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Solvent-tolerant DNase-producing gram-negative bacteria: Isolation characterisation and optimisation

Pranali Mahajan, Akshat Singh & Seema Sambrani*

Department of Life Sciences, Somaiya Vidyavihar University, Vidyavihar 400077 Mumbai, Maharashtra, India

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Solvent-tolerant microbes are an upcoming group of microorganisms capable of surviving and functioning in environments rich in organic solvents. Their enzymes are highly stable in the presence of organic solvents and are designed to mediate cellular and metabolic processes in an environment rich in solvents. Among these, DNases (Deoxyribonucleases) have gained significant attention for their diverse applications in molecular biology, diagnostics, and therapeutics due to their ability to degrade DNA. They are used in genomic DNA removal, biofilm disruption, and in the treatment of diseases such as cystic fibrosis and autoimmune disorders. The discovery of solvent-tolerant DNases with high thermal and pH stability enhances their potential for industrial applications, particularly in non-aqueous or extreme environments. This study focuses on isolating and characterising novel organic and aliphatic solvent tolerant bacteria producing DNase from the unique ecosystem of the Arabian Sea coast in India. These strains were screened for DNase activity using DNase plates and DPA test. Molecular identification of the strains was done by MALDI-TOF mass spectroscopy and 16s rRNA sequencing. The ammonium sulfate precipitation method was used to purify and characterise the enzyme. Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the purified enzyme with a molecular weight of 31kDa displayed maximum activity at 70°C and pH 9, sustaining good viability and activity in an environment of organic solvents such as diethyl ether, petroleum ether and methanol. Optimisation studies involving different carbon/nitrogen sources, pH, temperature, metal ions, and salt concentrations further enhanced enzyme production and stability. These findings underscore the significance of marine extremophiles as sources of industrially relevant enzymes. To the best of our knowledge, this is the first report of DNase activity in aliphatic solvent-rich environments, with promising implications for biotechnological and pharmaceutical applications.

Keywords: Marine extremophiles, Non-aqueous enzymology, Extracellular nucleases, Organic solvent stability, Solvent resistant enzymology

Organic solvents are widely recognised for their toxic effects on microorganisms, primarily due to their tendency to accumulate in cell membranes and disrupt integrity[1]. Owing to their antimicrobial qualities, these solvents have found broad applications such as industrial solvents, food preservatives, permeabilisation agents, and disinfectants[2]. Extensive research has explored how organic solvents induce cellular damage, with findings indicating that they compromise membrane structure and bilayer stability, impairing metabolic processes and leading to cell death[3].

Despite these toxic effects, certain microbial species-referred to as organic solvent-tolerant (OST) organisms have evolved the capacity to survive and function in solvent-rich environments. These OST bacteria represent a distinct group of extremophiles capable of withstanding high concentrations of toxic solvents[4]. "The first OST bacteria, *Pseudomonas putida*, was reported in 1989 by Innou & Horikoshi, demonstrating the ability to grow in the presence of 50% toluene[5-8]". While many of these tolerant species belong to Gram-negative genera such as *Pseudomonas*, similar traits have also been observed in Gram-positive bacteria like *Bacillus* and *Rhodococcus*[3].

Gram-negative bacteria can flourish in extremely high concentrations of hazardous organic solvents as they have evolved a variety of exclusive adaptive mechanisms. These mechanisms include the expression of shock proteins (e.g. phage shock proteins), the release of solvent-associated membrane vesicles, active efflux of toxic compounds via specialised efflux pumps, reinforcement of cell envelopes to enhance rigidity and reduce permeability, and the activation of membrane repair pathways[8].

Unlike conventional aqueous reactions, the utilisation of organic solvents as reaction media for various enzymatic processes provides desirable benefits for biocatalysts. However, such applications require enzymes that retain activity and structural ability in the presence of organic solvents. Unfortunately, most enzymes are inherently labile and prone to losing activity in non-aqueous environments. To combat this challenge, several strategies, including substrate engineering, medium engineering, and protein engineering, have been developed to enhance enzyme stability and activity in organic solvents[9].

Correspondence:
E-mail: seema.sambrani@somaiya.edu

Nonetheless, enzymes that are inherently solvent-stable would offer considerable benefits for synthetic and industrial processes, prompting increased interest in OST microorganisms as potential sources of such biocatalysts.

Solvent-tolerant enzymes represent a unique class of catalysts that maintain functionality in solvent-saturated environments. Solvent tolerant enzymes are of increasing interest for industrial applications particularly due to their ability to retain activity in non-aqueous environments where most conventional enzymes fail. This includes potential for use in bioremediation, pharmaceuticals, and fine chemical synthesis, where high substrate solubility and minimal microbial contamination are desired[10]. Notably, enzymes from extremophiles, such as those inhabiting cold or saline marine environments, often exhibit inherent stability in organic solvents and extreme conditions[11,12] Among them, DNases (EC 3.1.21.1) are of particular interest due to their wide-ranging applications in both biomedical and molecular biology fields. Extracellular DNases are vital enzymes with a broad spectrum of biological and industrial roles, including the degradation of chromosomal material and biofilm disruption. Their application is increasingly significant in molecular biology and diagnostics due to their capacity to efficiently hydrolyze DNA under varying environmental conditions[13,14]. These extracellular enzymes cleave DNA by hydrolyzing phosphodiester bonds[14,15]. In addition to their biological roles in processes such as apoptosis and actin regulation, DNases are used in clinical contexts, including therapies for cystic fibrosis and autoimmune diseases like systemic lupus erythematosus[14]. DNase I is extensively employed in molecular biology for genomic DNA removal, RNA purification, plasmid digestion in in vitro transcription, DNase footprinting, and nick translation assays [14, 16]. This study aims to isolate OST bacterial strains capable of producing DNase under solvent-rich conditions and to optimize culture parameters for enhanced enzyme production and activity.

Materials and Methods

Collection and isolation of the cultures

Water sample was collected from the Arabian Sea at Juhu, Mumbai (19.10000000°N and 72.83000000°E), Maharashtra, in sterile plastic bottles and processed in the lab. This sample was serially diluted to reduce microbial population and facilitate the isolation of microorganisms. The diluted samples were then spread-plated on DNase agar plates, a selective medium designed to promote the growth of microorganisms capable of producing or tolerating DNase enzymes. The plates were incubated at room temperature for 48 hours to allow visible colony formation. To obtain a pure culture, the obtained isolates were further subcultured.

Screening of DNase producing organisms

After the isolation of microorganisms, they were qualitatively screened for DNase production by spot inoculation onto small DNase agar (Brand: Himedia; Catalog number: M482) Petri plates. The plates were incubated at room temperature for 48 hours, followed by flooding with 1 N Hydrochloric acid (HCl). This step was crucial for triggering a biochemical reaction indicative of DNase enzyme production. Bacteria capable of producing DNase enzymes indicated by formation of clear zones or halos and were selected for the following experiments[17].

Selection of the most productive solvent tolerant microorganism

Several organic solvents, including toluene, benzene, benzyl chloride, diethyl ether, petroleum ether, acetone, glacial acetic acid, iso propyl alcohol, iso octane, ethanol, and methanol (Loba Chemie), were utilised to screen for the strain with broad stability in organic solvents. This was conducted to investigate the impact of both organic and aliphatic solvents on bacterial activity and enzyme production. This analysis can provide insights into whether variations in solvent structures influence the enzyme production capabilities of the bacteria.

Culture identification using MALDI-TOF

The selected strains were identified by MALDI-TOF mass spectroscopy (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight). It is an efficient analytical technique for quick and precise identification of microbial cultures. It combines matrix-assisted laser desorption/ionisation, where a matrix absorbs laser energy to ionise microbial molecules, with time-of-flight analysis, which measures the time ions take to reach the detector to determine their mass-to-charge ratio (m/z). The process involves preparing the sample with a matrix solution, irradiating it with a laser to produce ions, and performing mass spectrometric analysis to generate a unique mass spectrum that identifies the microbial species.

DNase assay

The DNase assay utilising the diphenylamine test is a crucial technique in molecular biology and biochemistry for quantifying DNase enzyme activity. This assay involves preparing samples containing the DNase enzyme, mixing them with a DNA substrate, and terminating the reaction at specific intervals using a termination reagent. The addition of diphenylamine reagent to the reaction mixture reacts with deoxyribose fragments from DNA degradation, forming a blue-coloured complex. After incubating the mixture at 100°C for 20 minutes, the absorbance was measured at 595 nm. The absorbance correlates with the amount of deoxyribose released, allowing the quantification of DNase activity, with higher absorbance indicating greater enzyme activity.

Carbon and Nitrogen source optimization

To optimize DNase broth, various carbon sources like mannitol, maltose, fructose, and lactose were tested to enhance microorganism growth and enzyme production[18]. Similarly, nitrogen sources such as tryptone, yeast extract, beef extract and casein were varied. DNase production was computed at the end of the cultivation period to determine the best carbon and nitrogen sources that support high yield of DNase. Studies show that selecting appropriate nitrogen sources, including peptones and hydrolysates, enhances microbial growth and productivity. This optimisation identifies the best conditions for microorganism metabolism and enzyme production[14,19].

Effect of pH and temperature on DNase activity

This test aimed to enhance the growth and productivity of microorganisms in the broth by identifying the most favourable pH value for their metabolic processes. Through the manipulation of pH levels, we sought to pinpoint the pH point that offers the most conducive conditions for bacteria to produce the DNase enzyme. The pH spectrum explored during this process ranged from 6 to 10 and the temperature range was a spectrum from 4°C, representing a low-temperature condition, to room temperature 25°C, 37°C, and an elevated temperature of 70°C[12,14,19].

Effect of metal ion on DNase activity

Iron is vital for cell functions like respiration and DNA synthesis; its availability affects cell growth rates, morphology, and differentiation. Metal ion testing, focusing on $FeSO_4$, $CuSO_4$, EDTA, $MgSO_4$ and $ZnSO_4$, explored their impact on DNase enzyme activity and DNA degradation efficiency, aiming to identify optimal conditions for these interactions[20,21].

Enzyme purification

After a 75% ammonium sulfate salting-out procedure, crude extracts underwent dialysis in potassium phosphate buffer (pH 7). The extracts were placed in dialysis bags, which allow small molecules to pass through while retaining proteins. The buffer was continuously replaced to remove salts and contaminants. The progress of dialysis was monitored using Nessler's reagent, which detects ammonia or ammonium ions. A negative result from Nessler's reagent indicated successful removal of salts, resulting in a concentrated and purified protein solution. This process ensures the effective purification of proteins by eliminating unwanted contaminants.

SDS-PAGE

SDS-PAGE was carried out using 12% polyacrylamide. The molecular weight of the enzyme was measured by comparing the mobility of various molecular weights as standard markers[22].

Scanning electron microscopy (SEM)

SEM analysis of bacterial cells post-solvent exposure begins with culturing and exposing cells to the solvent, fixing them with glutaraldehyde, dehydrating using ethanol, and mounting on SEM stubs. In the SEM chamber, images were captured to observe structural changes like cell shrinkage and membrane disruption. Quantitative analysis via software tools measure parameters such as cell size and surface roughness, providing insights into the effects of solvent exposure on bacterial cell structure.

Results and Discussion

This study highlights the potential of Gram-negative bacteria isolated from the Arabian Sea to produce DNase enzymes and thrive in solvent-rich environments. These findings showcase the ecological and biotechnological significance of extremophilic microorganisms, particularly in marine ecosystems acclaimed for their dynamic conditions and microbial diversity[7,23].

Screening of bacterial isolates

Various bacteria were isolated from ecologically dynamic Arabian Sea in India. The Arabian Sea, renowned for its ecological richness and diversity, serves as an ideal setting for the discovery of novel

Fig. 1 — DNase activity of selected bacterial strains (Culture 1 and culture 2) on DNase plates after flooding with 1N HCl.

enzymatic functionalities within microorganisms possessing specialised adaptations to this distinct environment diversity[7,15]. The isolates were tested for DNase activity on DNase agar by flooding the plate with 1 M HCl. After diluted HCl is added onto the plate, the isolates showing DNase activity will hydrolyze DNA present in the medium into nucleotides, which will be indicated by clear zones near those colonies. This is because individual nucleotides dissolve in diluted HCl, but DNA does not, and precipitates[17]. (Fig. 1). Among the 15 isolated cultures, eight of them showed potential for DNase production, out of which the best two cultures were selected for further screening and characterisation.

Identification of cultures

The selected cultures were identified using MALDI-TOF MS analysis and 16S rRNA sequencing. In MALDI-TOF mass spectrometry, the score quantifies the similarity between the mass spectrum of an unknown microorganism and reference spectra in a database, thereby indicating the confidence level of the identification. The strains were identified as *Proteus mirabilis* and *Klebsiella pneumoniae* after the 16s rRNA sequence result was uploaded to the database and examined for the homology alignment (Table 1). Morphological and biochemical characterisation of the isolated strains also showed a resemblance to these results.

Solvent tolerance and microbial adaptations

The bacterial strains, identified as *Proteus mirabilis* and *Klebsiella pneumoniae*, exhibited exceptional tolerance to various organic solvents, a property critical for applications in biotechnological processes requiring robust enzyme systems. The ability of the selected strains to maintain growth and DNase activity in an environment of various organic solvents demonstrated their tolerance towards the solvents. Interestingly, the DNase enzymes retained activity when exposed to organic solvents such as diethyl ether, petroleum ether, toluene, chlorobenzene (Table 2), which underscores their potential for use in non-aqueous enzymatic processes, where traditional enzymes often lose activity. This aligns with previous studies where solvent-resistant strains of *Pseudomonas aeruginosa* and *Bacillus subtilis* retained enzyme activity in similar solvents, but the dual tolerance of both *Proteus mirabilis* and *Klebsiella pneumoniae* observed here is rare and expands the known range of solvent-stable bacteria[21,22.]

Organic solvents are known to disrupt microbial cell membranes by dissolving membrane lipids and altering permeability, ultimately leading to metabolic failure and cell death. However, organisms that thrive in such conditions often exhibit unique structural modifications and biochemical defenses, such as efflux systems and stress response proteins[24-26]. The ability of *P. mirabilis* and *K. pneumoniae* to survive in solvents like diethyl ether and petroleum ether suggests underlying polyextremophilic traits, such as enhanced membrane integrity and oxidative stress resistance[27].

Table 1 — MALDI-TOF MS analysis results of selected cultures

Culture Number	Organism (Best Match)	Score
Culture 1	*Proteus mirabilis* 13210_1 CHB	2.094
Culture 2	*Klebsiella pneumoniae* sp. *pneumoniae* 9295_1 CHB	2.330

Table 2 — Growth of bacterial isolates in different solvents

Solvent	Culture 1	Culture 2
Toluene	+	+
Benzene	+	+
Chlorobenzene	+	+
Diethyl ether	+	+
Petroleum ether	+	+
Iso Octane	+	+
Acetone	-	-
Acetic acid	+	+
Isopropyl alcohol	-	-
Ethanol	-	-
Methanol	-	-

[‘+’ Growth observed; ‘-’ No growth observed]

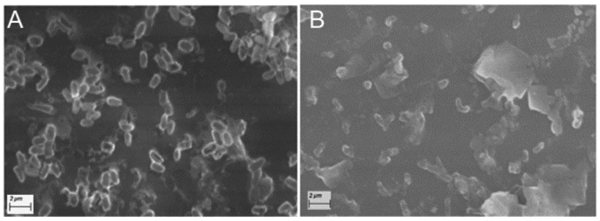

Fig. 2 — Scanning electron microscopy images of (A) Culture 1; (B) culture 2.

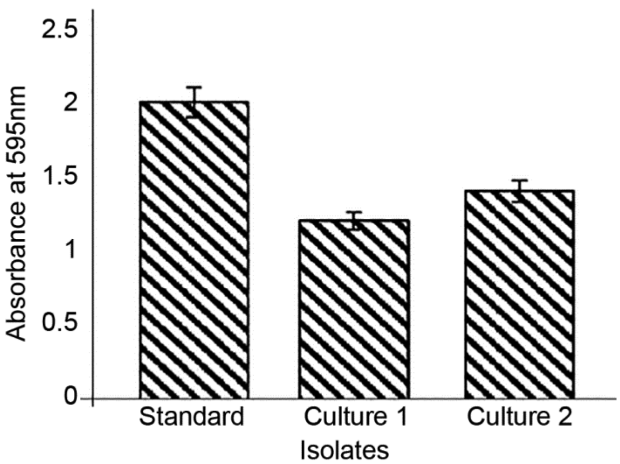

Fig. 3 — Dnase activity of isolates compared to standard DNase.

SEM analysis revealed significant structural adaptations, such as morphological changes and the presence of halos around the bacteria indicating an increased peptidoglycan layer. The rod-shaped bacteria exhibited curved and rounded structures after solvent exposure, likely a protective response to reduce membrane stress (Fig. 2). These modifications align with previous findings that solvent-tolerant bacteria reinforce their cell walls to mitigate solvent toxicity, reduce permeability, and maintain membrane integrity[3,8]. It also showed an increase in cell size which has been reported in previous studies[6]. Structural adaptations such as cell rounding and increased peptidoglycan thickness have been reported as typical responses in polyextremophilic bacteria[27] and are believed to enhance cell rigidity and reduce membrane permeability. These findings are consistent with our SEM observations.

The increased peptidoglycan layer observed in this study likely acts as a physical barrier to solvent entry, preserving cellular functionality under stress conditions. Similar mechanisms have been reported in solvent-tolerant bacteria like *Pseudomonas putida*, which utilise efflux pumps, membrane remodelling, and stress proteins to counteract solvent effects[1, 8]. Gaining knowledge of these adaptations opens possibilities for using these organisms in industrial settings and offers important insights into microbial resilience.

The DNase activity assay (Fig. 3) revealed that both bacterial isolates exhibited significant enzyme activity, though lower than the standard DNase. This suggests that while the isolates produce DNase, their efficiency may be influenced by factors such as enzyme stability, substrate affinity, or production conditions. The moderate DNase activity of the isolates compared to the standard enzyme suggests further optimisation is required. Similar patterns were observed by Devi *et al.* who reported higher activity only after supplementing metal cofactors and optimising incubation conditions.Similar findings have been reported, highlighting the need for optimisation in microbial DNase production[28].

Optimization of DNase production

Culture medium composition plays a vital role in modulating bacterial growth and enzyme productivity. While complex media containing yeast extract or tryptone support high yields, defined media provide better reproducibility for scale-up[29]. To overcome limitations posed by the instability of native enzymes in organic media, protein and enzyme engineering strategies such as site-directed mutagenesis and directed evolution have been developed to enhance catalytic efficiency and solvent stability[30]. However, naturally occurring solvent-tolerant enzymes, such as the DNase characterised in this study, offer a more straightforward and eco-efficient solution for biocatalysis in solvent-rich processes. Fructose emerged as the optimal carbon source for DNase production (Fig. 4A), consistent with studies demonstrating that microorganisms preferentially metabolize certain sugars for efficient energy generation and enzyme synthesis[28]. Among nitrogen sources, tryptone supported the highest DNase activity, followed by beef extract and yeast extract (Fig. 4B). This agrees with reports indicating that organic nitrogen sources enhance microbial growth and productivity due to their rich amino acid content[31]. The preference for fructose and tryptone as optimal carbon and nitrogen sources parallels findings in *Myroides profundi* and *Staphylococcus aureus* strains, which demonstrated higher DNase yields under similar conditions[14,32].

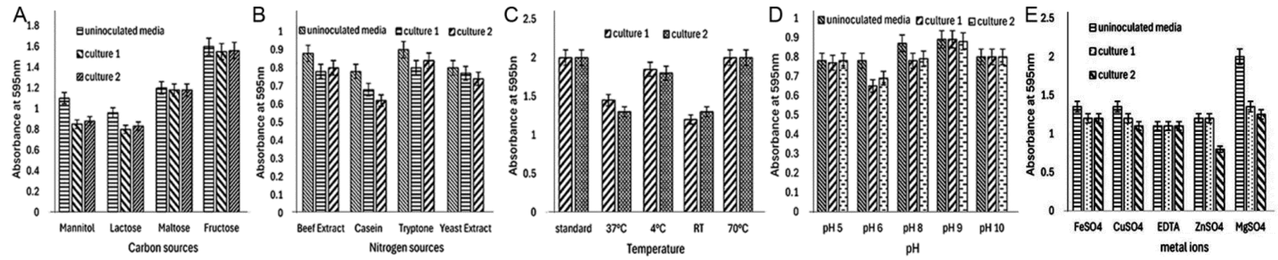

Fig. 4 — (A) Effect of carbon sources on the DNase production of selected bacterial isolates (B) Effect of nitrogen sources on the DNase production of selected bacterial isolates (C) Effect of temperature on DNase production of selected bacterial isolates. (D) Effect of pH on DNase production of selected bacterial isolates. (E) Effect of metal ions of DNase activity.

Enzyme activity and stability

The DNase enzymes exhibited optimal activity at 70°C and pH 9, highlighting their thermostable and alkaline-tolerant nature (Fig. 4C & 4D). These properties are highly desirable for industrial applications, particularly in harsh processing environments (Rawat). In contrast, Deshmukh & Shanker reported extracellular DNase production by *Streptomyces thermonitrificans* with optimal activity at 40 °C and pH 7.0[33], suggesting that the DNase characterised in our study exhibits significantly higher thermal and pH tolerance, making it more suitable for industrial applications under extreme conditions.The DNase isolated from *T. pretiosum* expressed in *Pichia pastoris* displayed robust degradation activity comparable to that of bovine pancreas DNase I. Similarly, the Gram-negative isolates studied here showed substantial activity at 70°C and pH 9, outperforming conventional DNase enzymes in terms of thermal stability. This thermostability can be attributed to structural features such as divalent ion-binding residues and conserved catalytic motifs (yu). Alkaline-tolerant enzymes are sought after for applications requiring non-neutral pH conditions, such as detergent formulation and wastewater treatment[34].

Studies have emphasised the influence of metal ions like Mg^{2+} and Fe^{2+} on DNase activity, likely due to their cofactor roles in nucleolytic reactions[29]. The enhanced activity of DNase in the presence of Mg^{2+}, Fe^{3+}, and Cu^{2+} ions suggest their role as cofactors (Fig. 4E), stabilizing the enzyme structure and promoting catalysis, a phenomenon widely observed in metalloenzymes[15].

The SDS-PAGE analysis of the dialysed extract from various isolates yielded a consistent and intriguing observation: each isolate exhibited a distinct band on the gel with a common molecular weight of approximately 31 kilodaltons (kDa) (Fig. 5). While most reported DNases fall within the 38–45 kDa range[33], the smaller 31 kDa enzyme found here suggests a potentially unique isoform. Its high activity at 70°C and pH 9 surpasses previous DNases like those from *Streptomyces thermonitrificans*, which functioned optimally at 40°C and pH 7.This consistent banding pattern at 31 kDa across different isolates suggests the presence of a protein or protein complex of particular significance within these microbial samples[33]. DNase has been previously reported to have a spectrum of molecular weights from 38 to 45 kDa[14]. Several studies have demonstrated that enzymes from organisms like *Pseudomonas aeruginosa* and *Listeria monocytogenes*

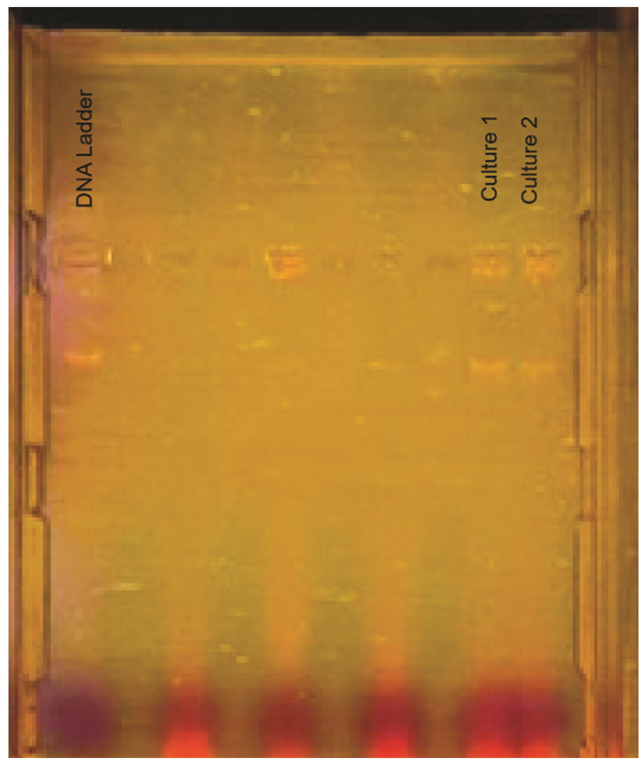

Fig. 5 — SDS-PAGE of the enzyme purified using ammonium sulphate precipitation technique.

exhibit strong tolerance to polar and non-polar solvents while maintaining catalytic functionality, suggesting their applicability in processes such as biodiesel production, detoxification, and waste treatment[19,22,35]. The DNase from this study, with its tolerance to aliphatic solvents and high-temperature operation, could similarly contribute to sustainable biotechnological applications.

These results contribute novel insights into enzyme functionality in solvent-rich marine environments. The ability of these DNases to remain active under thermal, alkaline, and solvent stress underscores their potential as biocatalysts in molecular biology, environmental DNA degradation, and synthetic chemistry. Future studies on protein structure and gene regulation will be crucial in harnessing these enzymes for commercial use[11,30].

Conclusion

Two marine isolates, *Proteus mirabilis* and *Klebsiella pneumoniae*, obtained from the Arabian Sea coast of India, demonstrated notable tolerance to both aliphatic and aromatic solvents while maintaining DNase production. The purified enzyme (31 kDa) exhibited optimal activity at 70 °C and pH 9, retained substantial activity in the presence of diethyl ether, petroleum ether, and methanol, and showed improved yields upon optimisation of carbon/nitrogen sources, pH, temperature, and metal ions. These results confirm the objective of identifying robust DNase enzymes with high thermal, alkaline, and solvent stability, underscoring their potential in non-aqueous biocatalysis, environmental DNA degradation, and pharmaceutical applications.

Future research aimed at understanding and enhancing these properties could unlock new possibilities for their application in diverse scientific and industrial fields. This study is among the first to report on DNase activity in aliphatic solvent-rich environments, expanding the known applications of such enzymes. The unique properties of these enzymes, including their solvent tolerance and stability under extreme conditions, make them promising candidates for various industries, such as pharmaceuticals, bioremediation, and synthetic biology. However, several aspects warrant further exploration. Structural studies using techniques such as X-ray crystallography or molecular dynamics simulations could provide insights into the enzyme's stability and activity in solvent-rich environments. Additionally, genetic and proteomic analyses could reveal the molecular mechanisms underlying solvent tolerance and DNase production, paving the way for engineering more robust and efficient enzymes.

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Conflict of interest

The authors declare that they have no conflict of interest.

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