable for the detergent, food, and pharmaceutical sectors.

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Author Contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by V.R. Majithiya. The first draft of the manuscript was written by V.R. Majithiya. The author S.D. Gohel analyzed the data and reviewed and edited the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

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Data Availability The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval This article does not contain any studies with animals or human participants.

Consent to Participate The authors agreed to participate in this work.

Consent for Publication We declare that the information in this manuscript has not been published elsewhere nor is it under consideration by any other journal. Furthermore, it is the consensus of all authors to submit this manuscript for possible publication in ABB.

Competing Interests The authors declare no competing interests.

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