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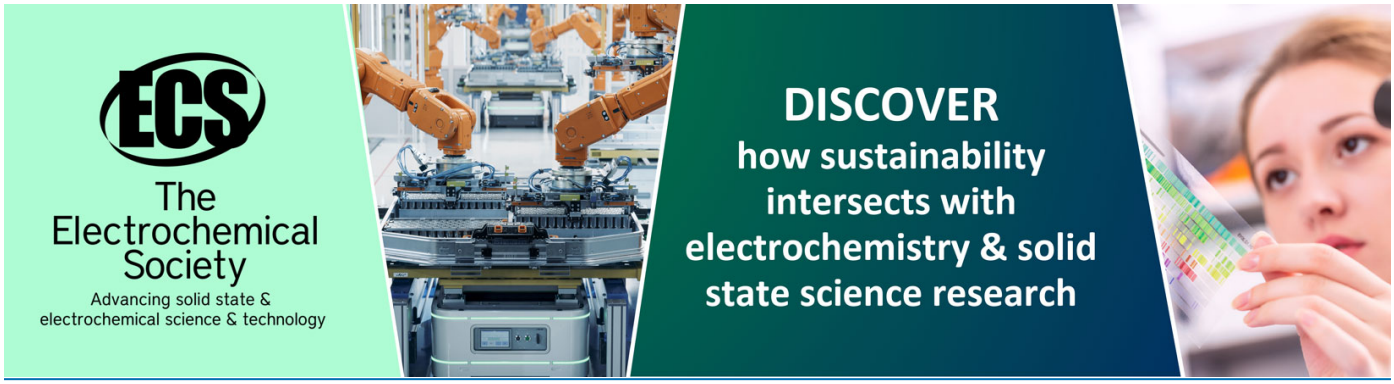

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Pigment from *Streptomyces bellus* MSA1 isolated from marine sediments

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Abstract. The existing study is purposeful on the intracellular pigment extraction from actinomycetes isolated from Kovalam Beach regions of Chennai, Tamil Nadu, India. Only one actinobacterial isolate showed pigmented growth out of total 4 isolates. Ethyl acetate as the solvent was used in cell disruption technique for the extraction of intracellular pigments. UV-Visible spectrophotometry, FT-IR spectroscopy, HPLC and GC-MS were used for the partial characterization of the pigment. The extracted pigment was applied for the preparation of lip balm and assessing its textile dyeing property. In addition, the extracted pigment was analysed for antioxidant, antibacterial activity, MTT assay and haemolytic activity. On optimization, dextrose and maltose were the best carbon sources. The finest nitrogen sources were found to be casein and peptone. The optimum temperature range was 35°C -40°C and optimal pH was found to be between 6.0 and 8.0. The obtained results showed potent antioxidant activity and found to be non-toxic to human erythrocytes.

1 .Introduction

The Actinomycetes are one of the Gram positive bacteria and are having extreme G+C (>55%) accumulation in their DNA. In some research reported that actinomycetes are recognized as prokaryotic organisms. The significant cultural characteristics of actinomycetes were found to be pigment production [1].In the dyestuff industry, the synthetic dyes are toxic and it is replaced by the natural dyes. Indigo (from plants), Cochineal (from animals), ocher (from minerals) are three kinds of natural dyes. Natural dyes can afford the great desired substitute to the chemical dyes. It is simple and effortless than the other natural pigment. In an industrial scale, pigment extractions from actinomycetes are not in practice. Moreover, proper protocols are not available for the fermentation and the recovery of pigments. When compare to the pigments from plants and animals sources microbial pigments afford a good choice. Bio pigments are extracted from living entity. Due to the potential health hazard effects, natural pigments are favored over synthetic colorants [3]. Natural pigments extracted from plants are frequently limited, uneven, highly priced, and need more difficult and tedious process for production (Kim et al., 1999). In contrast, pigment from microbes can be easily formed in sufficient amount, are cost effective and have a simpler extraction and purification process (Parskh et al., 2000). The range of shades that can be possible obtained is more varied. Currently, the whole world is looking towards the usage of traditional products and adopting a natural approach towards life through increased usage of biological products. For healthy life, public are paying attention in natural food, herbal medicines and conventional practices. Higher demands were seen for the products from biological substances like plants and microorganisms. Recently, bio-cosmetics are pushing through the cosmetics industry in the world and the demand for the bio-cosmetics is rising and quite significant [4]. Hence, it may be assumed that incidence of such atypical

strains which produces pigment with consistency is of research interest, further more if it is obtained from an atypical or relatively less exploited habitat. The current study is about the characterization and the isolation of the pigmented actinomycetes and the applications of the extracted pigment.

2. Materials and methods

2.1 Marine soil sample collection

A Kovalam beach region of Chennai, Tamil Nadu, India was selected for soil sample collection. By using sterile polythene bag soil samples were collected and transported under controlled conditions. [6].

2.2. Isolation

The samples were pretreated with $CaCO_3$. And one gram of marine soil sample was used for the serial dilution. Diluted samples were inoculated into different plates. For the isolation of marine actinomycetes starch casein agar and nutrient agar were used (Usha et al., 2011). To reduce contamination the media was supplemented with cycloheximide (25 µg/ml), nalidixic acid (25 µg/ml) and incubated at 28˚C for 7 to 10 days [7]. All the plates were examined for actinomycetes colonies based on morphology and colors of pigmentation. And the pure culture of actinomycete isolate was maintained in starch casein agar plates for further studies [8].

2.3. Identification of test organism

For the preparation of stock culture, the isolated pure colonies were inoculated in starch glucose broth for 7-12 days. The test organism was identified and confirmed by standard tests [9].

2.4. Morphological Characters

The culture was grown on starch casein agar plate for 3 to 14 days. Morphological characterization was implemented with a magnified lens on actinomycete strains. Aerial mycelium, aerial color, nature of colony, pigmentation, size and reverse side color were observed under the microscope.

2.5. Scanning Electron Microscopy

Under the scanning electron microscope (SEM), spore surface and its morphology was observed. [10].

2.6. Biochemical and Molecular characterization

The universal primers were used for the PCR amplification of 16SrDNA. Big dye terminator cycle sequencing kit was used for the sequencing of DNA. For the homology search the sequence was administered to BLAST analysis and submitted to the GenBank database.

2.7. Pigment and Crude Extraction

The natural pigment producing marine actinomycetes colonies were selected for the production. According to (Priyanka et al., 2015) the production media was prepared. The broth pH was adjusted to PH 7.2.To obtain the extracellular pigment from actinomycetes, the broth was placed on a rotary shaker. The pigmented broth was centrifuged for 15 min at 5000rpm. The supernatant was collected and pellet was discarded. This step was repeated twice [11]. The supernatant was filtered using Whattman qualitative

filter paper. The supernatant was suspended to different polarity solvents such as ethyl acetate, chloroform, methanol, ethanol in ration1:1 [12].

2.8. Screening of marine pigmented actinomycetes for antimicrobial activity

For the screening of antibacterial activity, the purified marine pigmented actinomycetes were subjected to cross streak method [13]. The test pathogens are *Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi , Staphylococcus aureus and Bacillus subtilis.* For control the plates were streaked with test organism without pigmented actinomycetes

2.9. Antibacterial activity of pigment.

Well diffusion method was used to test the antibacterial activity of pigment. The human bacterial pathogens (*Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella typhi*) were swabbed on nutrient agar. The crude pigment concentration was increased from100µLto 150µLthe zone of inhibition was measured after 5 days of incubation [14].

2.10. Antioxidant activity

The 2, 2-diphenyl-1-picrylhydrazylradical scavenging activity (DPPH) was carried out by the method [15]. For the examination of DPPH radical scavenging activity, crude extracts were prepared at different concentrations.

2.11. Hemolytic Assay

In this assay, 5 mL of blood containing equal amount of Alseiver solution was centrifuged at 15000 rpm for 30 minutes. After centrifugation at 1500 rpm for 5 min, pellet with RBC was washed with 0.75% saline and the supernatant was discarded. The 0.5mL of cell suspension with normal saline was mixed in the sample. The mixture was incubated for 30 min at 37°C and then centrifuged at 1500 rpm for 10 minutes. The supernatant containing free hemoglobin was measured using UV-Vis spectrophotometer at 540nm. Distilled water was used as minimal hemolytic controls and phosphate buffer saline was used as maximal [17].

2.12. MTT cell proliferation assay

The crude extract treated HeLa cells were subjected to Cell Quanti-MTT cell viability assay kit (Bioassay Systems) for the detection of cytotoxic activity. The cells employed with 0.1% of DMSO or wells containing only culture medium as control. The graph plotting, the mean and the IC50 value were deliberated according to Rakshanya et al (2011) [18].

2.13 Optimization of media components

2.13.1. Basal Medium

The medium containing MgSO4.7H2O (0.5gm/l), FeSO4.7H2O (0.01gm/l), NaCl (0.5gm/l)), K2HPO4 (2.28gm/l) without C and N sources were used for optimization.

2.13.2. Carbon Source

Starch casein broth was preparedwith 0.2% casein (standard nitrogen source),pH was adjusted to 7.2 before sterilization in 4 different conical flask. The basal medium at 1% w/v concentration with dextrose, sucrose, maltose & fructose were used as a carbon sources to be tested. After inoculation, the flasks were placed on incubation. At the time of incubation continuous aeration and agitation wasrequired.The cultures wereanalyzedoptically for pigment intensity after 10 days. The filtered biomass weight was measured. To remove the moisture completely the biomassfiltrate was subjected to hot air oven before weighing.The DPPH scavenging activity was estimated by measuring OD value at 517 nm [19].

2.13.3. Nitrogen Source

The effect of *nitrogen Source was optimized equivalent to the* effect of *Carbon Source. Here* casien, yeast extract, Peptone &asparagine were used. The methodology for the determination of nitrogen source was followed according to Palanichamy et al (2011).The OD was measured at 517 nm to detect the activity of DPPH scavenging.

2.13.4. pH

According to Palanichamy et al (2011) the pH effect for optimization was done. Pigment intensity and biomass were calculated. The DPPH scavenging activity was estimated by measuring OD value at 517 nm.

2.13.5. Temperature

Starch casein broth were inoculated,as above,and incubated at 30°C, 35°C, 40°C and 45°C. Intensity of pigment and dry weight of biomass were noticed after 7-10 days The OD value of cultured broth was calculated at 517 nm and the DPPH scavenging activity was estimated.

2.14. Thin Layer Chromatography

Using a capillary tube the crude extract was loaded on pre coated TLC plates. The developing solvent was standardized and poured into the chromatography tank that was saturated in the mobile phase. For equilibration the chromatography column was kept for 10 min in the solvent. The plate was dried and pigment spots were visualized under normal light. Rf values were calculated and compared with the standard Rf values.

2.15. HPLC analysis

The pigment was analyzed by high-performance liquid chromatography (HPLC). The elution of metabolite was done. The rate of flow for the elution was 1.0 mL $min^{-1}$and analyzed at 250 nm with C18 column (3.0mm×300mm).

2.16. UV-Visible Spectrophotometry

By UV-Visible spectrum, the λ max of the isolated pigment was observed and analyzed. The absorbance range of the extracted pigment was detected by using λ max. Ethyl acetate solvent was used for dissolving extracted pigment. By using UV-visible spectrometer the analysis were made in the range of 200-800 nm

2.17. GC-MS analysis

The GC- MS analysis was done according to the Dastager et al methodology. [2] .The GC –MS spectrum analysis was compared with GC-MS NIST library.

2.18. Fourier-Transform Infrared Spectroscopy (FT -IR Spectroscopy)

Fourier Transform Infrared Spectroscopy was done. To determine the presence of functional group in the ethyl acetate extract, the important IR bands (OH, C-N, N-H, C-H,C=C,C-H,C-O), symmetric, asymmetric and stretching frequencies were studied and analyzed.

2.19. Applications of Bio-Pigment

2.19.1. Bio-lip balm

Lanolin, coconut oil and shredded bee wax were mixed for the bio- lipstick preparation. The bowl was kept in water bath until the wax melts entirely and all the ingredients are consistently mixed. The pigment was added to it to impart colour. The preliminary stability test includes colour, odour, appearance and spread ability of lip balm. The formulation was residential and evaluated in 3 days at room temperature (28 ℃) and oven temperature (50 ℃) [20].

2.19.2. Textile Dyeing

The extracted pigment was used for dyeing the wool, absorbent cotton, and thread. Ferrous sulphate and copper sulphate were used as pre-mordant for the samples. By using MLR 1:50, the wool, absorbent cotton, and thread were dyed in colored filtrate. The dyeing time and the incubation temperature were found to be 45 min and at 70-80°C. The samples were washed with cold water after dyeing.

3. Results & Discussion

3.1Morphological & biochemical characterization

The marine soil samples were collected from Kovalam beach regions of Chennai, Tamil Nadu of India. And 5 different actinomycetes were isolated. Among, which one isolate showed a light pink colourwhitish aerialmass, which turned grey on ageing (Fig. 1& 2).For the isolation of actinomycetes starch casein agar were used.Among these media, starch casein agar enriched more number of actinomycete isolates. Cross streaking was done to identify the bioactivity against pathogens. Further it was sub cultured and maintained to perform the further experiments.Gram staining was performed and the slide was observed under the light microscope in which spores and hyphae were observed (Fig.3).The test was done in order to confirm the presence of gram positive actinomycetes .Figure 4 represents the spore morphology of MS1 isolate under SEM. Based on the biochemical characterization; it is confirmed as *Streptomyces* sp. which is a gram-positive actinomycetes. The test results showed positive for casien hydrolysis , starch hydrolysis , glucose , lactose , sucrose , mannitol whereas it showed negative results for Voges Proskaucer test (VP), ONPG test , Arabinose , Sorbitol and Raffinose.

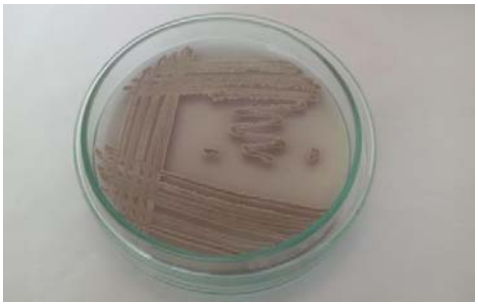


Figure 1.pure culture of *Streptomyces bellus*VITMSA1

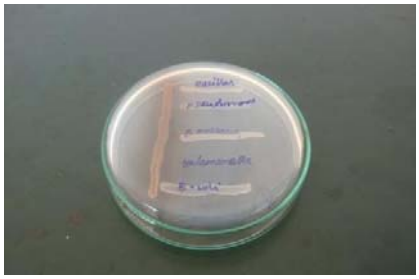


Figure 2.cross streak assay

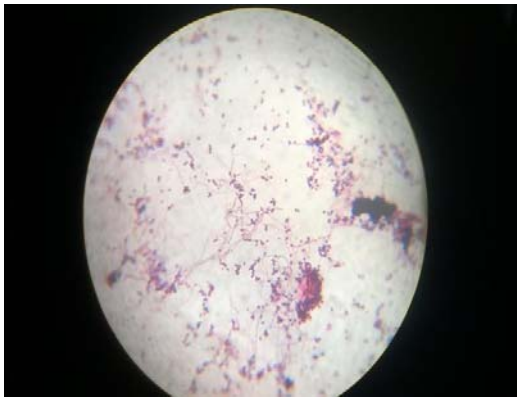


Figure 3.Morphology of the *Streptomyces bellus* MSA1(100X)

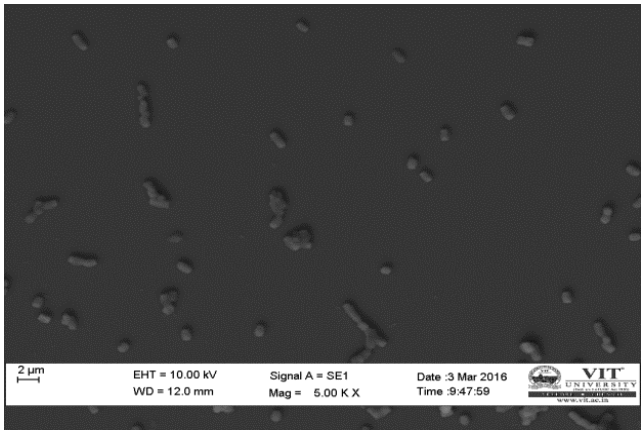


Figure 4.Micrograph of scanning electron microscope: spores of the actinomycete isolate VIT MSA1

3.2 Molecular characterization

Using 16S r-RNA sequencing, the molecular characterization was done. The BLAST results revealed the isolate VITMSA1 was found to be 99% similar to *Streptomyces bellus*(Fig.5).

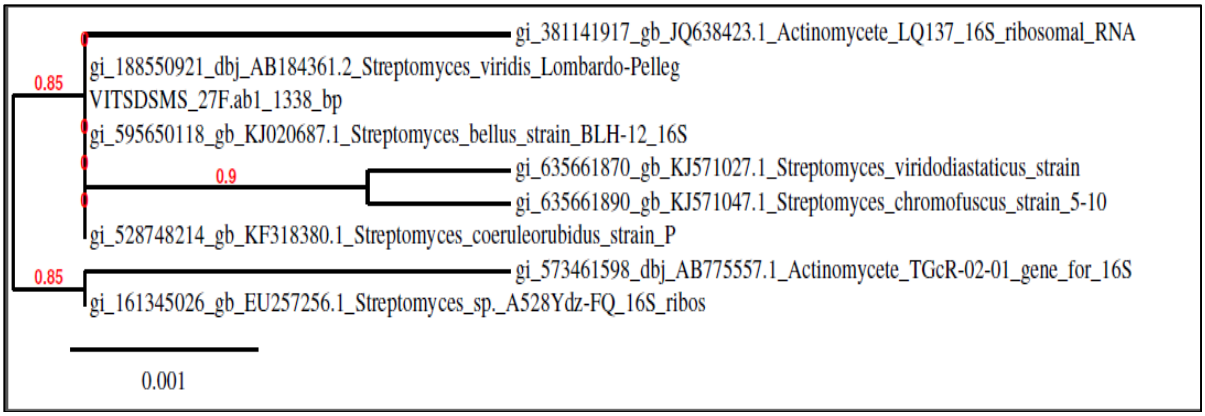


Figure 5. Phylogenetic tree of *Streptomyces bellus* MSA1

3.3. Antibacterial activity of crude pigment extract

The ethyl acetate extracts of *Streptomyces bellus*MSA1 was prepared. Antibacterial activity of crude pigment was observed against all tested pathogens. Maximum zone of inhibition was observedagainst *Escherichia coli*, *Staphylococcus aureus*, *Bacillus.* sp., *Pseudomonas aeruginosa* was measured. The zone of inhibition against *Escherichia coli* and *Bacillus.*sp. Was found to be 8mm and 7mm which was more than the standard chloramphenicol. Figure.6 represents the antibacterial activity of crude extract against the pathogens. *Escherichia coli* and *Staphylococcus aureus* showed maximum activity in existing study followed by *Bacillus.*sp and *Pseudomonas aeruginosa*. The significant antibacterial activity was showed by crude pigment against 4 pathogenic organisms. Based on the results, it was obvious that pigment extract showed excellent anti-bacterial activity against *Staphylococcus aureus* and *Escherichia coli* strains. The inhibitory concentration was found to be 150µL.

E.coli *Bacillus* sp. *Staphylococcus aureus*

Pseudomonas sp.

Figure 6.Antibacterial activity of crude pigment extract

3.4Antioxidant activity

Ethyl acetate extract of *Streptomyces bellus*MSA1 in crude form showed 82% DPPH activity which was quite good when compared with the standard that is ascorbic acid. (Fig.7) In addition, radical scavenging activity performed with different concentration of ethyl acetate extract also showed good result. The percentage inhibition ranges from 82% to 66%. [21]

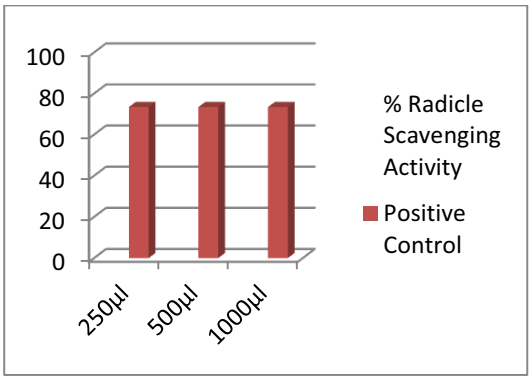


Figure 7.Antioxidant activity

3.5 Optimization

In all the examined carbon sources, the organism was able to grow. The highest biomass production (0.291gm/100 ml) was observed in medium supplemented with dextrose. In dextrose and maltose, highest pigment intensity was observed. Pigment intensity was less in other carbon sources such as sucrose and fructose than the dextrose and maltose. Casien and peptone were the greatest nitrogen sources out of all

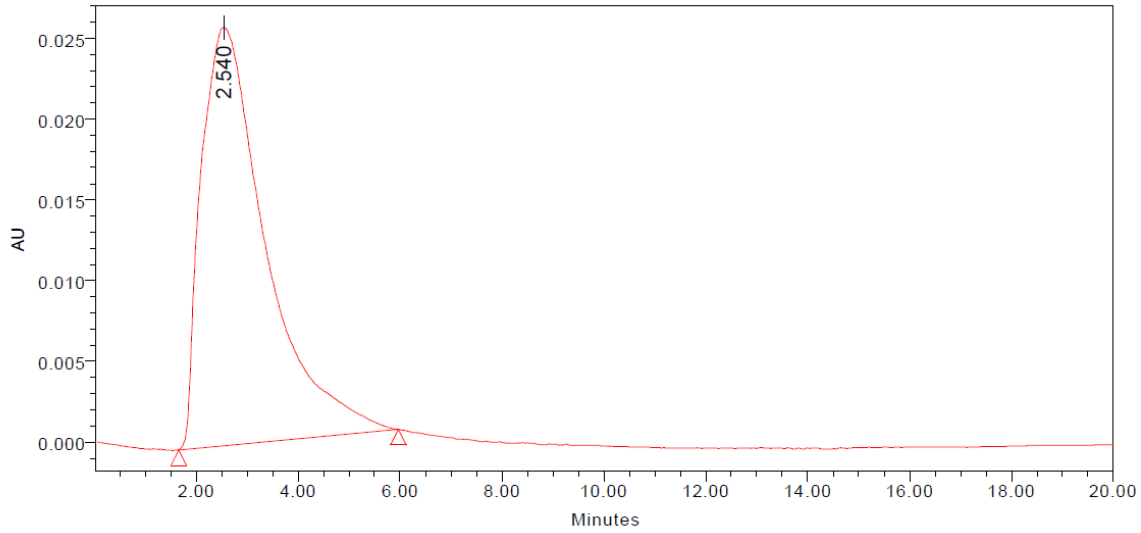
tested nitrogen sources. For the pigment production, the casein and peptone were the best nitrogen sources. Highest biomass yield of casein was found to be 0.382gm/100 mL. Maximum growth was observed in medium supplemented with asparagine and yeast extract, but the pigment production was not found significant. Maximum growth and pigment production was documented at pH 6 -8 and at 40°C.Previous reports of *Streptomyces* sp. also revealed a much similar results [13]. Optimum pigment production was observed in carbon source, dextrose [22]. In the present study dextrose and casein were the best carbon & nitrogen source for the pigment production.

3.6. Purification

An R_f value of 1.07 was obtained in thin layer chromatography with Butanol: acetic acid: distilled water (6 : 2 : 2;v/v).

3.7. HPLC analysis

HPLC chromatogram for secondary metabolite from *Streptomyces sp.* MSA1 was done. Retention time of the peak was found to be 2.54 min. (Fig.8)



Peak Results

	Name	RT	Area	Height	Amount	Units
1		2.540	2274011	25893		

Figure 8. HPLC chromatogram

3.8.GC-MS analysis

Figure 9 denotes the chromatogram of ethyl acetate extract of the metabolite. Eight peaks were observed. All the peaks correspond to bioactive compounds. The retention peaks were perceived maximum at 16.21.(fig.9)

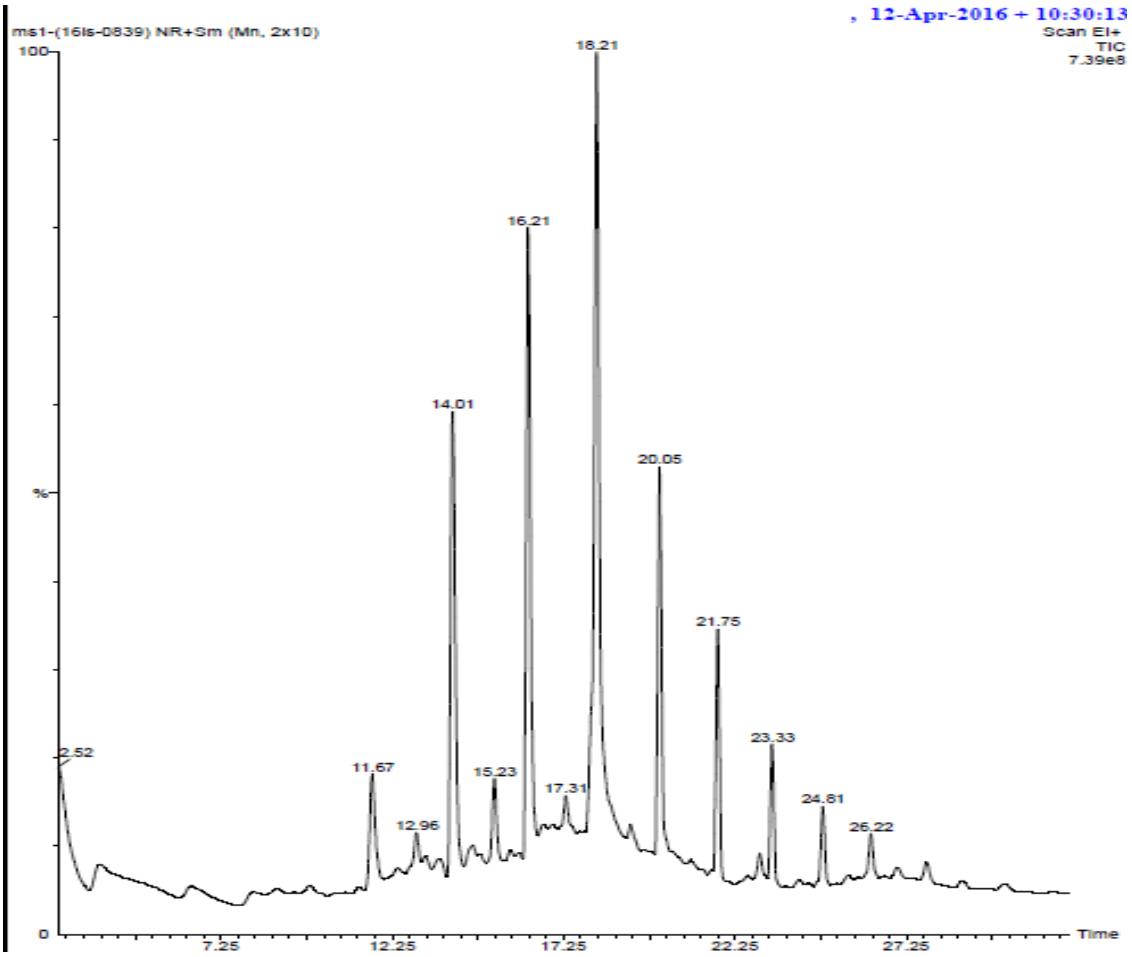


Figure 9. GC-MS spectrum of ethyl acetate extract

3.9. FTIR

For the ethyl acetate extract, FT-IR absorption in KBr (Fig. 10) was influenced by sharp peaks at 1737.86 cm'l, 1236.37 cm'l and 1045.42 cm'l. The bond C=O stretch which corresponds to functional group ester, saturated aliphatic dominates the IR spectra. The bonds C–N stretch, C-H oop, C–Cl stretch and C–Br stretch which corresponds to functional groups aliphatic amines, aromatics and alkyl halides are present in equal amount in the IR spectra.(Fig.10)

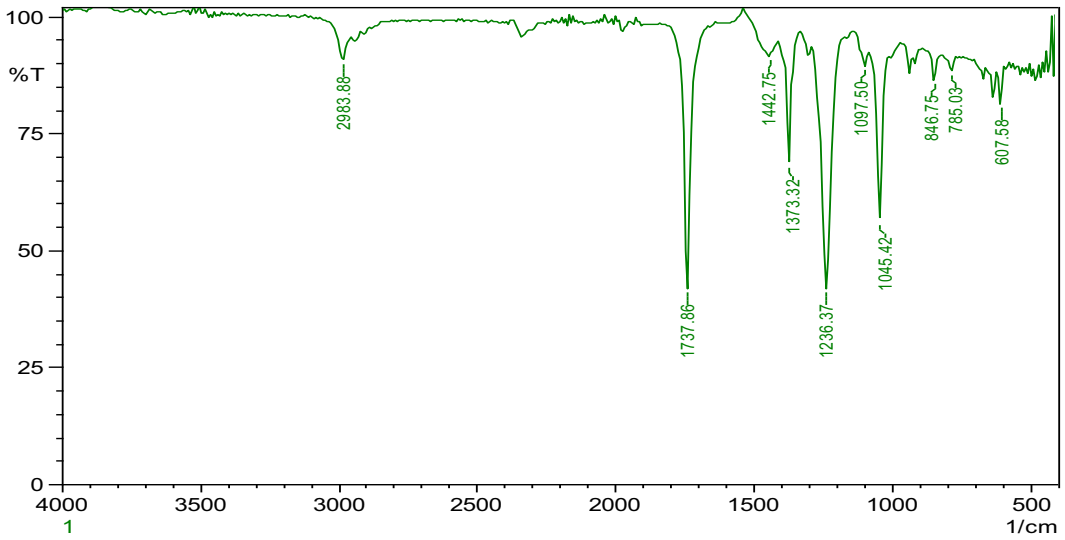


Figure 10. The FTIR spectrum of pigment extracted from *Streptomyces bellus* MSA1

4. Conclusion

Natural products represent a fundamental task in the spot of therapeutics. It is widely used to treat abundant diseases. Bioactive compounds and their related moieties have historically been incredible as a source of therapeutic agents. For the unique and typical molecules of microbial origin, world researchers are paying attention on marine sources for new & effective drugs. Most of researches are dedicated to explore novel bioactive molecules from actinomycetes. So the chances of finding rare species of actinobacteria are more which increases the scope of this study. This research has to be led to characterize the actinomycetes as potential bio active agents. There are so many antibiotics, bioactive metabolites and pigments are successfully extracted & commercialized. There are adorable biological types for the progress of conceivable drugs with precise cellular targets. Hence metabolites from marine actinomycete *Streptomyces bellus* explore the remarkable chemical diversity of nature molecules in the pursuance for new drugs.

Acknowledgements
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References

[1] Ali M, Keera A, Abeer, Helmy M, Samia A E H, Nadia K A, Ahmed and El-Hennawi H M 2011 *Res J Chem Sci* 1**5** 22-28.

[2] Dastager S G, Wen-Jun Li A, Dayanand, Tang S K, Tian X P, Zhi X Y, Xu L H and Jiang C L 2006 *African Journal of Biotechnology* **5** 11

[3] Mapari S A S, Nielsen K F, Larsen T O, Frisvad J C, Meyer A S and Thrane U 2005 Curr Opin Biotechnol **16** 109-238.

[4] Kim, Chang-Ho, Kim and Hong S I 1999 *Process Biochemistry* 35 **5** 485-490.

[5] Carvalho D, Cesar J, Soccol C R, Babitha S, Pandey A and Wojciechowski A L 2008 In Current *Developments in Solid-state Fermentation* (Springer New York) pp. 337-355 .

[6] Mapari S A, Nielsen K F, Larsen T O, Frisvad J C, Meyer A S and Thrane U 2005 Current Opinion in Biotechnology **16**(2) 231-8.

[7] Williams S T, Goodfellow M, Alderson G, Wellington E M, Sneath PH and Sackin M J 1983 *Microbiology* **129**(6) 1743-813.

[8] Saadoun I, Hameed KM, Moussauui A 1998 Microbios **99**(394) 173-9.

[9] Devi D K 2011 *J. Microbiol.Biotech. Res* **2** 74-79.

[10] Vasanthabharathi V, Lakshminarayanan R and Jayalakshmi S 2011 *African Journal of Biotechnology* **10** (54) 11224.

[11] Amsaveni R, Sureshkumar M, Vivekanandhan G, Bhuvaneshwari V, Kalaiselvi M, Padmalochana K and Preethikaharshini J 2015 J. Biosci. Nanosci **2(**2) 24-8.

[12] Velmurugan, Palanivel, Seralathan, Kannan K, Balachandar, Lakshmana P, Chae J C and Oh B K 2010 *Carbohydrate Polymers* **2** 262-268.

[13] Ramesh, Subramani, and Mathivanan N 2009 *World Journal of Microbiology and Biotechnology* **2** 2103-2111.

[14] Williams S T and Flowers T H 1977 *Microbios* **20** 99-106.

[15] Suthindhiran K and Kannabiran K 2009 *American Journal of Infectious Diseases* **5** 90- 98.

[16] Quadri, Raziuddin S and Agsar D 2012 *World J Sci Technol* **2(**2) 8-12.

[17] Priya, Jayaprakash A, Sagadevan E, Dhanalakshmi P, Kumar S S, Karthikeyan V and Arumugam P 2012 *Journal of Modern Biotechnology* **1** 63-69

[18] Rakshanya, Usha, Shenpagam H N, and Devi D K 2011 *J. Microbiol. Biotech. Res* **2** 74-79

[19] Palanichamy V, Hundet A, Mitra B and Reddy N 2011 *International Journal of Plant, Animal and Environmental Sciences* **1** 158-170.

[20] Wijittra A, Tanasupawat S, Amnuoypol S and Suwanborirux K 2006 *Thai J Pharm Sci* **30** 49-56.

[21] Alihosseini F and Sun G 2011 In *Functional Textiles for Improved Performance, Protection and Health* (Woodhead.391-Publishing Limited Cambridge UK) pp. 375

[22] Vasanthabharathi V R, Lakshminarayanan and Jayalakshmi S (2011) *African Journal of Biotechnology* **10** 54 :11224.