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ANTIBACTERIAL AND ANTICANCER FATTY ACID PRODUCED FROM MARINE BACILUS SUBTILIS AVSC3: ISOLATION, CHARACTERIZATION AND BIOLOGICAL ACTIVITY

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ABSTRACT: It turns out that there is a tremendous amount of wealth below the surface of the ocean waiting to be discovered. The ocean environment is capable of solving many unsolved questions and is also home to an undiscovered hoard of mysteries. Seawater collected from Suryalanka marine sediments in Guntur, Andhra Pradesh, India was used to extract coastal soil samples. Selected bacterial strains were isolated using the spread plate technique, the bacteria with biological activity were screened using antibacterial activity, and the 16S rRNA sequencing was utilised to identify the bacteria. Metabolite which is biologically active was created in a defined setting (incubation, temperature, pH, Nacl concentration, and carbon amino acid and nitrogen sources). TLC was used to purify crude extract, which was then eluted with hexane to separate it on a column. Spectrally characterised purified fraction was examined for antibacterial and anticancer properties. Out of the 23 isolates, isolate AVSC3 exhibits a noticeable inhibitory activity against test organisms. This was established through a characterization of the test organism, Bacillus subtilis AVSC3, with a GenBank accession number of GEO:M21745. MN386243. To produce bioactive metabolites, an ideal incubation time is 60 hours, 35°C, pH 7, 0.5% NaCl, and 1% glucose and peptone. The antibacterial property of the pure fraction was found to be hexadecanoic acid, with a molecular formula of C16H32O2, which has spectral characteristics characteristic of this molecule. 14.2 mm-long Coli followed by Staphylococcus The 13.7-mm-diameter aureus, the 9-mm-diameter M.luteus, the 8.1-mm-diameter S.pyogenes, the 4.6-mm-diameter P. vulgaris, and the 4.4-mm-diameter S.typhi all demonstrated significant antimicrobial activity against MCF-7 cell lines, with the strongest effect observed at 25µg.

KEY WORDS: Hexadecanoic acid, Marine Bacillus subtilis AVSC3,Anticancer, Characterization, MCF-7 cell line, Antibacterial etc.

1. INTRODUCTION

In different ecosystems, marine biota is typically found in marine sediment, oceans, other marine surfaces, and living forms in the water (Wilson and Stevenson 1980, Nair and Simidu 1987,

Austin, 1992). In addition to novel chemical structures, these substances represent a diverse reservoir of unexplored compounds, besides unique biological activities (Sepcic et al., 2011). Because microbes have been thought to be an important and underutilised resource for bioactive compounds that have significant clinical value, they have remained largely overlooked (Rosenfeld and Zobell, 1947, Grein and Meyers, 1958).

Some kinds of marine bacteria are heterogeneous bunches of microscopic organisms that are able to tolerate a range of environmental conditions such as high and low temperatures, pressure, salinity, and pH. (Rampelotto, 2010). The genus Bacillus is generally more demanding for resources and space, which gives them a competitive advantage over other species. molecules, and the mechanisms by which marine organisms are different from land-dwelling ones are responsible for the development of different bioactive metabolites (Jensen, P.R.; Fenical, 1994, Feling et al., 2003). Competition was faced by several different bioactive compounds, all of which had been genetically engineered to oppose one another (Sayem et al., 2011 & Paul et al., 2007).

Several Bacillus species produce different classes of antibiotics, which has been demonstrated. Nearly 8% of the genome is used to synthesise antibiotics (Chen et al., 2007 & Kunst et al., 1997). Melent'ev et al. (2006) reported that Bacillus species are the most effective fungal and bacterial bacteria at competing with different microbes (Hagelin et al., 2004). The diverse classes of bioactive compounds that bacilli produced include various types of lipoamides, lipopeptides, fatty acids, polypeptides, polyketides, macrolactones, and isocoumarins (Hamdache et al., 2013; Baruzzi et al., 2011). (Hamdache et al., 2013 & Baruzzi et al., 2011).

The clinical properties of saturated fatty acids have a total of 14, 16, and 18 carbon atoms in a straight chain, with 14, 16, and 18 carbon atoms in a straight chain having different clinical properties. Several studies done by Paul et al., concluded that omega-3 fatty acids, specifically the long-chain omega-3s, such as eicosapentaenoic acid and docosahexaenoic acid, block cell proliferation in the prostate and breast cancer. Antihypertensive, antioxidant, hemolytic, hypocholesterolemic, antiandrogenic activities (Markkas and Madhuramozhi., 2015). A promising antibiotic producing isolate from B. subtilis is A.V.SubtilisAVSC3. The investigation sought to discover which bacterial pathogens the compound is effective against and to discover the antibacterial and anticancer properties on several clinical pathogens and cell lines.

2. MATERIALS AND METHODS

Sediment soil samples were collected from Suryalanka, Andhra Pradesh, India. Peptone, Beef extract, Sodium chloride and Agar were obtained from HiMedia Laboratories (Mumbai) Ltd and used as preliminary screening medium. Pathogenic strains were obtained from Microbial Type Culture Collection centre (MTCC). Breast cancer cell line (MCF-7) was obtained from the National Center for cellular Sciences (NCCS), Pune, India.

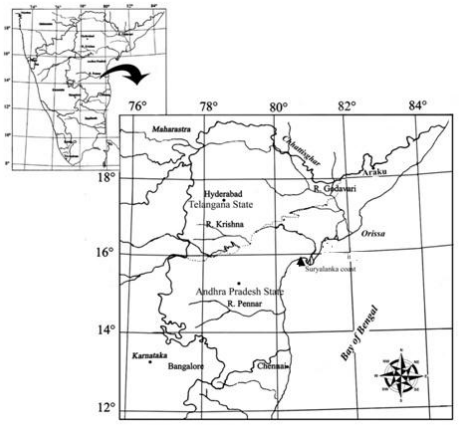


Figure 1: Study Area - Surya Lanka coast of Bapatla, Andhra Pradesh

2.1 Isolation of marine bacteria

Marine soil sediment was collected from Surya Lanka coast of Bapatla, Andhra Pradesh (Fig.1) and the samples were stored in polythene covers under chilled condition for further experiments. Marine bacteria were isolated by using serial dilution technique. The soil samples were serially diluted (10-1 to 10-9) and the dilutions were inoculated on Beef extract- peptone agar media (Lanhong Zheng *et al.,* 2014). The inoculated plates were incubated at 37ºC, for 24-48 hours. Morphologically different bacterial colonies were selected from10-5 to 10-7 dilutions. The experiment was done in triplicate.

2.2 Molecular Identification of bioactive compounds producing strain

AVSC3 strain was isolated and the pure cultures were maintained. 16S rRNA partial gene sequencing was opted for molecular identification of AVSC3. (Syed *et al.,* 2019)

2.3 Antibacterial activity of crude extract

Escherichia coli (MTCC 1696),*Salmonella typhi* (MTCC 8587), *Proteus vulgaris* (MTCC7299), *Micrococcus luteus*, *Streptococcus pyogenes* and *Staphylococcus aureus* (MTCC 3160)were selected as target pathogens. Streptomycin was used as positive and DMSO as negative control. 24 hours culture of test organisms was inoculated by spreading on NAM plates. 6-mm wells were punched in the medium and 60 µl of the crude extract of AVSC3 was loaded into each well and incubated for 24-48 hours at 37 °C. Diameter of each zone in millimeters was measured after incubation (Balouiri *et al.,* 2016).

2.4 Optimizing the media for growth and anti bacterial activity of AVSC3

Optimization was carried out at for different parameters like incubation period, Temperature, pH, NaCl concentration, carbon sources, nitrogen sources and amino acids. The growth of the isolate was determined by measuring OD at 540 nm and antibacterial activity by the presence or absence of zone (Syed *et al.,* 2019).

2.5 Extraction of crude

Culture broth was centrifuged at 10,000 rpm for 15 minutes. The supernatant was collected and equal volume of ethyl acetate was added and vigorously shaken for 20mins. Extraction was repeated with equal volume of ethyl acetate and organic layer was evaporated in a rota evaporator. The crude was processed for further screening tests. (Nandhini SU *et al.,* 2018).

2.6 Isolation of bioactive metabolite by TLC

Sample was diluted and loaded on commercially available TLC plate (Silica gel 60) plates (Merck, Germany). Solvent ratio used for the separation of the compounds was Chloroform: methanol (4:1, v:v). One spot was revealed under UV-trans-illumination at 365 nm. (Zeeshan *et al.,* 2012).

2.7 Column chromatography

Sample was loaded into column packed with silica gel (Himedia 100-200 mesh) and eluted by chloroform: methanol (4:1) solvent system. DMSO was added to the purified fraction and used for further analysis.

2.8 Antibacterial activity of pure compound

Antibacterial activity was carried out with purified compound using well diffusion method as above discussed.

2.9 Anticancer activity

Purified compound obtained from AVSC3 was assessed for in vitro cytotoxicity by MTT assay against MCF-7 cell line. 100 µl media was loaded in 96 well with a density of 10,000 cells per well and incubated for 24 h with Doxorubicin as standard. The cells were exposed to various concentrations of the test compounds for 48 h. 10 µl of MTT solution was added to each well and incubated at 37 °C for 4 hours. At the end of incubation, 200 µl of DMSO was added to each well and absorbance was noted at 570 nm. The mean % of cell viability in relation to untreated cells

was estimated from data of triplicates (Venkanna *et al.*, 2014). Growth inhibition was calculated using the formula:

% inhibition = 100 (control-treatment) Control

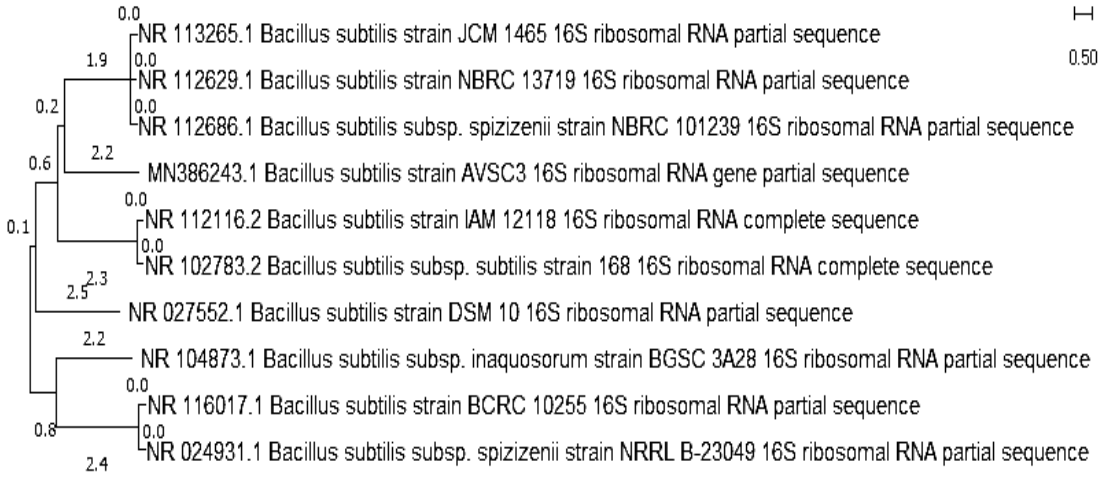
2.10 Spectroscopic Characterization and Elucidation of the Structure

The pure fraction obtained from AVSC3showing efficient antimicrobial and anticancer activity was used for structural elucidation. LC-MS (Liquid chromatography)is an effective technique in the profiling the compounds with short run times (Nagy et al. 2004), so in this study we used LC-MS for preliminary identification of pure compound. The UV spectrum was determined on UV visible spectrophotometer. IRspectra (4000-200 cm'1) was recorded on model Nicolate Megna FTIR-550 spectrophotometer in the form of KBR pellets. Mass spectra were recorded using MASS Water Quattro Micro Mass spectrometer. 'H NMR spectra were obtained in CDCL3 using NMR Bruker Avance-H 300/Hz spectrometer. The data obtained in these analytical techniques was used for the structural elucidation of bioactive compound.

3. RESULTS

3.1 Isolation and Molecular identification of bioactive compound producing bacteria

In present study a total of 23 bacterial strains were isolated of which AVSC3 showed antibacterial as well as anticancer activities.16S rRNA gene sequence of AVSC3 revealed the isolate as *Bacillus subtilis* strain and deposited in GEN BANK, NCBI as *Bacillus subtilis* AVSC3 with GenBank accession no. **MN386243**(Fig. 2)**.**



3.2 Antibacterial activity of crude extract

Zones of inhibition produced by the crude extract weremeasured using agar well diffusion method. As shown in Table1, the extract exhibited interesting antibacterialactivitiy against *Escherichia coli* and *Staphylococcus aureus*, higher activity against Gram-negative *coli* than that of Gram-positive *cocci*.

3.3 Optimizing the media for growth and anti bacterial activity of AVSC3

When grown in nutrient broth at different incubation hours results showed that the isolate has shown maximum growth and antibacterial activity after 60hrs incubation and 35°C at pH7, maximum results were found at 0.5% NaCl concentration, AVSC3(1.45) Maximum growth and antibacterial activity of found when the broth was supplemented with Glucose and peptone.

3.4 Isolation of bioactive metabolite by TLC and column chromatography

The crude extract obtained a single spoton TLC platet with Rf value 0.79(Fig. 3). Single fraction was obtained in column chromatography which was collected and stored for further analysis.

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Figure 3: crude extract obtained a single spoton TLC platet with Rf value 0.79

3.5 Antibacterial activity of pure compound

The column collected pure fraction was dissolved in DMSO and tested for its antimicrobial activity against gram negative and gram positive Bacteria by agar well diffusion method by using DMSO as control. The results indicated in the plates (Fig. 4)has shown highest antibacterial activity against *Escherichia coli* with zone of inhibition 14.2mmin diameter, followed by *Staphylococcus aureus* 13.7 mm, *Micrococcus luteus* 9 mm, *Streptococcus pyogenes*8.1 mm, *Proteus vulgaris* 4.6 mm, *Salmonella typhi* 4.4 mm.

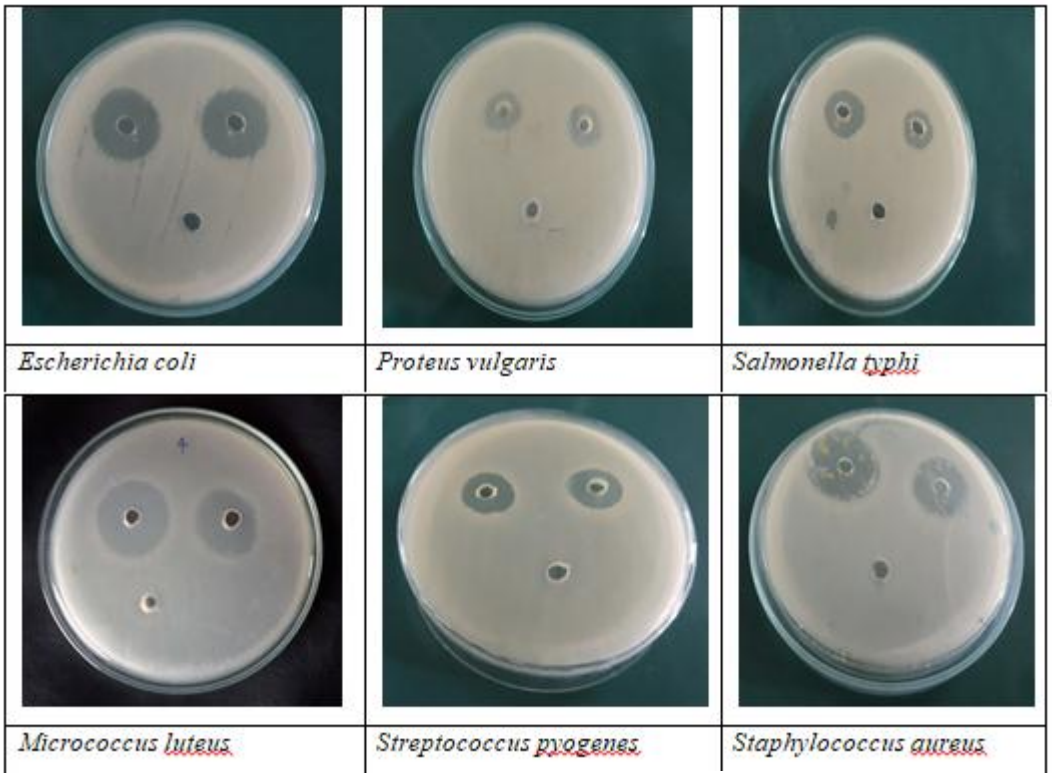

Figure 4: Plates showing antibacterial activity against *Escherichia coli* with zone of inhibition

3.6 Anticancer activity

As clearly shown in (Fig. 5), the effect of purified compound on MCF-7 tumour cell line exhibited a reasonable degree of anticancer activity. The compound has shown inhibition of 32.88% at 5 µg concentrations, 58.28% inhibition at 10µg, 68.98% at 10µg, 59.09% at 50 µg and 37.97% at 100 µg concentrations respectively. Maximum inhibition was seen at 25µg. The inhibition of the cells was gradually increased with increasing the compound concentration from 5-25µg with 53.76 IC_{50}

(µg).

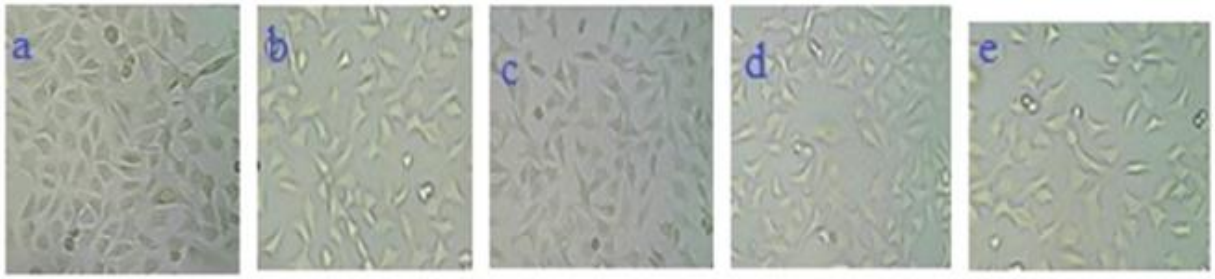

Figure 5: Effect of purified compound on MCF-7 tumour cell line exhibited a reasonable degree of anticancer activity

3.7 Spectroscopic Characterization and Elucidation of the Structure of pure compound

The LC-MS result acquired indicated a peak with the highest M/Z value of 256 which suggested to be the molecular mass of the compound. Going by this observation and also the molecular mass of the compound is (256.3), we could predict that the compound may be a fatty acid (Fig. 6).

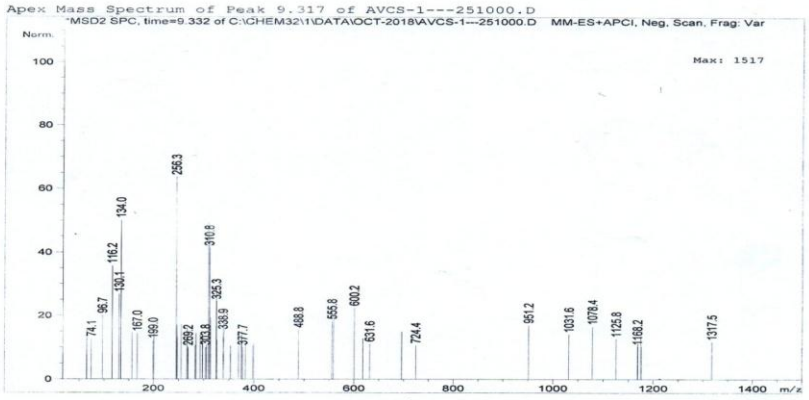

Figure 6: Molecular mass of the compound

The Fig. 7 shows the band of pure fatty acid compared to the standard. The compound showed an absorption band with a peak at 218 nm which illustrated the presence of fatty acid.

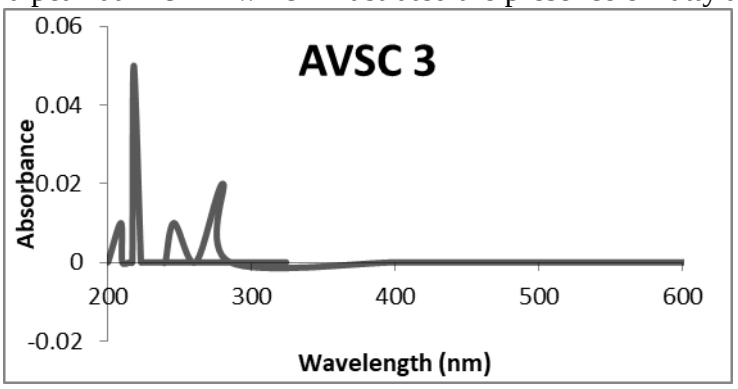

Figure 7: Band of pure fatty acid compared to the standard

Infrared (IR) spectroscopic analysis (Fig. 8), absorptions band at 3604.54 cm-1 indicates a characteristic of OH stretching , 2850.31 cm-1 reveals as aliphatics (CH3) stretching, 1702.06 cm-1 is due to the carbonyl (C=O) group. These absorption frequencies resemble the data published by Lunn and Theobald, (2006).

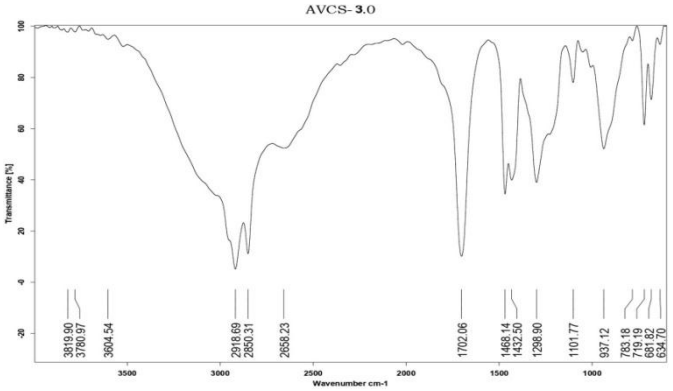

Figure 8: Infrared (IR) spectroscopic analysis absorptions band

The 1HNMR spectrum (Fig. 9)(400MHz, CDCl3) of the compound revealed a long peakat δ 1.25, 28H of the long chain was obvious for one proton. The 1HNMRspectrum showed a long peak at δ 2.32 which could be attributed to methylene group. The singlet at δ 10.93 was indicates an OH in COOH. The triplet of three protonsat δ 0.89 could be a terminal methyl group. The above mentioned spectral features are in closeagreement to those observed for saturated fatty acid according to the research carried out by Guillen and Ruiz (2001). Hence the isolated pure compound is hexadecanoic acid with molecular formula of C16H32O2 which belongs to the series of compound CnH2nO2 that is fatty acid.

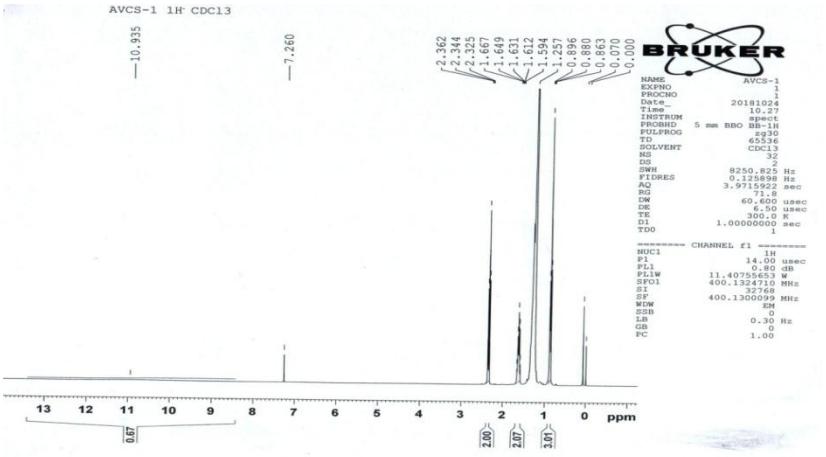

Figure 9: The 1HNMR spectrum

4. DISCUSSION

There are different bioactive metabolites created by marine bacteria that have novel modes of activity. These microbes have a tremendous amount of potential for developing effective management strategies to combat human, animal, and plant pathogens by means of biotechnology (Mondol MAM et al., 2013). Bacillus in the marine environment has gained prominence as an antibiotic recently. It was discovered by Ramasubburayan et al. (2014) that a range of marine bacteria strains, including those found in B. licheniformis, B. pumilus, B. mojavens, B. subtilis, and B. firmus, exhibit antibiotic properties. We isolated a potent strain of marine bacteria known as AVSC3, with antibiotic and anticancer properties, and designated it as B.subtilisAVSC3, which has a unique accession number of MN386243. The final yield is greatly affected by everything that relates to nutrition, physical conditions, and recovery and purification techniques (Sharp et al.1989). Incubation, temperature, pH, and growth medium significantly affected the final yield in this experiment. For the greatest yield of viable cells, incubation time should be 32-48 hours

(Korsten and Cook, 1996). After 60 hours of incubation, the isolate AVSC3 had reached its peak growth and antibacterial potency. 30°C temperature and pH 7.0 were the optimum growth parameters in the studies done by Abdurrahman et al., (2016), and in contrast, 35°C temperature and pH 3 were the optimum temperature and pH levels for the production of antibacterial compound production for BacillusSAFR-032. At a concentration of 0.5 percent, both growth and antibacterial activity reached their maximum values. Peptone and glucose supplementation results in enhanced growth and antibacterial activity. The results of the study done by Das et al. (2014) state that B. subtilis AN11 isolated from Bhitarkanika mangroves inhibited the growth of both gramme positive and gramme negative bacterial pathogens.

Omega-3 fatty acids, especially DHA, are well known for their anti-bacterial and anti-fungal properties. While these six fatty acids have antibacterial properties, others have shown that hexadecenoic, stearic, oleic, linoleic, and linolenic acids are also well known to have antimicrobial activity (McGaw et al.2002). Antimicrobial, antioxidant, and anticancer activities were found in the derivatives of lipids, such as hexadecanoic acid, octadecenoic acid, L-(+)-ascorbic acid 2,6-dihexadecanoate, mono (2-ethylhexyl) ester 1,2-benzenedicarboxylic acid, γ-sitosterol, and sigmasterol, derived from marine sources and plants (Karthikeyan et al., 2014). The secondary screening for Saccostrea glomeratare revealed that the fraction that contained N-hexadecanoic acid and other fatty acids had potential antimicrobial and antitumor properties (Karthikeyan et al., 2014). The researchers isolated hexadecanoic acid from neem oil and found that it completely inhibited pathogenic bacteria like Staphylococcus aureus, Escherichia coli, and Salmonella sp (Zhong-hui et al., 2010). Gram-negative and gram-positive pathogenic bacteria are also targets for this chemical (Fig. 4).

The researchers conducted an in vitro MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxicity assay of the bacterial strain B. subtilis subsp. subtilis RG, and found that it has strong cytotoxicity against human breast adenocarcinoma cells (MCF-7) with an IC50 value of 46.64µg ml-1 after 48 hours of incubation. Compounds with lower IC50 values often have higher cytotoxic activity, according to the research by Kosanic et al. (2012). The hexadecenoic acid derived from the larva of Protaetia brevitarsis is associated with apoptosis induction in colon 26 cells, which indicates that this fatty acid plays a prominent role in inducing apoptosis. It was also shown to have cytotoxic activity against MCF-7 cell lines with an Ic50 value of 53.76 and an overall inhibition of 68.98% at 25µg for the hexadecanoic acid that was isolated from our isolate of Bacillus subtilis AVSC3.

5. CONCLUSION

In summary, the findings of the current investigation on select bacteria showed there is a great opportunity to find novel bioactive compounds with potent antibacterial activity. Bacillus subtilis has earlier been studied for its role in synthesising fatty acids, but the biological activities of those fatty acids are extremely limited. It appears that the antimicrobial and anticancer potential of hexadecanoic acid produced by the Bacillus subtilisAVSC3 has been discovered. There is significant potential for future research in drug development, given the presence of such valuable and hard-to-find species in the marine environment. Cancer treatment, using fatty acids in novel ways, would create a new pathway for changing public health.

6. ACKNOWLEDGMENTS

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