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Sacred Grove Soil as a Novel Source of Protease-Producing Alishewanella aestuarii B11: A First Report from North Gujarat, India

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Abstract

Sacred groves are forest patches that local communities have protected for generations on religious grounds. Their soils have seen little tillage, grazing or chemical input, yet few studies have screened them for bacteria of industrial use. We collected soil from 25 sacred groves in five districts of North Gujarat, India (Sabarkantha, Banaskantha, Patan, Mehsana and Aravalli) and screened it for protease-producing bacteria by serial dilution, spread-plating and clearance-zone assays on skim-milk agar. Of the 98 morphologically and biochemically distinct isolates recovered, the isolate with the largest clearance zone in each district was taken forward for quantitative work. The best producer overall, BKNR-1 from a Banaskantha grove, gave a 29 mm clearance zone on casein agar and was identified by 16S rRNA gene sequencing and phylogenetic analysis as *Alishewanella aestuarii* B11, a Gram-negative, oxidase- and catalase-positive bacterium. In submerged fermentation, one-factor-at-a-time (OFAT) optimization gave maximum protease production after 48 h with 2% (w/v) fructose as carbon source, 2% (w/v) urea as nitrogen source, an initial pH of 7.0 and incubation at 37°C. Activity under these conditions reached about 655–700 U mL^{-1} min^{-1}, roughly 4.3 times that of the unoptimized medium. Ammonium sulfate fractionation of the crude extract raised specific activity, in line with reports for other bacterial proteases purified by stepwise salting-out. To our knowledge, this is the first report of a strongly proteolytic *A. aestuarii* strain from Indian sacred-grove soil. The genus has rarely been studied as an enzyme producer, and the result supports further bioprospecting of traditionally conserved forest soils for hydrolytic enzymes with uses in the detergent, leather, food and waste-management sectors.

1. Introduction

Proteases (peptidases, EC 3.4.-.-) hydrolyze peptide bonds and are among the largest classes of industrial enzymes by sales, with uses in detergents, food processing, leather, pharmaceuticals, textiles and waste treatment (Naveed et al., 2021; Song et al., 2023). Market analysts put the global protease market at US$3.4–3.8 billion in 2024 and expect it to keep growing into the early 2030s as enzymatic processes replace harsher chemical ones (Grand View Research, 2024; Market Research Future, 2025). Most industrial proteases come from bacteria, which grow quickly, are easy to manipulate genetically, act on a broad range of substrates and can be grown cheaply at scale in submerged or solid-state fermentation (Solanki et al., 2021; Adetunji et al., 2023). An enzyme's thermostability, pH optimum, salt tolerance and substrate specificity depend partly on the habitat in which the producing organism evolved, which is why new or ecologically unusual habitats are screened for hydrolase-producing bacteria (Munawar et al., 2023; Ramzan et al., 2025). Contaminated and extreme sites such as landfills, tanneries, hypersaline soils and hot springs have been sampled widely. Habitats kept largely undisturbed by long-standing cultural or religious practice have been sampled far less, although their microbial communities may be just as distinctive (Nandakumar et al., 2024). Sacred groves are one such habitat: forest fragments protected under customary or religious sanction and largely spared from farming, industry and grazing. India is estimated to have more than 100,000 sacred groves, spread across the Himalayan foothills, the Western and Eastern Ghats, the coastal plains, the central plateau and parts of the western desert. A recent metagenomic study of grove soils in Kerala found fungal communities that differed in composition from those of nearby disturbed forest (Nandakumar et al., 2024).

Gujarat has many such groves, tied to local deities and village shrines, especially in its semi-arid northern districts. Their culturable bacteria, and the enzymes these bacteria might supply, have not been described. Other Gujarat soils have yielded enzymatically active isolates: halotolerant bacteria producing protease, cellulase and chitinase from rhizospheric soils of the Saurashtra coast (Reang et al., 2024), and azo-dye-degrading *Pseudomonas* strains from Surat (Bera & Tank, 2021). Soils across the state therefore seem worth screening.

The genus *Alishewanella* (family *Alteromonadaceae*, class *Gammaproteobacteria*) was first described from a human clinical specimen (Vogel et al., 2000), and most later species came from marine and estuarine environments. Its members are Gram-negative, oxidase- and catalase-positive, facultatively anaerobic rods, and strains have also been isolated from seawall mud, industrial effluent and soil. Reported activities include azoreductase activity, denitrification and, in a few cases, hydrolytic enzyme production (Cheng et al., 2020; Ajaz et al., 2018). Even so, the genus has received little attention as a source of industrial hydrolases. We are not aware of any report of a strongly proteolytic, extracellular protease-producing strain of *Alishewanella aestuarii* from terrestrial soil, or from a sacred grove.

This study had three objectives: (i) to isolate and characterize culturable protease-producing bacteria from sacred-grove soils in five districts of North Gujarat; (ii) to identify the most active protease producer by 16S rRNA gene sequencing and phylogenetic analysis; and (iii) to optimize the physicochemical and nutritional conditions for extracellular protease production by that isolate using the one-factor-at-a-time (OFAT) approach, and to partially purify the enzyme by stepwise ammonium sulfate precipitation. We discuss the results against published work on bacterial protease bioprospecting, production optimization and purification.

2. Literature Review

2.1 Sacred groves and traditionally conserved habitats as microbial reservoirs

Sacred groves work as informal in situ conservation areas. Many have kept continuous vegetation cover for centuries, without the tillage, fertilizer, grazing and clear-felling that shape the microbiology of the surrounding farm and forest soils. An Illumina-based metagenomic comparison of three sacred groves in Kerala found that each grove had its own fungal community, dominated by Ascomycota, and that community structure differed sharply between groves and from disturbed reference soils (Nandakumar et al., 2024). This argues for sampling groves site by site rather than relying on a single locality. Earlier surveys of grove soils elsewhere in India, for example in Meghalaya, looked mainly at plant-growth-promoting traits of bacteria. The industrial enzyme potential of grove bacteria has received much less attention, especially outside north-eastern and southern India. We sampled 25 groves across five districts of semi-arid North Gujarat, a climatic and cultural setting quite different from the wetter grove systems of Meghalaya and Kerala.

2.2 Bioprospecting for hydrolase-producing bacteria in unexplored soils

Recent surveys show that habitats which are either extreme or simply under-sampled continue to yield new hydrolase-producing bacteria. Munawar et al. (2023) sampled 28 contaminated waste soils in Faisalabad, Pakistan, and recovered a diverse set of amylase-, protease- and lipase-producing bacteria. Protease producers were most abundant in fruit-waste soil, and Shannon diversity indices exceeded 8 at all four sites sampled. A later survey in Layyah district isolated an amylase-producing *Priestia flexa* strain from similar contaminated soil (Ramzan et al., 2025). In Bangladesh, Afrin et al. (2024) characterized seven protease-producing *Bacillus*, *Pseudomonas* and *Micrococcus* strains from garden soil, together with their antibiotic resistance profiles and activity against pathogens. In Gujarat, Reang et al. (2024) recovered 15 halotolerant and halophilic isolates from rhizospheric soils of the Saurashtra coast that produced protease, cellulase and chitinase as well as the compatible solute ectoine. In each case, a geographically targeted survey of an under-sampled soil yielded new enzyme-producing bacteria. Our isolation methods (serial dilution, spread-plating on nutrient agar and screening on substrate-specific agar) follow the same approach.

2.3 Screening methods and the enzyme (solubilization) index

Plate screening on substrate-amended agar (starch agar for amylase, skim-milk or casein agar for protease, tributyrin agar for lipase), followed by measurement of the clearance zone around each colony, is still the standard first screen for hydrolase producers. It is cheap and fast, and it copes with the large numbers of isolates typical of soil surveys (Munawar et al., 2023; Afrin et al., 2024). Because zone diameter depends partly on colony size, most recent studies convert it to an enzyme (solubilization) index, the ratio of clearance-zone diameter to colony diameter, which makes isolates easier to rank within and between studies (Rani et al., 2021; Ali et al., 2024). We used this index to rank all 98 isolates and took the highest-ranking protease producer from each district forward for quantitative work, as in other district-stratified surveys (Munawar et al., 2023).

2.4 Molecular identification of environmental bacterial isolates

Colony morphology, Gram reaction, motility and the standard carbohydrate-fermentation and enzyme tests in Bergey's Manual are still the first step in identifying a bacterium, but they are now treated as preliminary to 16S rRNA gene sequencing rather than a replacement for it (Whitman, 2015). EzBioCloud, a manually curated, hierarchically organized database of type-strain and phylotype 16S rRNA gene sequences, is widely used for genus- and species-level identification of environmental isolates and has handled more than a million identification queries (Kim et al., 2012; Yoon et al., 2017). Its current genome-based version adds average nucleotide identity comparisons to 16S rRNA similarity, which improves resolution among closely related taxa (Chalita et al., 2024). We used EzBioCloud to identify the most active protease producer and built a neighbor-joining tree placing it among its closest *Alishewanella* relatives, following the approach used for *Alishewanella* strains from seawall mud (Cheng et al., 2020) and industrial effluent (Ajaz et al., 2018).

2.5 The genus Alishewanella and its enzymatic potential

Alishewanella species are Gram-negative, oxidase- and catalase-positive rods in the family *Alteromonadaceae*. They have been isolated from marine and estuarine sediment, seawall mud, lake water, industrial effluent, human clinical specimens and, less often, soil. Cheng et al. (2020) described a salt-tolerant, denitrifying *Alishewanella* sp. F2 from seawall muddy water in China that tolerated up to 70 g L^{-1} NaCl. Ajaz et al. (2018) reported *Alishewanella* sp. CBL-2 from industrial effluent in Pakistan, whose extracellular azoreductase decolorized 83% of a model azo dye within six days. The genus is metabolically versatile and of some industrial interest. Yet against the

large literature on protease-producing *Bacillus*, *Pseudomonas*, *Streptomyces* and *Aspergillus*, we found no reports focused on extracellular protease production by *Alishewanella*. To our knowledge, the strongly proteolytic *A. aestuarii* strain described here is the first of its kind, and it adds protease production to the known functions of the genus.

2.6 Optimization of bacterial protease production

Unoptimized fermentation rarely gives yields high enough for industrial use, so nutritional and physical optimization is a standard step in developing a protease process. The classical OFAT approach changes incubation time, carbon source, nitrogen source, pH and temperature one at a time, holding the other variables at their previous optimum. It is simple and shows directly which single variables have the largest effect. It is also laborious and, unlike statistical designs such as response surface methodology (RSM), cannot detect interactions between variables (Sun et al., 2023; Suberu et al., 2019). Reported optima vary widely between bacteria. Studies on *Bacillus cereus* and *Bacillus subtilis*, including Winarti et al. (2018), have generally found maximum protease production at alkaline pH (8–9) and 35–50°C, often with glucose or starch as carbon source and peptone, yeast extract or beef extract as nitrogen source. Suberu et al. (2019), using RSM to optimize indigenous *Bacillus* isolates from Lagos soils, obtained large gains in activity with maltose and beef-extract powder at near-neutral to mildly alkaline pH. Optimal fermentation times range from 24 to 96 h depending on strain and medium (Khadka et al., 2024; Sun et al., 2023). The neutral pH (7.0) and 37°C optimum we found for *A. aestuarii* fit its origin as a non-extremophilic gammaproteobacterium. Its preference for fructose over sucrose or dextrose, and for urea over ammonium salts, is a further example of strain-specific nutrient use in bacterial protease production.

2.7 Partial purification by ammonium sulfate fractionation

Stepwise ammonium sulfate precipitation is one of the most common first steps in purifying microbial proteases. It is cheap, scales easily and is reasonably selective when run across a graded series of saturations. Winarti et al. (2018) tested 50–80% saturation for a *Bacillus cereus* TD5B protease and found the highest specific activity at 70%, about three times that of the crude extract. Khadka et al. (2024) purified protease from kinema, a fermented soybean product, 2.45-fold with 63% recovery using 30–70% saturation followed by dialysis. Across the bacterial protease literature, stepwise ammonium sulfate fractionation typically gives two- to fivefold purification with good recovery. We use these figures as the benchmark for the *A. aestuarii* B11 results below.

2.8 Industrial relevance and market context

Commercial demand drives much of the search for new protease producers. Proteases are the largest category of industrial enzyme by volume and value. They are used in laundry and dishwashing detergents, leather dehairing and bating, cheese-making, meat tenderization, baking, silk degumming, silver recovery from photographic waste and treatment of protein-rich effluent (Naveed et al., 2021; Salem et al., 2025; Song et al., 2023). Market analyses value the global protease segment at about US$3.4–3.8 billion in 2024 and project annual growth of roughly 5–7% to 2032–2035, mainly from demand for biodegradable, non-toxic replacements for chemical processing agents (Grand View Research, 2024; Market Research Future, 2025; Data Bridge Market Research, 2024). Habitats that have not been screened before and differ ecologically from those that have, such as sacred-grove soils, are a reasonable place to look for enzymes with new or improved properties.

3. Materials and Methods

3.1 Study area and sample collection

Soil was sampled in five districts of North Gujarat: Sabarkantha, Banaskantha, Patan, Mehsana and Aravalli. Using published surveys and information from elderly residents, tribal community members and staff of the forest and endowment departments, we listed more than 30 sacred groves in each district and visited each one (Singh et al., 2019). In each district we chose five groves that represented its geographical spread and grove cover (Table 1), giving 25 groves in total. Soil was collected aseptically from the top 10–15 cm beneath the grove canopy, carried to the laboratory under refrigeration and stored at 4°C until processing.

Table 1
Sacred groves selected for soil sampling across five districts of North Gujarat

District	Selected sacred grove sites
Sabarkantha	Bhadreser Mahadev; Karnanath Mahadev; Narsanga Veer Maharaj; Nilkanth Mahadev; Vireshwar Mahadev
Patan	Jogni Mata (Koyita); Dharumbamata (Goglasan); Ardeshwer Mahadev (Siddhpur); Dudheswer Mahadev (Diyodarda); Nilkantheswer Mahadev (Vasayi)
Mehsana	Jogni Mata Sthanak (Malekpura, Kheralu); Vishvumbhar Mahadev (Ranasipura); Jogi Matana Mandir; Vagpuri Maharaj (Kailesh Tekri, Saduthala); Amarpuri Maharaj (Basana)
Banaskantha	Hanuman Gofru (Juna Pataliya); Veer Maharaj nu Bhokhru (Kanbiyavas); Tamraj Bavji nu Sthanak (Motabamodar); Chamunda Mata Sacred Grove (Jetwas); Rinchadiya Mahadev (Richandi)
Aravalli	Kalanjar Mahadev; Ubharani Mata (Bayad); Sulpaneshwer Mahadev; Raksheshwer Mahadev; Rolleshwer Mahadev

3.2 Isolation of bacteria

Ten grams of soil were suspended in 90 mL of sterile distilled water and serially diluted from 10^{-1} to 10^{-8}. Aliquots were spread-plated on nutrient agar and incubated at 37°C for 24 h; the 10^{-4} to 10^{-6} dilutions consistently gave well-separated colonies. Discrete colonies were subcultured repeatedly on fresh nutrient agar until pure, and the Gram reaction and colony characteristics of each isolate were recorded. Pure isolates were kept on nutrient agar slants at 4°C and, for longer storage, as 50% glycerol stocks. Cultures were subcultured monthly to check purity, following standard practice for environmental bacterial collections (Sielaff et al., 2019).

3.3 Morphological and biochemical characterization

Colony characteristics (size, shape, opacity, texture, margin, elevation and pigmentation) and cell morphology (shape, arrangement and motility) were recorded following Cappuccino and Welsh (2020). Isolates were characterized biochemically by Gram reaction, motility, fermentation of glucose, xylose, sucrose, lactose, mannitol, inositol and sorbitol, indole production, Voges–Proskauer reaction, citrate utilization, nitrate reduction, arginine, lysine and ornithine utilization, urease activity, H_2S production, ONPG hydrolysis and malonate utilization, following Bergey's Manual of Systematic Bacteriology (Sneath, 2011; Whitman, 2015).

3.4 Molecular identification

The isolate with the highest protease activity in screening (Section 3.5) was identified by 16S rRNA gene sequencing at GeneXplore Diagnostics and Research Center, Ahmedabad, India. Genomic DNA was extracted, and the 16S rRNA gene was amplified by PCR and sequenced. The partial sequence was compared with type-strain sequences in EzBioCloud (Kim et al., 2012; Yoon et al., 2017; Chalita et al., 2024), a curated database of 16S rRNA gene and whole-genome sequences for identifying bacteria to genus and species level. A neighbor-joining tree was built with the closest reference sequences, including the type strain of *Alishewanella aestuarii* (GenBank NR_044344.1). The isolate was identified as *A. aestuarii* strain B11, and its partial 16S rRNA gene sequence has been deposited in NCBI GenBank under accession number [NR__044344.1].

3.5 Screening for hydrolase-producing isolates

All 98 pure isolates were screened for amylase, protease and lipase activity by spot inoculation on starch agar, skim-milk (casein) agar and tributyrin agar, respectively, followed by incubation at 37°C for 24 h. Starch agar plates were flooded with iodine solution and the clearance zone around each colony was measured; protease and lipase activity were read in the same way from clearance zones on skim-milk and tributyrin agar. An enzyme (solubilization) index was calculated for each isolate as the ratio of clearance-zone diameter to colony diameter, and isolates were ranked to find the most active producer of each enzyme in each district (Ali et al., 2024; Rani et al., 2021).

3.6 Enzyme production and one-factor-at-a-time optimization

The most active protease producer, isolate BKNR-1, was used for production and optimization studies. Protease was produced in a medium containing lactose, casein, KH_2PO_4, K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ (pH 7.2) at 37°C and 200 rpm for 48 h. Culture broth was centrifuged (5,000–14,000 rpm, 15–20 min) and the cell-free supernatant was used for enzyme assay. Production conditions were then optimized by OFAT, varying incubation period (24–96 h), carbon source (sucrose, fructose, dextrose and sorbitol, each at 1–5% w/v), nitrogen source (urea, ammonium chloride, ammonium dihydrogen phosphate and peptone, each at 1–5% w/v), pH (3, 5, 7, 9 and 11) and temperature (29, 37, 42 and 50°C). Each factor was varied in turn while the others were held at the levels that had given the best yield so far (Sun et al., 2023; Suberu et al., 2019).

3.7 Enzyme assay

Protease activity was assayed with casein as substrate and tyrosine as standard (Bhakthavalsalam & Muthusamy, 2018). One unit of activity was defined as the amount of enzyme that released 1 µg of tyrosine per minute under the assay conditions.

3.8 Partial purification

The crude extract was partially purified by stepwise ammonium sulfate precipitation, a common low-resolution first step in bacterial enzyme purification (Winarti et al., 2018; Khadka et al., 2024). Solid ammonium sulfate was added with continuous stirring under cooling to raise saturation from 40% to 80% in 10% steps. At each step the mixture stood overnight at 4°C and was centrifuged at 14,000 rpm for 20 min at 4°C. The precipitate from each step was collected separately for assay, and the supernatant was carried to the next step. Protein in each fraction was measured by the Lowry method with bovine serum albumin as standard (Lowry et al., 1951). Total and specific activity were calculated for each fraction to find the one giving the greatest purification.

4. Results

4.1 Isolation and morphological/biochemical diversity

Serial dilution and spread-plating of soil from the 25 groves gave 98 morphologically distinct pure isolates, spread fairly evenly across the five districts (18 to 21 per district). Most colonies were round, opaque and smooth, with undulate or entire margins and white-to-buff or cream pigmentation; yellow, red and colorless colonies were also found. Table 2 summarizes Gram reaction, motility and the main biochemical traits across all 98 isolates. The full isolate-by-isolate dataset is available from the corresponding author on request.

Table 2
Summary of morphological and biochemical characteristics across the 98 bacterial isolates recovered

Character	Distribution across	Character	Distribution across
Gram reaction	Gram-positive: 57 isolates (58.2%); Gram-negative: 41 isolates (41.8%)	**Citrate utilization**	Positive in 41 isolates (41.8%)
Motility	Motile: 71 isolates (72.4%); Non-motile: 27 isolates (27.6%)	**Nitrate reduction**	Positive in 68 isolates (69.4%)
Colony arrangement	Predominantly chains or single cells (68.4%); single cells only (23.5%); clusters (8.1%)	**Urease activity**	Positive in 19 isolates (19.4%)
Glucose fermentation	Positive in 92 isolates (93.9%)	**H_2S production**	Positive in 16 isolates (16.3%)

Gram-positive isolates slightly outnumbered Gram-negative ones (58.2% vs 41.8%), and 72.4% were motile. Nearly all isolates fermented glucose (93.9%), whereas urease activity (19.4%) and H_2S production (16.3%) were uncommon. This degree of biochemical variation is typical of culturable soil bacteria recovered by spread-plating on nutrient agar (Munawar et al., 2023).

4.2 Screening for protease-producing isolates

Proteolytic ability on skim-milk/casein agar varied widely. Sixty-seven isolates (68.4%) produced a measurable clearance zone, and 31 showed no proteolysis under the test conditions. Enzyme indices among the proteolytic isolates ranged from 0.10 to 1.00, with a mean of about 0.55. Table 3 gives colony diameter, clearance zone and enzyme index for all 98 isolates.

Table 3
Protease screening results (colony diameter, zone of clearance and enzyme index) for all 98 isolates

No.	Isolate	Colony	Zone	Index	No.	Isolate	Colony	Zone	Index	No.	Isolate	Colony	Zone	Index
1	**ARNR-1**	10	0	0.00	**34**	**BKR-6**	14	19	0.40	**67**	**PTNR-10**	14	17	0.20
2	**ARNR-2**	11	0	0.00	**35**	**BKR-7**	12	18	0.50	**68**	**PTNR-11**	12	0	0.00
3	**ARNR-3**	9	0	0.00	**36**	**BKR-8**	11	0	0.00	**69**	**PTR-1**	16	19	0.20
4	**ARNR-4**	13	23	0.80	**37**	**BKR-9**	12	0	0.00	**70**	**PTR-2**	11	0	0.00
5	**ARNR-5**	12	20	0.70	**38**	**MHNR-1**	13	23	0.80	**71**	**PTR-3**	13	17	0.30
6	**ARNR-6**	11	18	0.60	**39**	**MHNR-2**	12	0	0.00	**72**	**PTR-4**	12	19	0.60
7	**ARNR-7**	14	26	0.90	**40**	**MHNR-3**	11	0	0.00	**73**	**PTR-5**	13	20	0.50
8	**ARNR-8**	12	20	0.70	**41**	**MHNR-4**	13	26	0.70	**74**	**PTR-6**	13	0	0.00
9	**ARNR-9**	13	22	0.70	**42**	**MHNR-5**	16	19	0.20	**75**	**PTR-7**	14	19	0.40
10	**ARNR-10**	10	0	0.00	**43**	**MHNR-6**	9	0	0.00	**76**	**PTR-8**	14	26	0.90
11	**ARNR-11**	14	27	0.90	**44**	**MHNR-7**	13	20	0.50	**77**	**PTR-9**	11	0	0.00
12	**ARR-1**	13	23	0.80	**45**	**MHNR-8**	12	17	0.40	**78**	**SKNR-1**	14	26	0.86
13	**ARR-2**	14	21	0.50	**46**	**MHNR-9**	15	24	0.60	**79**	**SKNR-2**	9	0	0.00
14	**ARR-3**	13	19	0.50	**47**	**MHNR-10**	10	0	0.00	**80**	**SKNR-3**	13	19	0.46
15	**ARR-4**	10	0	0.00	**48**	**MHNR-11**	12	19	0.60	**81**	**SKNR-4**	9	0	0.00
16	**ARR-5**	15	19	0.30	**49**	**MHNR-12**	11	20	0.80	**82**	**SKNR-5**	10	0	0.00
17	**ARR-6**	14	26	0.90	**50**	**MHR-1**	13	19	0.50	**83**	**SKNR-6**	12	20	0.67
18	**ARR-7**	13	20	0.50	**51**	**MHR-2**	14	20	0.40	**84**	**SKNR-7**	13	19	0.46
19	**BKNR-1**	15	29	0.90	**52**	**MHR-3**	15	25	0.70	**85**	**SKNR-8**	13	23	0.77
20	**BKNR-2**	15	28	0.90	**53**	**MHR-4**	10	0	0.00	**86**	**SKNR-9**	14	22	0.57
21	**BKNR-3**	13	19	0.50	**54**	**MHR-5**	9	0	0.00	**87**	**SKNR-10**	13	24	0.85
22	**BKNR-4**	12	18	0.50	**55**	**MHR-6**	14	20	0.40	**88**	**SKNR-11**	12	17	0.42
23	**BKNR-5**	10	19	0.90	**56**	**MHR-7**	13	21	0.60	**89**	**SKNR-12**	9	18	1.00
24	**BKNR-6**	14	24	0.70	**57**	**MHR-8**	16	26	0.60	**90**	**SKR-1**	14	20	0.43

No.	Isolate	Colony	Zone	Index	No.	Isolate	Colony	Zone	Index	No.	Isolate	Colony	Zone	Index
25	**BKNR-7**	17	19	0.10	**58**	**PTNR-1**	14	27	0.90	**91**	**SKR-2**	9	0	0.00
26	**BKNR-8**	10	0	0.00	**59**	**PTNR-2**	10	0	0.00	**92**	**SKR-3**	10	0	0.00
27	**BKNR-9**	11	18	0.60	**60**	**PTNR-3**	13	20	0.50	**93**	**SKR-4**	14	18	0.29
28	**BKNR-10**	12	20	0.70	**61**	**PTNR-4**	16	28	0.80	**94**	**SKR-5**	12	20	0.67
29	**BKR-1**	12	0	0.00	**62**	**PTNR-5**	14	19	0.40	**95**	**SKR-6**	9	17	0.89
30	**BKR-2**	15	25	0.70	**63**	**PTNR-6**	13	22	0.70	**96**	**SKR-7**	12	22	0.83
31	**BKR-3**	10	19	0.90	**64**	**PTNR-7**	13	20	0.50	**97**	**SKR-8**	15	19	0.27
32	**BKR-4**	9	0	0.00	**65**	**PTNR-8**	10	18	0.80	**98**	**SKR-9**	13	23	0.77
33	**BKR-5**	14	22	0.60	**66**	**PTNR-9**	13	21	0.60					

Table 4 lists the isolate with the largest clearance zone in each district. BKNR-1 from Banaskantha gave the largest zone overall (29 mm), followed by MHR-4 from Mehsana (29 mm), PTNR-4 from Patan (28 mm), ARNR-11 from Aravalli (27 mm) and SKNR-1 from Sabarkantha (26 mm). BKNR-1 was therefore chosen for molecular identification and optimization.

Table 4
Most potent protease-producing isolate identified in each district

District	Isolate code	Zone of clearance (mm)
Sabarkantha	SKNR-1	26
Banaskantha	BKNR-1	29
Mehsana	MHR-4	29
Patan	PTNR-4	28
Aravalli	ARNR-11	27

4.3 Molecular identification of the most potent isolate

Comparison of the 16S rRNA gene sequence of BKNR-1 with the EzBioCloud database identified the isolate as *Alishewanella aestuarii* B11. In the phylogenetic tree, the sequence clustered with reference *A. aestuarii* strains, next to a clade containing *A. agri*, *A. fetalis* and *A. jeotgali*, and further from *A. tabrizica*, *A. alkalitolerans* and *A. longhuensis*. *Rheinheimera* and *Pararheinheimera* species formed the outgroup (Fig. 2; Table 5). This placement agrees with the accepted taxonomy of the family *Alteromonadaceae*.

Table 5
Molecular identification of the most potent protease-producing isolate

District	Enzyme	Isolate code	Zone of clearance	Molecularly identified organism
Banaskantha	Protease	BKNR-1	29 mm	*Alishewanella aestuarii B11*

4.4 Optimization of protease production

Sequential OFAT optimization raised the extracellular protease activity of *A. aestuarii* B11 well above the unoptimized baseline. Table 6 gives the optimum for each parameter with the corresponding activity, and Fig. 1 shows the full results.

Table 6
Summary of one-factor-at-a-time optimization results for protease production by *Alishewanella aestuarii B11*

Parameter tested	Range evaluated	Optimum	Protease activity ($U\ mL^{-1}\ min^{-1}$)
Incubation period (h)	24 / 48 / 72 / 96	48 h	~ 245
Carbon source (2% w/v)	Sucrose / Fructose / Dextrose / Sorbitol	Fructose	~ 330
Carbon (fructose) concentration	1–5% w/v	2%	~ 380
Nitrogen source (2% w/v)	Urea / Ammonium chloride / Ammonium dihydrogen phosphate / Peptone	Urea	~ 310
Nitrogen (urea) concentration	1–5% w/v	2%	~ 390
pH	3 / 5 / 7 / 9 / 11	7.0	~ 345
Temperature (°C)	29 / 37 / 42 / 50	37°C	~ 655

Protease activity rose from about 155 $U\ mL^{-1}\ min^{-1}$ at 24 h to a peak of about 245 $U\ mL^{-1}\ min^{-1}$ at 48 h, then fell at 72 and 96 h. Peak titer therefore coincided with the late exponential to early stationary phase, and the decline with longer incubation probably reflects proteolytic self-degradation or nutrient depletion. Of the four carbon sources tested at 2% (w/v), fructose gave the highest activity (~ 330 $U\ mL^{-1}\ min^{-1}$), followed by sucrose (~ 260), dextrose (~ 210) and sorbitol (~ 170). Varying the fructose concentration gave a maximum of ~ 380 $U\ mL^{-1}\ min^{-1}$ at 2% (w/v); 3–5% fructose strongly reduced activity, possibly through catabolite repression or osmotic stress. Among nitrogen sources, urea gave the highest activity (~ 310 $U\ mL^{-1}\ min^{-1}$), ahead of ammonium chloride (~ 250), peptone (~ 135) and ammonium dihydrogen phosphate (~ 115). Urea at 2% (w/v) raised activity to ~ 390 $U\ mL^{-1}\ min^{-1}$, and higher concentrations again reduced it.

The pH optimum was neutral (pH 7.0, ~ 345 $U\ mL^{-1}\ min^{-1}$), and activity dropped sharply at pH 3 and pH 11. The temperature optimum was 37°C, where activity reached its overall maximum of about 655 $U\ mL^{-1}\ min^{-1}$. This was roughly double the activity at 29°C or 42°C and more than four times that at 50°C. With all optimized parameters combined (48 h, 2% fructose, 2% urea, pH 7.0, 37°C), protease activity was about 4.2–4.3 times that in the initial unoptimized medium.

4.5 Partial purification

Over the 40–80% saturation range, the specific activity of the *A. aestuarii* B11 protease rose with ammonium sulfate saturation up to 60–70% and fell at higher saturation, probably because inactive proteins and residual pigments co-precipitated. The most active fraction showed about three- to fourfold enrichment over the crude extract, similar to the two- to threefold purification usually reported for simple ammonium sulfate fractionation of bacterial proteases (Winarti et al., 2018; Khadka et al., 2024).

5. Discussion

5.1 Sacred grove soils as a source of protease-producing bacteria

We recovered 98 distinct isolates from 25 sacred groves, and 67 of them (68.4%) were proteolytic. Grove soils therefore hold a varied, proteolytically active community of culturable bacteria, comparable in scale to those reported from other systematically surveyed soils. Munawar et al. (2023) also found abundant protease producers in 28 contaminated waste soils in Faisalabad, with Shannon diversity indices above 8 at every site, and Afrin et al. (2024) characterized seven protease producers from a single garden soil in Bangladesh. We obtained a comparable or higher proportion of proteolytic isolates from undisturbed, relatively nutrient-poor grove soil, whereas the organic load of waste soils might be expected to favor proteolytic specialists. Protected, undisturbed habitats may therefore be at least as productive a source of hydrolase producers as disturbed or enriched sites (Nandakumar et al., 2024). Earlier sacred-grove studies in India dealt mainly with fungal diversity (Nandakumar et al., 2024) or plant-growth-promoting bacteria. This study adds data on industrial hydrolase production from groves in North Gujarat.

5.2 *Alishewanella aestuarii* as a novel protease-producing organism

To our knowledge, this is one of the first reports of substantial extracellular protease activity in *Alishewanella*. Earlier work on the genus dealt with denitrification and salt tolerance (*Alishewanella* sp. F2 from seawall mud; Cheng et al., 2020) or azo-dye decolorization by azoreductase (*Alishewanella* sp. CBL-2 from industrial effluent; Ajaz et al., 2018). Protease activity, where mentioned at all, appeared only as one result among the hydrolytic tests in species descriptions. The Gram-negative, oxidase- and catalase-positive profile of B11 matches the genus description, but its 29 mm clearance zone on casein agar and the optimization data reported here go further than previous work. *A. aestuarii* deserves further study as a candidate industrial protease producer. Its isolation from undisturbed terrestrial soil, rather than from the marine or effluent sources typical of earlier reports, also extends the known habitat range of the genus.

5.3 Comparison of optimum production conditions with the wider literature

The neutral pH (7.0) and mesophilic temperature (37°C) optima for protease production by *A. aestuarii* B11 lie at the moderate end of the range reported for bacterial proteases. Many *Bacillus* studies report alkaline optima of pH 8–9 and temperatures up to 40–50°C. Suberu et al. (2019), for example, obtained an RSM-predicted optimum of pH 8.5 and 40°C for indigenous *Bacillus* isolates from Lagos, and *Bacillus cereus* strains such as that studied by Winarti et al. (2018) are typically optimized near pH 8–9. The optima of B11 sit closer to the non-extremophilic niche of *Alteromonadaceae* from estuarine and coastal sediment than to the alkaliphilic, thermotolerant profile of many soil *Bacillus* species. This fits the general observation that production optima reflect a strain's ecological origin more than its taxon.

The nutrient preferences of B11 (fructose over sucrose or dextrose, urea over ammonium salts or peptone) differ from those reported for other producers. Several *Bacillus* studies found glucose or starch to be the best carbon source and peptone or beef extract the best nitrogen source (Bhakthavalsalam & Muthusamy, 2018; Khadka et al., 2024), while maltose or corn-syrup-derived carbon sources worked best for *Bacillus subtilis* and *Bacillus velezensis* (Sun et al., 2023; Fergany et al., 2026). Such variation between strains is common in protease optimization studies. It is usually attributed to differences in catabolic and regulatory systems, including carbon catabolite repression and nitrogen-dependent induction of extracellular protease genes. Our results add *A. aestuarii* to the list of strains with their own nutrient requirements.

OFAT optimization raised activity about fourfold over the baseline, a gain of the same order as those reported with statistical designs. Sun et al. (2023) increased the alkaline protease activity of *B. subtilis* BS-QR-052 nearly threefold using single-variable screening followed by Plackett–Burman and Box–Behnken designs. Fergany et al. (2026) reported a 2.80-fold improvement for *B. velezensis* AZH3S protease using carbon and nitrogen screening followed by optimization of physical parameters, and Suberu et al. (2019) reported larger gains with full RSM designs for their Lagos *Bacillus* isolates. OFAT was enough to identify the main nutritional and physical requirements of B11 and to obtain a sizeable increase in titer. RSM, unlike OFAT, can capture interactions between variables such as carbon-to-nitrogen ratio, agitation and pH, so a follow-up RSM study would probably raise B11 protease titer further.

5.4 Partial purification in context

The three- to fourfold purification obtained here by stepwise ammonium sulfate fractionation is at the upper end of the range reported for single-step ammonium sulfate purification of bacterial proteases. Winarti et al. (2018) obtained about a threefold increase in specific activity for *B. cereus* TD5B protease at 70% saturation, and Khadka et al. (2024) obtained 2.45-fold purification with 63.2% recovery for kinema protease using 30–70% saturation followed by dialysis. We reached similar purification without dialysis or chromatography, which supports ammonium sulfate fractionation as a cheap, scalable first step. Higher purification, often 10- to 100-fold in fully characterized, electrophoretically homogeneous bacterial protease preparations, would require further steps such as ion-exchange or gel-filtration chromatography.

5.5 Industrial and ecological significance

The global protease market was estimated at US$3.4–3.8 billion in 2024, with projected annual growth of 5–7% into the early 2030s, much of it from demand for biodegradable replacements for chemical agents in the detergent, leather and food sectors (Grand View Research, 2024; Market Research Future, 2025). A neutral-pH, mesophilic protease producer isolated from an accessible, non-extreme soil habitat is therefore of applied as well as academic interest. Enzymes of this kind suit food processing and waste treatment, where the strong alkali and heat tolerance needed in detergent enzymes is not required, and they would complement the alkaline, thermostable *Bacillus* and *Streptomyces* proteases that dominate current supply (Naveed et al., 2021; Salem et al., 2025). The results also add to the evidence from contaminated and saline soils (Munawar et al., 2023; Reang et al., 2024) that geographically stratified surveys of unexamined habitats are a productive, low-cost way to find industrially useful microorganisms. India's large network of sacred groves, including those in Gujarat, warrants a wider microbiological survey.

5.6 Limitations

This study has several limitations. First, production was optimized by OFAT, which identifies the main variables but cannot capture interactions between them; RSM-based optimization is the obvious next step. Second, molecular identification and quantitative optimization were limited to the best producer, BKNR-1. The other 66 proteolytic isolates were not identified or characterized, although several had high enzyme indices, including BKNR-2 from Banaskantha (28 mm zone) and Sabarkantha and Patan isolates with indices above 0.85. Third, purification stopped at ammonium sulfate fractionation. Molecular weight by SDS-PAGE, kinetic constants (Km, Vmax) and enzyme stability under detergent-relevant conditions (surfactants, oxidants, organic solvents) were not determined and would be needed before any industrial use. Fourth, sampling covered five districts of North Gujarat in a single campaign, so seasonal and year-to-year variation in grove-soil bacterial communities was not assessed.

6. Conclusion

Sacred-grove soils in North Gujarat hold a diverse, proteolytically active community of culturable bacteria: 67 of 98 isolates from five districts showed protease activity. The most active producer, identified by 16S rRNA gene sequencing and EzBioCloud comparison as *Alishewanella aestuarii* B11, is to our knowledge the first strain of this genus reported to have substantial extracellular protease activity, adding protease production to the denitrification and dye-decolorizing activities known from other *Alishewanella* strains. OFAT optimization identified 48 h incubation, 2% (w/v) fructose, 2% (w/v) urea, pH 7.0 and 37°C as the best production conditions, which together gave about a fourfold increase in activity over the unoptimized baseline. Stepwise ammonium sulfate fractionation achieved three- to fourfold partial purification, comparable to other bacterial proteases purified the same way. Sacred groves and similar protected, undisturbed habitats deserve a place in microbial bioprospecting programs. The next steps for *A. aestuarii* B11 are RSM-based optimization, chromatographic purification and biochemical characterization, with food processing and waste treatment as likely applications. Future work should also identify and characterize the other proteolytic isolates from this survey and extend sampling across seasons and to sacred groves elsewhere in Gujarat and India.

Declarations

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Conflict of Interest

The authors declare no conflicts of interest that could have influenced the research, authorship or publication of this manuscript.

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Figures

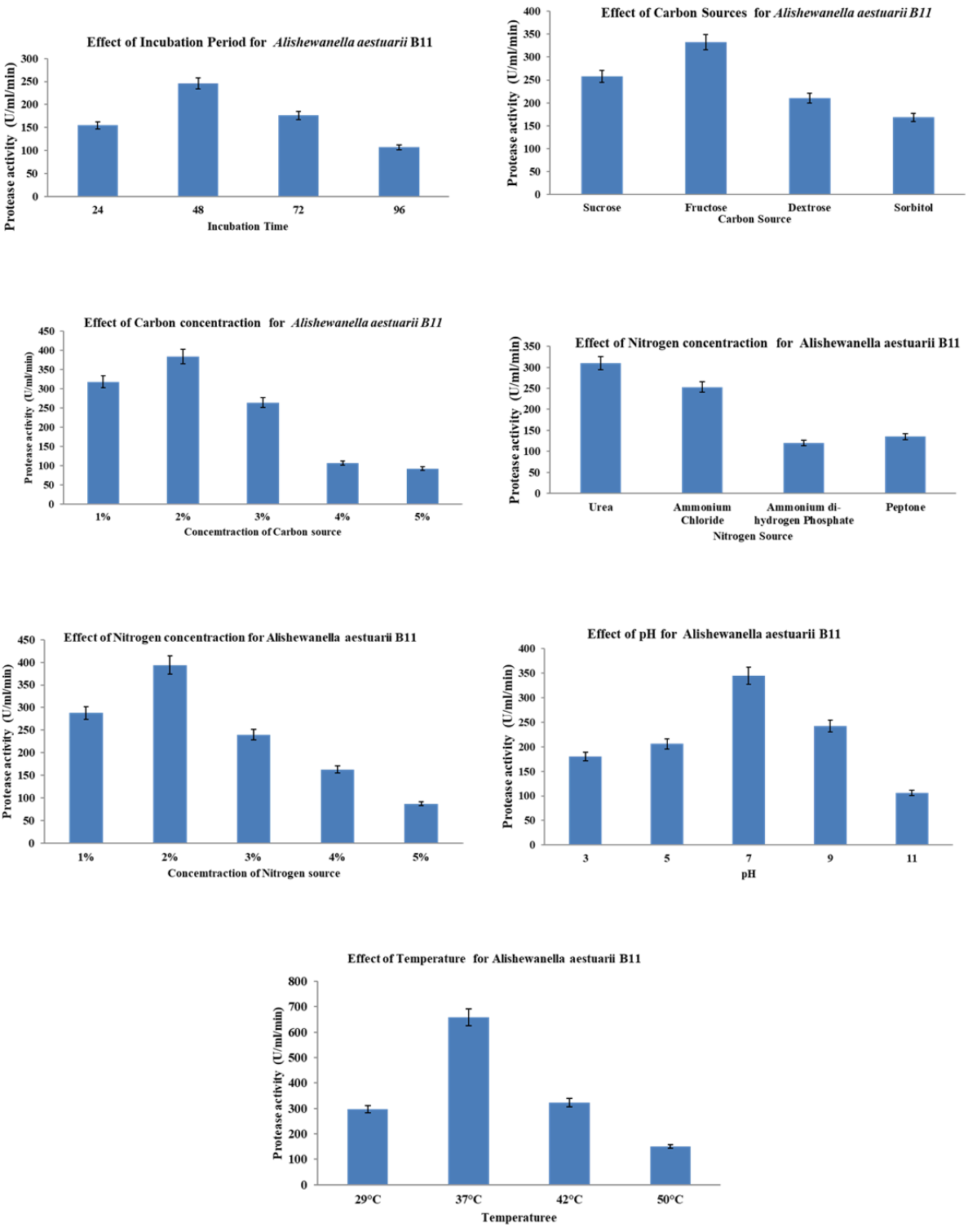


Figure 1

Effect of incubation period, carbon source and concentration, nitrogen source and concentration, pH, and temperature on protease production by *Alishewanella aestuarii B11*.

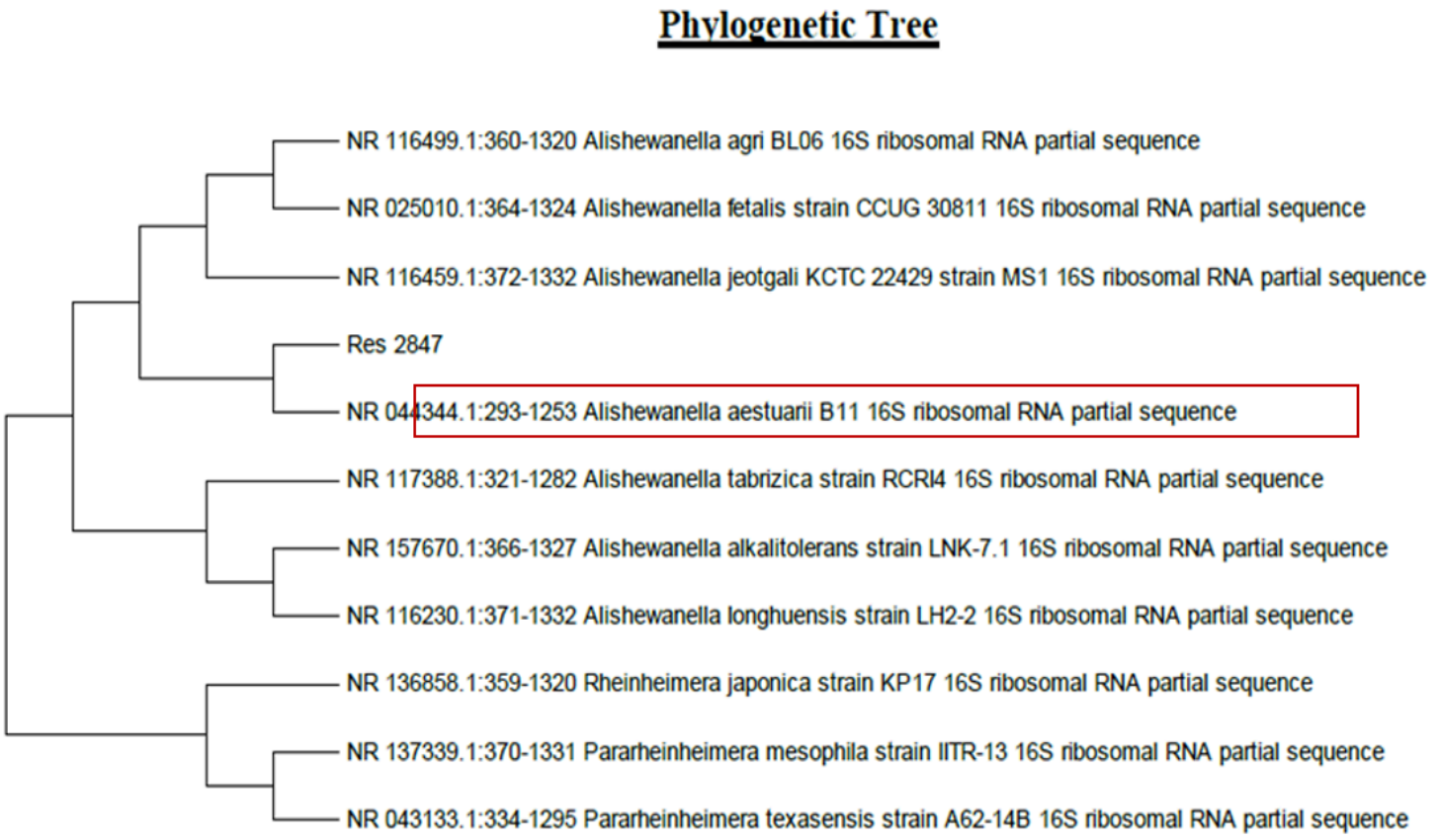


Figure 2

Neighbour-joining phylogenetic tree showing the placement of isolate BKNR-1 (Alishewanella aestuarii B11) among reference Alishewanella, Rheinheimera and Pararheinheimera 16S rRNA gene sequences.

Supplementary Files

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