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Cellular fatty acid composition, protein profile and antimicrobial activity of *Bacillus* sp., isolated from fish gut

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PEER REVIEW

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Comments

Generally, this is a potential research work in which authors have demonstrated the antimicrobial activity of *Bacillus* sp. from fish gut in protein profile and cellular fatty acids. The activity was assessed based on cultural, morphology, physiology, biochemical characteristics and 16S rRNA sequencing. *Bacillus* sp. was found to be an important antibacterial agent.

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ABSTRACT

Objective: To purify and partially characterize the antimicrobial compounds from bacteria *Bacillus* sp., isolated from fish gut.

Methods: Protein and fatty acids were isolated from the bacteria and checked for the presence of antibacterial activity. Protein has been purified to apparent homogeneity from the supernatants of culture by means of ammonium sulphate precipitation followed by dialysis. Fourier transform infrared spectroscopy analyses were performed for proteins to identify the functional groups.

Results: Protein showed an apparent molecular mass 56, 47 and 39 kDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis. Fatty acids were extracted and subjected to gas chromatographic analysis.

Conclusions: The antimicrobial activity of the bacteria might be due to the presence of fatty acids and proteins which holds promise for the development of new drugs.

KEYWORDS

Fatty acid, Protein, Antimicrobial activity, GC–MS, Fish gut, PCR

1. Introduction

There has been a rapid worldwide increase in pathogenic bacteria that are resistant to multiple antibiotics. The alarming increase and spread among pathogens of bacterial resistance to all clinically useful antibacterial agents has been one of the most serious public health problems in the past decade[1]. Bacteria have been especially useful to the pharmaceutical industry for their seemingly unlimited capacity to produce secondary metabolites with diverse chemical structures and biological activities. Searching for novel bacteria constitutes an essential component in the natural product–based drug discovery. The importance of this antibiotic lies in its broad–spectrum activity against microorganisms, with low toxicity against mammalian

cells[2]. The interest in marine microorganisms as a source of new antibacterial compounds has risen in the last decades due to the increasing resistance of pathogens to available antibiotics. Marine microorganisms could be a source of metabolites that might not be observed in terrestrial ones[3]. Rosenfeld WD, *et al.*[4] accomplished the first investigations on a marine bacterium producing antibacterial compounds against Gram–positive bacteria. Since then many marine bacteria have been shown inhibition against varied microorganisms. *Pseudomonas* sp. are known to produce different active metabolites. It is a Gram–negative, rod shaped, asporogenous and monoflagellated bacterium that has an incredible nutritional versatility. It is able to catabolize wide range of organic molecules including organic compounds such as benzoate. Furthermore, it

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is able to produce rhamnolipids, quinolones, hydrogen cyanide, phenazines, and lectins[5].

In the present work we describe the isolation of a bacterial strain from fish guts, which generates an antimicrobial agent. The identification of this strain is based on the cultural, morphology, physiology, biochemical characteristics and 16s rRNA sequencing. The bioactive substance was isolated, purified and biological activities were determined.

2. Materials and methods

2.1. Bacterial strains and their characterization

The intestines of several fresh marine fishes were collected and their guts were excised and grinded with mortar and pestle. Then they were diluted by serial dilution technique and spreaded onto nutrient agar plates. Strains were incubated at 37 °C for 48 h and then different types of colonies were isolated and streaked on new plate to obtain homogenous culture. Twenty strains were isolated and purified and checked for the presence of antibacterial activity. Only one potential strain from among these was selected and biochemically characterized based on Bergey's manual of systematic bacteriology.

2.2. Antibacterial susceptibility testing

The isolated strains were tested for the presence of antibacterial activity against human pathogens. The cells and cell components were separated and checked for the presence of antibacterial activity. The substances that showed antibacterial activity were then collected and purified for further studies.

2.3. Gas–liquid chromatography of cellular fatty acids

Bacillus sp., strain was mass cultured in 100 mL of glucose, yeast extract, peptone broth (GYP broth) and incubated overnight at 35 °C[6]. The culture broth was centrifuged to obtain a satisfactory cell pellet. Lysis of cells through saponification, methylation of fatty acids and extraction of the methyl esters into the organic phase were achieved according to modified Bligh and Dyer method. The samples were then processed on a gas–liquid chromatograph (GC–17 A, Shimadzu, Tokyo, Japan) equipped with flame ionization detector and split injector, using TC–70 capillary column (GL science, Tokyo, Japan). The fatty acid ester peaks were identified and calibrated with stranded fatty acids. Data given are averages of at least three determinations.

2.4. Protein concentration determination

Protein concentrations were determined with 50 mL of samples by the method of Bradford with Coomassie protein

assay reagent according to manufacturer's instructions. Bovine serum albumin was used to construct a standard curve[7].

2.5. Ammonium sulphate precipitation

Following cultivation of *Bacillus sp.*, cells were removed by centrifugation (5000 ×r/min) and 40%, 50%, 60%, 70%, and 80% saturation was reached by slow addition of solid ammonium sulphate and held overnight at 4 °C with stirring. Samples were pelleted by centrifugation (12000 ×r/min). The collected fractions were then dissolved in 0.01 mol/L phosphate buffer (pH 7.0) and dialysed in 1 kDa cut–off membrane against the same buffer at 4 °C overnight.

2.6. Polyacrylamide gel electrophoresis of protein

The precipitate was collected and partially purified using dialysis against phosphate buffer (pH 8.0). The samples were then resuspended in sample buffer (0.5 mol/L tris, glycerol, 10% sodium dodecyl sulfate, 2% mercaptoethanol, 0.05% bromophenol blue), loaded onto gels, and electrophoresed at 200 V for about 4 h. polyacrylamide gel (12%) with sodium dodecyl sulphate were used.

2.7. Fourier transform infrared spectroscopy (FTIR) analysis

The partially purified proteins were subjected to FTIR analysis for the identification of functional groups using Fourier transformer infrared spectrophotometer (Shimadzu, Japan).

2.8. 16s rRNA based identification of bacterial species

The genomic DNA extracted from the gut bacterial strain was PCR amplified for 16S rRNA genes using the universal bacterial primers Eubac27F (5'–AGA GTT TGA TCM TGG CTC AG–3') and 1492R (5'–GGT TAC CTT GTT ACG ACT T–3'). This primer combination amplifies a 1500 bp 16S rRNA fragment[8]. Amplification reaction was performed in a 0.2 mL optical–grade PCR tube (Tarsons, India). Fifty nanogram of DNA extract was added to a final volume of 50 µL of PCR reaction mixture containing 1.5 mmol/L MgCl₂, 1× Reaction buffer (without MgCl₂) (Fermentas), 200 µmol/L of each dNTPs (Fermentas), 100 pmol/L of each primer and 1.5 UI *Taq* DNA polymerase (Fermentas). PCR was performed in an automated thermal cycler (Lark Research Model L125+, India) with an initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 52 °C for 45 seconds, extension at 72 °C for 90 seconds and final extension at 72 °C for 10 min. PCR product was run on 1% agarose in TAE buffer [40 mmol/L tris, 20 mmol/L acetic acid, 1 mmol/L EDTA (pH 8.0)] to confirm that the right product (1500 bp) was formed. The PCR product was purified using the QIAGEN PCR purification kit for sequencing and further analysis.

2.9. Sequence similarities and phylogenetic analysis

The BLAST program (www.ncbi.nlm.nih.gov/blast) was employed in order to assess the degree of DNA similarity. The phylogenetic tree was displayed using the TreeView program.

3. Results

3.1. Isolation and identification of gut bacteria

By using serial dilution technique the colonies were isolated and screened for their presence of antibacterial activities. Out of all isolated strains only one strain showed maximum zone of inhibition and it was utilized for further studies. The potential strain was identified using Bergey's manual of systemic bacteriology. The strain was identified as *Bacillus* sp., using standard biochemical methods.

3.2. Antibacterial activity

The strain was tested against ten different human pathogens (Table 1). The protein and fatty acids were separated and checked for the presence of antibacterial activity. Of those pathogens tested the strain showed maximum zone of inhibition to the pathogen *Klebsiella oxytoca* and *Vibrio cholera* and minimum zone of inhibition to the pathogen *Salmonella paratyphi* and *Staphylococcus aureus* (Figure 1).

3.3. Cellular fatty acids analysis

The fatty acids extracted using Bligh and Dyer methods were subjected to GC–MS to identify the composition. The MS is the first commercial system that takes into account, the presence or absence of fatty acids in aerobic microorganisms. Long chain components 16–21 carbon atoms were identified in the bacterial extracts of *Bacillus* sp. Linolenic acid and stearic acid were found in higher percentage and docosahexaenoic acid, eicosapentanoic acids were found in traces (Table 2).

Table 1

Antibacterial activity of protein isolated from *Bacillus* sp.

S. no	Pathogen name	Zone of inhibition (mm)
1	<i>Escherichia coli</i>	11
2	<i>Salmonella typhi</i>	10
3	<i>Klebsiella pneumonia</i>	8
4	<i>Vibrio parahemolyticus</i>	–
5	<i>Klebsiella oxytoca</i>	12
6	<i>Proteus mirabilis</i>	10
7	<i>Vibrio cholera</i>	12
8	<i>Streptococcus pneumonia</i>	10
9	<i>Staphylococcus aureus</i>	6
10	<i>Salmonella paratyphi</i>	7

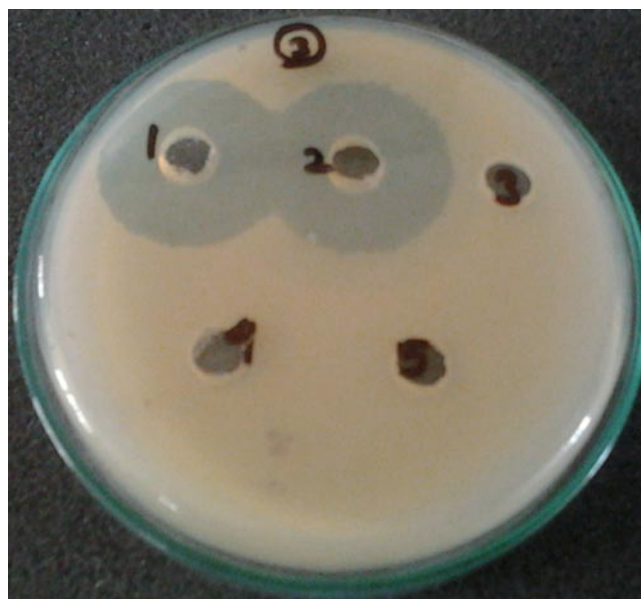


Figure 1. Antibacterial activity of *Bacillus* sp. strain.

Table 2

Fatty acids profile for the strain *Bacillus* sp.

Fatty acids	Percentage
Palmitic acid (16:0)	1.5125
Margaric acid (17:0)	0.4272
Stearic acid (18:0)	3.1974
Oleic acid (18:1)	1.2396
Linolenic acid (18:2)	3.4105
Alpha linolenic acid (18:3)	2.0672
Monocytic acid (18:4)	0.5154
Eicosapentanoic acid (20:5)	In traces
Docosahexaenoic acid (22:6)	In traces

3.4. SDS–PAGE analysis

The proteins were precipitated using ammonium sulphate at 60% concentration and it was dialysed against phosphate buffer at pH 7.0. The partially purified proteins were lyophilised and electrophoresed at 12% concentration of gel. Three discrete bands were seen with the molecular weight of 56, 47 and 39 kDa (Figure 2).

3.5. Spectroscopic characteristics of the antimicrobial agent

The infrared (IR) spectrum indicated that there is a typical N–H group, the $\nu=2955\text{ cm}^{-1}$ indicated that methyl group, the $\nu=1577\text{ cm}^{-1}$ correspond to a primary amine; the $\nu=1238\text{--}1086\text{ cm}^{-1}$ peaks showed the existence of different C–C group. The UV absorption peaked at 220.5, 230.0 and 260.0 nm (Figure 3 and Table 3).

3.6. Molecular phylogeny of the selected isolate

DNA was isolated and amplified using PCR. The 16s rRNA sequence of the isolate was compared to the sequence of *Bacillus* sp., in order to determine the relatedness of the isolate to these *Bacillus* strains. The phylogenetic tree (as displayed by the TreeView program) revealed that the strain isolated from fish gut is closely related to *Bacillus* sp. The most potent strain

evidenced an 99% similarity with *Bacillus* sp.

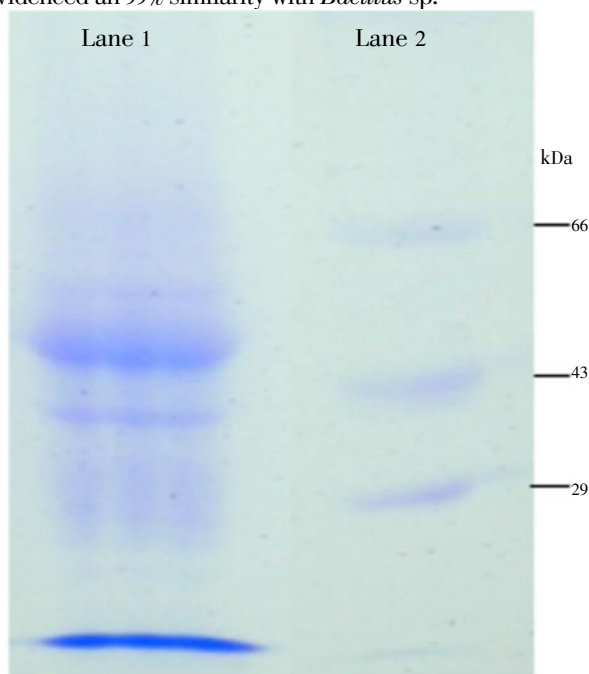


Figure 2. SDS –PAGE profile for protein isolated from *Bacillus* sp. Lane 1: Sample, Lane 2: Standard protein molecular weight marker.

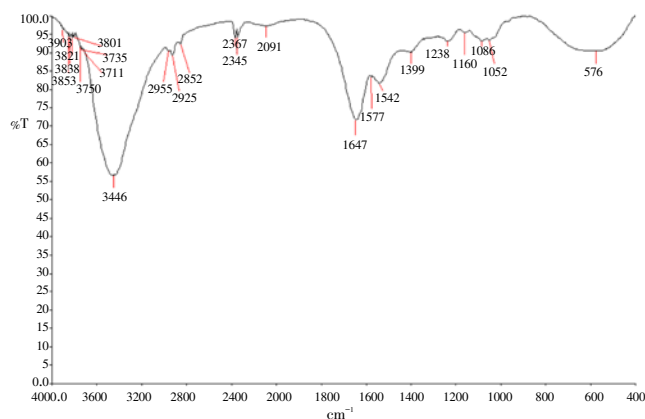


Figure 3. FTIR spectra analysis for protein isolated from *Bacillus* sp.

Table 3

FTIR spectral values of protein isolated from *Bacillus* sp.

S. No.	Range	Functional group
1	3000–3700	N–H
2	3711	N–H
3	3446	NH ₂
4	2955	Methyl C–H asymmetric/symmetric stretch
5	2925	Methyl C–H asymmetric/symmetric stretch
6	2852	Methylene C–H asymmetric stretch/symmetric stretch
7	2367	C≡C
8	2345	C≡N
9	2091	Transition metal carbonyls
10	1647	Alkenyl C=C
11	1577	Open chain azo –N=N–
12	1542	N–H
13	1399	Methyl–CH ₃
14	1238	Skeletal C–C vibration
15	1160	Skeletal C–C vibration
16	1086	Skeletal C–C vibration
17	1052	Cyclohexane ring vibrations
18	576	C–I aliphatic iodo compounds

4. Discussion

The increase in the frequency of multi-resistant pathogenic bacteria has created an urgent demand in the pharmaceutical industry for more rational approaches and strategies to the screening of new antibiotics with a broad spectrum of activity, which resist the inactivation process exploited by microbial enzymes[9]. Only one bacterial culture from twenty cultures was found to exhibit a wide spectrum of antimicrobial activities. Identification process has been carried out according to Bergey's manual of determinative bacteriology. The antimicrobial activities were determined and the results showed that the strain was maximum inhibitory to the pathogen *Klebsiella oxytoca* and *Vibrio cholera*, and minimum inhibitory to the pathogen *Salmonella paratyphi* and *Staphylococcus aureus*. Similar investigations and results were attained in previous studies[10]. Denaturing SDS–PAGE led to three bands of 56, 47 and 39 kDa. The cellular fatty acids were analysed using gas chromatography resulted in presence of polyunsaturated fatty acids. Bajpai VK, *et al.*[11] suggest that some of the bioconverted polyunsaturated fatty acids inhibit plant pathogens. The spectroscopic characteristics of the antimicrobial agent under study revealed that the IR spectrum peak $\nu > 3000 \text{ cm}^{-1}$ indicated that there is a typical amine group; the $\nu = 2955 \text{ cm}^{-1}$ peak revealed the presence of methyl group; $\nu = 1577 \text{ cm}^{-1}$ peak corresponded to the presence of primary amine; the $\nu = 1238\text{--}1086 \text{ cm}^{-1}$ peak showed the existence of different C–C group. The UV absorption spectrum showed the active compound typical absorbance's at wavelength 220.5, 230.0, 260.0 nm. Similar results were recorded by Atta HM[12]. In the view of all previously recorded data the strain *Bacillus* sp., bacteria has wide spectrum of antimicrobial activity[13]. Our data support the notion that *Bacillus* sp. should be further differentiated by the sequence analysis of DNA in order to resolve the appropriate taxonomic status. The sequence was submitted to NCBI and the accession number is KF420411[14].

Microorganisms are the potent producers of metabolic compounds used commercially as antibiotics and other novel drugs. The present study shows the present data focusing on microbial isolates which have the ability to produce antimicrobial agent. A further research would improve the antimicrobial agent production by *Bacillus* sp. against pathogenic microorganisms (Gram positive and Gram negative bacteria and unicellular and filamentous fungi).

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

To reduce or avoid the dependence on antibiotics, marine microorganisms have been considered as an effective alternative way to control bacterial infections. Many marine bacteria that can be able to catabolize wide range organic molecules including organic compounds such as benzoate have shown inhibition against varied microorganisms. Therefore, cellular fatty acid and protein profile contents play a major role in showing the antimicrobial activity of marine microorganisms (specially *Bacillus* sp., *Pseudomonas* sp., etc.). Therefore, there is need of new and potent antibacterial agents all over the world.

Research frontiers

The manuscript describes the importance of protein profile and cellular fatty acids were isolated from *Bacillus* sp. from fish gut, for the antimicrobial activity of the bacteria. The antimicrobial activity in the manuscript has shown as cultural, morphology, physiology, biochemical characteristics and 16S rRNA sequencing.

Related reports

Isolation of marine bacteria for 16S rDNA sequencing (*Briareum* sp., *Sinularia* sp., *Sarcophyton* sp., *Nephtheidae* sp., and *Lobophytum* sp. etc.) is reported to show antimicrobial effect. The literature data shows that they has evidence in providing high antimicrobial activity with antibacterial compounds of a marine bacteria.

Innovations and breakthroughs

Marine bacteria are known to produce wide range of compounds. Therefore, they are screened for secondary metabolites like antibiotic production. In this study, authors have demonstrated the antibacterial activity of *Bacillus* sp., in cellular fatty acid composition and protein profile.

Applications

This scientific study supports and suggests the use of bioactive substance of *Bacillus* sp. that is an useful antibacterial agent as a therapeutic agent.

Peer review

Generally, this is a potential research work in which authors have demonstrated the antimicrobial activity of *Bacillus* sp., from fish gut in protein profile and cellular

fatty acids. The activity was assessed based on cultural, morphology, physiology, biochemical characteristics and 16S rRNA sequencing. *Bacillus* sp. was found to be a important antibacterial agent.

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