

RESEARCH ARTICLE

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Studies on Production of Proteins from *Delftia* MW172212 Bacteria and their Antibacterial and Anticancer Applications

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Abstract

Salt pan water collected from Kovalm, Kanyakumari district, Tamil Nadu, used for bacterial isolation using Zobal marine agar. Red pigmented colonies were selected for further studies and labelled as KSB. Microscopic, macroscopic and molecular studies were performed for the identification of isolate and it is confirmed as *Delftia*. Gen Bank accession number obtained for the same and deposited as *Delftia* MW172212. The optimization studies concluded that optimum temperature was 40 °C, optimum pH was 8 and optimum lactose concentration was 20%. Acetone precipitation concentrates active compounds, increasing efficacy against Gram-negative bacteria, according to the *Delftia* sp. acetone-precipitated fraction, which had a maximal inhibitory activity against *E. coli* MTCC-1671 that was marginally greater than the pure isolate (17 mm). Neutral red assay showed significant inhibition against MCF-7 hence it is considered as anticancer compound.

Keywords: Salt Pan Water, Zobal Marine Agar, *Delftia*, Optimization, Antibacterial Study, Anticancer Study

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INTRODUCTION

Delftia is a diverse bacterial isolate having industrial and agriculture relevance. This species broadly distributed in all environments and isolated from different sources. The community of *Delftia* species are notable community of current research due to their less exposure and diverse applications.¹⁻⁸ They belong to the class of *Betaproteobacteria*. They are Gram-negative organisms isolated from different environmental niches including polluted water, soil, sea water, etc. Though it is having broad spectrum applications recent potential studies are reported for bioremediation. First time this bacterium reported from Netarland.⁹ In this study, bacteria are isolated from salt pan water, characterised, and then used for antibacterial and anticancer purposes.

MATERIALS AND METHODS

Sample collection

Using sterile bottles, a water sample was taken from Kovalam Saltpan in Kanyakumari, Tamil Nadu, and brought to the lab to be kept at 4 °C until it was needed.

Bacteria isolation

After serially diluting the samples with a 1M NaCl solution, they were plated on a Zobell marine agar plate (one litre contains 5,000 peptones). Ferric citrate: 0.100; NaCl: 19.450; $MgCl_2$: 8.800; Na_2SO_4 : 1.000; yeast extract: 7.6 ± 0.2 pH. After two weeks of incubation at 40 °C, the plates were checked for the development of halophilic bacterial isolates. Pure cultures were separated after morphologically distinct colonies that were seen on the plates were further streaked on halophilic agar plates.¹⁰

Characterisation and identification of isolate

Predominant isolate was characterised by microscopic study (Gram staining) and macroscopic studies-Biochemical studies and motility, according to Bergy's Manual of Determinative Bacteriology and by 16S rRNA gene sequencing.¹¹

Optimisation of parameters on the growth of bacteria

The process parameters such as incubation temperature, pH, salinity, carbon source and nitrogen source were optimized for the growth of bacteria.¹²

Optimization of temperature

After being inoculated into several tubes with nutritional broths (which included peptone,

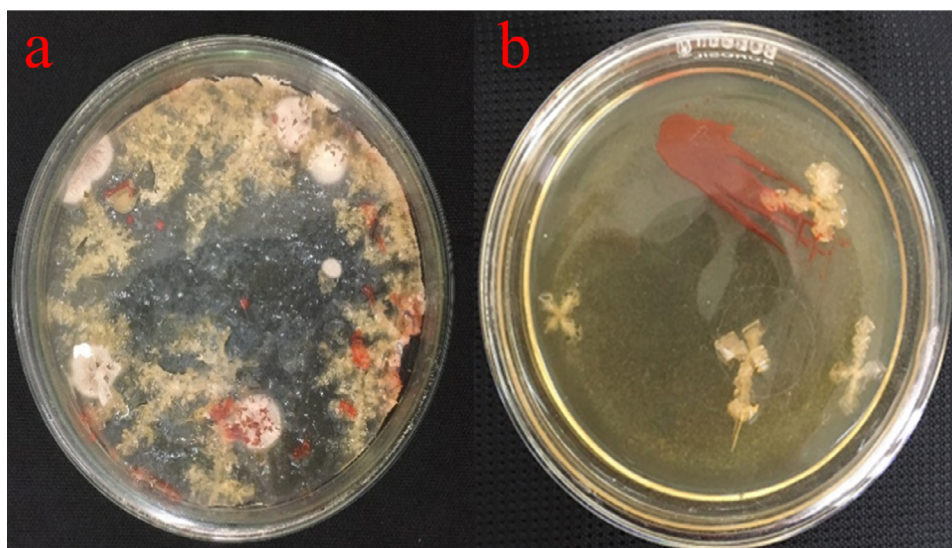


Figure 1. (a) Plate showing colonies of *Delftia* sp. Growing on agar plates, (b) isolated from salt water

NaCl and beef extract, the isolated bacteria was cultured for five days at 25, 37, and 40 °C. OD values of bacterial suspensions incubated at different temperatures were determined with colorimeter under 590 nm up to decline phase.

Optimization of pH

1N HCl and 1N NaOH were used to bring the pH of the liquid media down to pH 5, 6, 7, 8, and 9. After being injected into various broths, isolated bacterial cultures were cultured for five days at 40 °C. OD values of bacterial suspensions with different pH were determined with colorimeter under 590 nm.

Optimization of NaCl concentration

Nutrient broths were prepared with 10%, 15%, 20%, 25%, 30% and 35% NaCl, bacterial cultures were introduced in different broths and incubated for 5 days at 40 °C. OD values of bacterial suspensions with varying NaCl concentration were determined with colorimeter under 590 nm up to decline phase.

Effect of different carbon source

Nutrient broths were prepared with 20% NaCl, 0.3% beef extract and 0.5% different carbon sources (dextrose, lactose and sucrose). After being injected into various broths, isolated bacterial cultures were cultured for five days at 40 °C. OD values of bacterial suspensions with different carbon sources were determined with colorimeter under 590 nm up to its decline phase.

Effect of different nitrogen source

Nutrient broths were prepared with 0.5% peptone, 20% NaCl and 0.5% different N₂ sources. Isolated bacterial cultures were injected in different broths then incubated to 72 hours at 40 °C. OD values of bacterial suspensions with different nitrogen sources were determined with colorimeter under 590 nm up to its decline phase.

Mass scale cultivation of bacteria

Isolated culture was seeded in 150 ml of optimised nutrient broth medium in a 250 ml conical flask with optimised culture conditions in a shaker at 150 rpm.

Partial purification of protein

The culture filtrate of the incubated culture was obtained by centrifugation at 1000 rpm for 15 minutes. The protein in the culture filtrate precipitated with ammonium sulphate, and 2.9 g of ammonium sulphate was added to the supernatant while stirring continuously to achieve 100% saturation. The resulted precipitate allowed to centrifuged at 6000 rpm then the precipitate was resuspended in 10 ml of 0.05 M calcium chloride. The mixture was thoroughly stirred, and dialysis was conducted in tubular cellulose membrane against 0.05 M calcium chloride for 24 h at 4 °C. The precipitate then dissolved in 10 ml of 50 mM Tris-HCl (pH 7.5).¹³

Estimation of protein content

Using bovine serum albumin as the

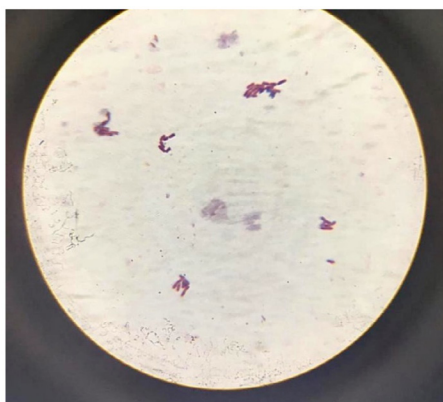


Figure 2. Gram-negative isolate

Table 1. Biochemical characteristics of the isolate

Biochemical tests	Results
Indole test	-
Methyl Red (MR) test	-
VP test	-
Citrate test	-
Urease test	-
Catalase test	-
Amylase test	+
Gelatinase test	-
Nitrate reduction test	-
Glucose fermentation	+
Lactose fermentation	+
Sucrose fermentation	+
Maltose fermentation	-

reference, Lowry's method¹⁴ was used to assess the protein content.

Antibacterial activity of partially purified protein

Antimicrobial activity of partially purified Delftin was evaluated against four different human pathogenic strains such as *E. coli*, *Pseudomonas* spp., *Bacillus* spp. and *Staphylococcus* spp., by agar well diffusion method. 10 µl of sample was added to the well of 6 mm diameter. Incubated at 37 °C for 24 hrs.

In vitro study-antitumor activity-neutral red assay

MCF-7 (Human Breast Adenocarcinoma) cells supplied by National Centre for Cell Sciences (NCCS). The culture medium made with 10% FBS, NaHCO₃, L-glutamine, and antibiotics. Cells were maintained in a 25 cm² tissue culture flask and incubated at 37 °C.

96 well plate analysis

Trypsinization was carried out on the cells with confluent monolayer, which were 48 h

old and suspended in 10% growth media. Then seeded into 96-well plates (volume of 100 µl with a concentration 5 × 10³). Finally, plates were incubated at 37 °C in a humidified 5% carbon dioxide incubator.

Compound stock preparation

1 mg of the compound was solubilized in 1 mL of Dulbecco's Modified Eagle Medium (DMEM) and the resulted solution was filtered via a 0.22 µm filter. Once the cells reached adequate confluency, they were trypsinized for two minutes using 0.025% (500 µl) trypsin in PBS/0.5 mM EDTA and transferred to T-flasks under sterile conditions. The sample (6.25 µl, 12.5 µl, 25 µl, 50 µl, and 100 µl in 500 µl DMEM) were incubated for 24 hours before adding 10 µl of neutral red. The samples the incubated at 37 °C for 3 h, then the cells were fixed with 200 µl ethanol-acetic acid solution, followed by extraction buffer. Absorbance was measured at 540 nm to calculate cell viability. The growth inhibition percentage was derived as follows.¹⁵

1)>SR1790-YSS03-RSR1_F12.ab1

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CC
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2)>SR1790-YSS03-RSF1_F11.ab1

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TGGGGAGCGCGCACGAGCGCGGAATGATGCTTGGCTGTAAATTACGTAATGGCCAAACGATCCG

Figure 3. Sequencing details of KSB

$$\% \text{ of viability} = \frac{\text{Mean Samples OD}}{\text{OD of control group (Mean)}} \times 100$$

the studies and labelled as KSB (Kovalam Salt Pan Bacteria).

RESULTS AND DISCUSSION

Isolation of bacteria

The microbiological investigations were carried out using standard plating methods. Zobal marine agar plate was used for the isolation.¹⁶ After two weeks of incubation, orange-red coloured colonies were dominant forming more than 50% noticed on halophilic agar (Figure 1). The red pigmented colonies were further considered for

Bacterial isolate identification

The isolate was identified through 16S rRNA gene sequencing, as well as by analysing its biochemical characteristics and examining its colony and cell morphology. It was discovered that the isolated colony was round, moderately big, orange-red in colour, smooth, elevated, and opaque throughout its whole edge. Macroscopic studies revealed that it is non-motile and biochemical characters are given in Table 1.

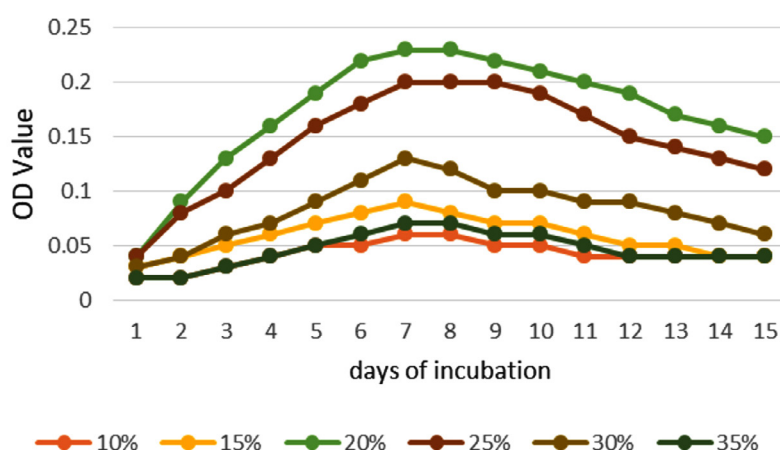


Figure 4. Optimum NaCl concentration

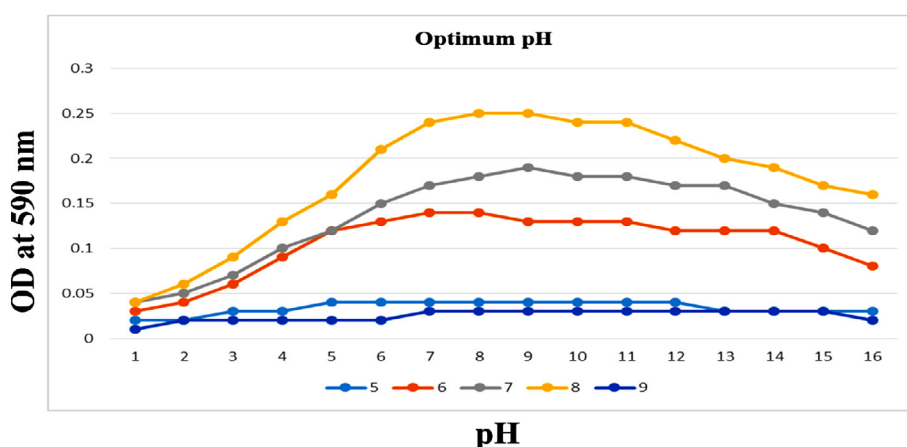


Figure 5. Optimum pH

Organism observed as Gram-negative (Figure 2) and molecular studies (Figure 3) details revealed that isolate belongs to *Delftia* genus. Gen Bank accession number obtained for the same and deposited as *Delftia* MW172212.¹⁷

Optimization of growth parameters

To enhance the growth and for the better production of metabolite external growth factors and media components were optimized. Bacterial isolate, *Delftia* was grown in wide range of salinity and optimum growth was achieved at 20%. The pH optimum for the growth of the organism was found to be 8. The temperature optimum for the growth was found to be 40 °C for *Delftia*. Growth was high in the medium containing lactose as the source of carbon and yeast extract

as the source of nitrogen.¹⁸ Results are given in Figures 4-8.

Estimation of protein content

By Lowry's method, protein content in partially protein was found to be 0.12 mg/ml (Figure 9). The sample was stored and used for further antibacterial and cell line studies.¹⁹

Identification of the bacterial isolates

Comprehensive morphological, biochemical, and 16S rRNA examinations established the isolate's identity.¹⁸ The bacterium is a non-sporulating, Gram-negative *bacillus* that appears as elongated rods, exhibits no catalase activity, hydrolyses starch, and remains negative for gelatin liquefaction. These diagnostic features,

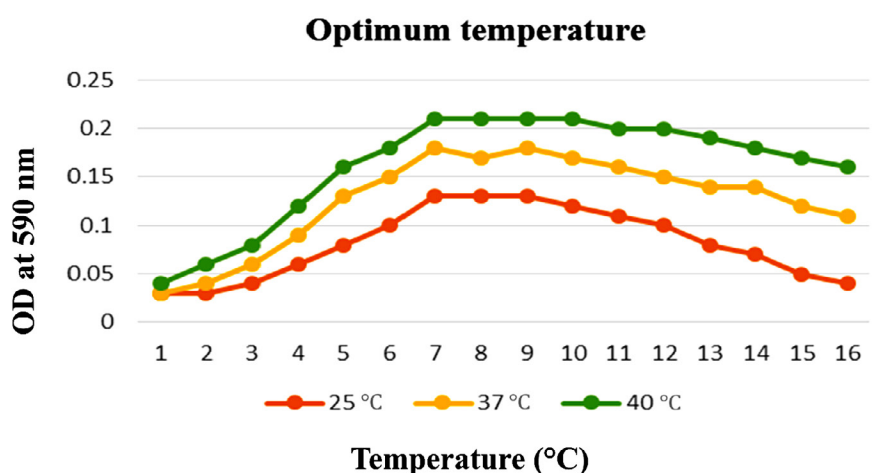


Figure 6. Optimum temperature

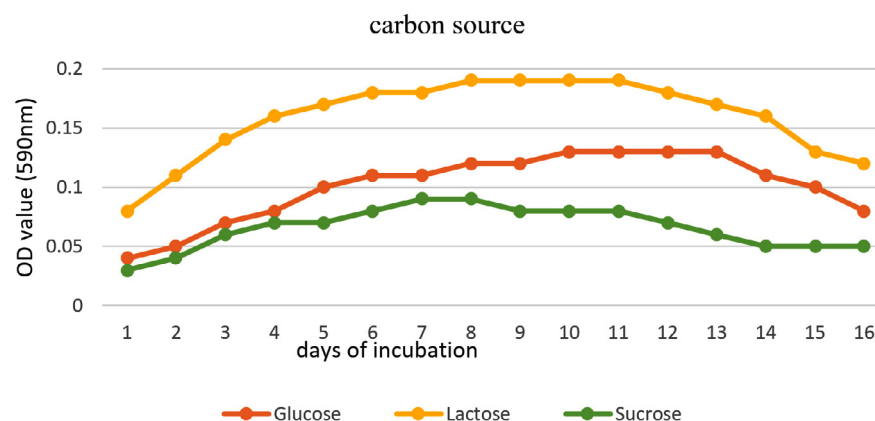


Figure 7. Effect of carbon source

together with sequence homology, warrant its placement within the genus *Delftia*. The isolate's 1,458 base pair 16S rRNA gene has been deposited in GenBank, where it is available under accession number/MW172212 (Figure 10).

Phylogenetic tree showing the relationship of *Delftia* sp. with 16S rRNA

The phylogenetic tree reinforces that the *Delftia* genus represents a distinct, monophyletic lineage within the family/Comamonadaceae, clearly separated from other related genera such as *Comamonas*, *Diaphorobacter*, and *Acidovorax*.²⁰⁻²⁴ It delineates two well-supported clades within *Delftia*-one encompassing *D.*

tsuruhatensis and *D. lacustris*, and the other comprising various *D. acidovorans* strains-highlighting significant intra-genus diversity and confirming previous genomic and ecological lineage differentiation. These findings corroborate the taxonomic classification based on 16S rRNA and whole-genome phylogenies, underscoring the evolutionary coherence and distinctness of *Delftia* in the *Betaproteobacteria* class.^{22,24}

The strain's designation as a possible lead for the discovery of new antibiotics is supported by its location within *Delftia*, which is corroborated by morphological and biochemical profiling as well as 16S rRNA gene sequencing. The isolate may be a potential lead for the

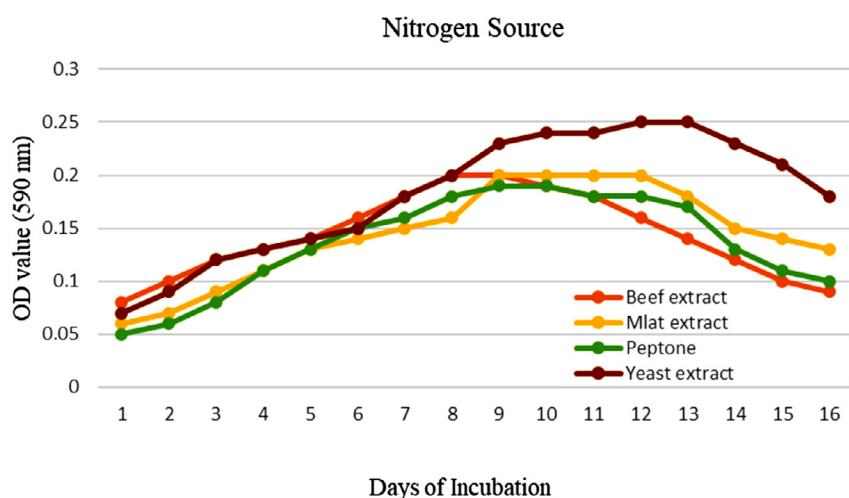


Figure 8. Effect of nitrogen source

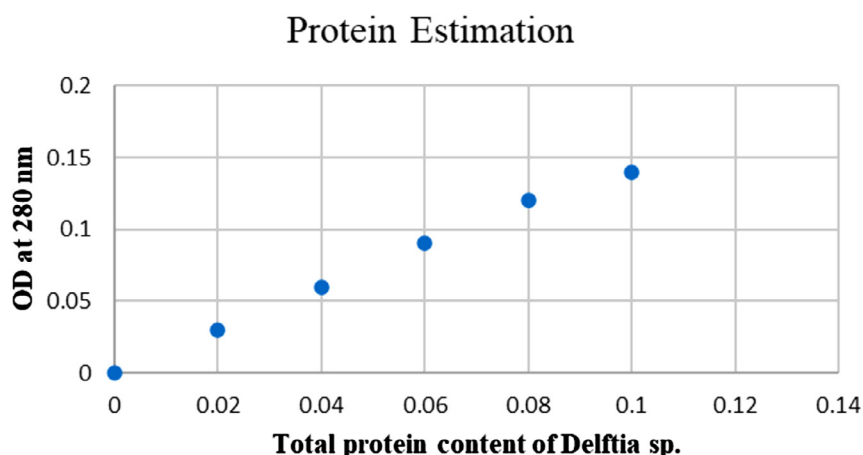


Figure 9. Protein Estimation

discovery of new antibiotics due to its notable suppression of *E. coli*, increased extract potency, and alignment with previously identified *Delftia* antimicrobial compounds. The study's emphasis on *in vitro* agar diffusion, however, emphasises the necessity of additional research to measure minimum inhibitory concentrations (MICs) and look into antibiotic synergy. Additionally crucial are the structural clarification and chemical characterisation of the acetone and Delftin precipitates. Therapeutic outcomes shall be established by assessing biosafety and *in vivo* effectiveness in mammalian models.

Antibacterial activity of partially purified protein

The partially purified protein extract of acetone and ethanol was tested for antibacterial activity against Gram-positive *Bacillus* sp., and *Streptococcus* sp., and Gram-negative bacteria, including *Pseudomonas*, and *E. coli*, (Figures 11 and 12). It showed significant antibacterial effects against these bacterial strains, leading to the conclusion that the separated protein must exhibit antibacterial activity against these four bacterial isolates.²⁰

Delftin activity of acetone fractions

The acetone-precipitated fraction from *Delftia* sp. demonstrated clear antibacterial effects, forming a maximum inhibition zone of 18 mm against *Escherichia coli* MTCC 1671 and a minimal zone of 10 mm against *Pseudomonas aeruginosa* MTCC 6538. In parallel, the naked isolate of *Delftia* sp. achieved an inhibition zone of 17 mm against *E. coli* MTCC 1671 and just 11 mm against *Streptococcus mutans* MTCC/896. These findings collectively highlight the notable antibacterial potential of both the acetone-precipitated fraction and the pure isolate, especially in targeting *E. coli* MTCC/1671 (Figure 11).

Delftin activity of ethanol precipitated samples

The ethanol-precipitated Delftin extract from the *Delftia* sp. isolate exhibited antimicrobial activity, with inhibition zones ranging from 10 mm against *Streptococcus mutans* MTCC 896 to a maximum of 16 mm against *Escherichia coli* MTCC 1671. Notably, the Delftin compound demonstrated its highest inhibitory effect against *E. coli* MTCC 1671, producing a 17 mm zone of inhibition, while showing the least effect against

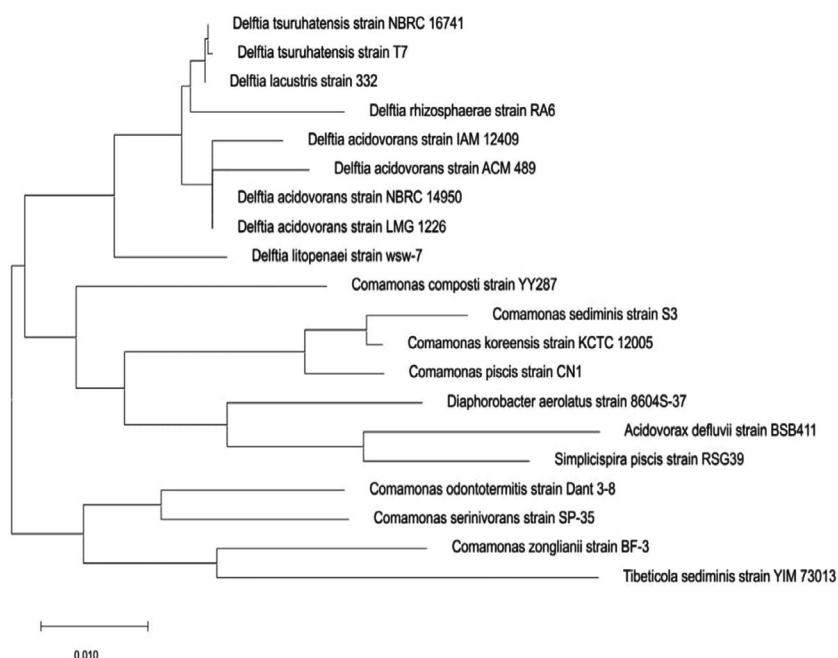


Figure 10. Phylogenetic tree showing the relationship of *Delftia* sp. with 16S rRNA

Bacillus subtilis MTCC 1134, with a 10 mm zone (Figure 12). This study presents novel insights into the antimicrobial potential of *Delftia* sp.,

comparing the effects of its crude isolate and solvent-precipitated extracts against a range of bacterial pathogens.

Acetone precipitation concentrates active compounds, increasing efficacy against Gram-negative bacteria, according to the *Delftia* sp. acetone-precipitated fraction, which had a maximal inhibitory activity against *E. coli* MTCC-1671 that was marginally greater than the pure isolate (17 mm).²⁰⁻²⁴ Purified Delftin had the maximum activity (17 mm) against *E. coli*, and its inhibition zones ranged from 10 mm to 16 mm, reflecting its selective antimicrobial ability.²⁰ The fact that *B. subtilis* (10 mm) exhibited the lowest activity suggests that Delftin has a

Table 2. Antitumor activity for partially purified protein

Sample	OD-I	OD-II	OD-III	Mean	%
Concen.				OD	Viability
(μ L)					
Control	0.1572	0.1556	0.1587	0.1572	100
6.25	0.1512	0.1508	0.1497	0.1506	94.78
12.5	0.1408	0.1410	0.1418	0.1412	89.82
25	0.1346	0.1346	0.1314	0.1335	84.94
50	0.1162	0.1144	0.1161	0.1156	73.52
100	0.0959	0.1055	0.1020	0.1011	64.33

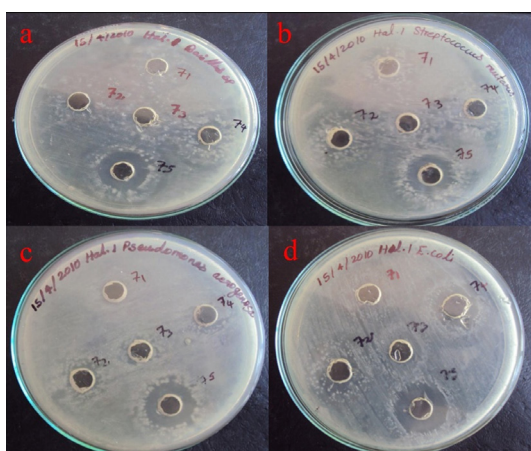


Figure 11. Antibacterial activity of Delftin extracted from *Delftia* sp. against a). *Bacillus* sp., b). *Streptococcus* sp., c). *Pseudomonas* sp., and d). *E. coli* sp. at varying concentrations using acetone as solvent

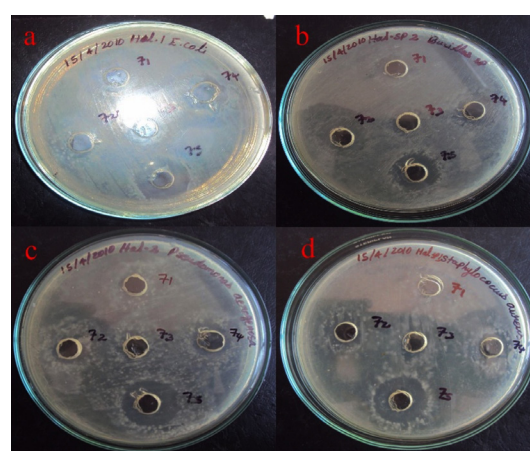


Figure 12. Antibacterial activity of Delftin extracted from *Delftia* sp. against a). *E. coli* sp., b). *Bacillus* sp., c). *Pseudomonas* sp., and d). *Staphylococcus* sp. at varying concentrations using ethanol as solvent

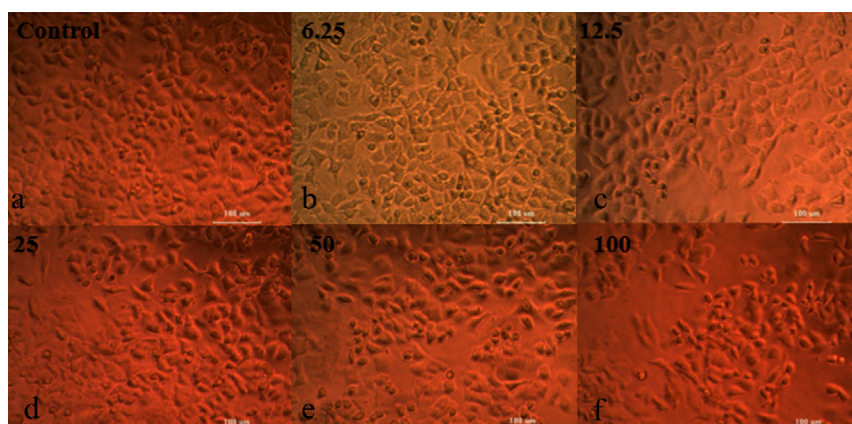


Figure 13. Determination of Cell Viability of MCF-7 by Neutral Red Assay for the various concentration of the protein a). Control; b). 6.25 μ L; c). 12.5 μ L; d). 25 μ L; e). 50 μ L; f). 100 μ L

predilection for certain Gram-negative bacteria. Baseline generation of antimicrobial metabolites is reflected in the inhibitory zones of the naked isolate.²¹

In vitro study-antitumor activity

The partially purified extracellular protein demonstrates antitumor activity against breast cancer cells. As the protein concentration increases, the cell viability percentage decreases (Figure 13 and Table 2).^{25,26} The neutral red cytotoxicity study was used to study the live cells. In this study the dye was absorbed and bound only by live cells, for dead cell nullified activity was observed.²⁷ The results clearly indicate that neutral red accumulation is directly related to number of viable cells. The neutral red solution pH was adjusted to 6.35 by adding KH_2PO_4 (1M). The LC50 value was determined to be between 137-149 μl , calculated using ED50 PLUS software.

CONCLUSION

The colony with red pigmentation were selected for further studies and named as KSB. Initial characterization was revealed that it is Gram-negative non-motile organism. IMViC studies negative for the organism, urease negative and gelatinase negative and amylase positive with zone of clearance. Optimization of media components and external factors were identified as 20% NaCl, pH 5, Temperature 40 °C, best carbon source lactose and yeast extract as nitrogen source. Partially purified Delftin had the maximum activity (17 mm) against *E. coli*, and its inhibition zones ranged from 10 mm to 16 mm, reflecting its selective antimicrobial ability and reported with antitumor activity against MCF-7 by Neutral Red assay.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

KP collected resources, performed data curation and supervised the study. APK applied methodology. RG performed formal analysis. Y, KP and KT wrote the manuscript. RG, KP, TM, KK and KT reviewed and edited the manuscript. All authors read and approved the final manuscript for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies on human participants or animals performed by any of the authors.

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