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REVIEW ARTICLE

Isolation, antibacterial testing from novel marine *actinomycetes*

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Abstract

This work was aimed at identifying marine *actinomycetes* and testing for their antibacterial properties. Water and sediment samples were collected from 10 different coastal regions of Visakhapatnam city, Andhra Pradesh, India. Hence, there were 125 actinomycete isolates using starch casein agar medium. These isolates were tested for antibacterial activity against 6 control organisms (*Bacillus cereus, B. subtilis, Staphylococcus aureas, Klebsiella pneumoniae, Pseudomonas aeruginosa and Escherichia coli* using cross-streak method. Out of the 125 isolates, 15 were active against all test bacteria, 52 were active against some of the test bacteria and 58 were not active against any of the test bacteria. The most promising isolates were subjected to secondary screening via the well diffusion method. Several experiments were done in order to establish the best environmental factors that would enhance the production of the antibacterial metabolite such as incubation time, temperature, pH, volume of inoculum, and carbon/nitrogen sources. Among all the isolate tested, the isolate J4 yielded the highest zone of inhibition at the optimised concentration. In this study, the authors establish that marine *actinomycetes* from coastal sediments could be a rich source of new antibiotics.

Keywords: Marine *actinomycetes*, Antibacterial activity, Optimization, Fermentation

Introduction

Originating from the Greek words "atkis," meaning "ray," and "mykes/mukes," meaning "fungi," the name "*Actinomycetes*" evolved. *Actinomycetes* are a kind of unicellular filamentous bacteria that produce spores and construct a filament network with several branches. Their role in imparting water's characteristic musty, earthy flavour and aroma has been known for some time. A huge colony, or mycelium, is the typical outcome of the filamentous and branching development patterns shown by the gram-positive, mostly anaerobic actinomycete. The G+C level of *actinomycetes* may range from low to high, with some having a level as high as 70% (Pudi et al. 2016).

Motility is provided by flagella when they are present. There are around a dozen suborders of *actinomycetes*. Many of these taxa are still waiting for a more firm taxonomy since their diversity has caused taxonomic instability. A combination of the Greek words atkis, meaning "a ray," and mykes, meaning "fungus," is formed by the term "*actinomycetes*" (Salim et al. 2017). Like fungus and bacteria, they share some traits. What distinguishes them from bacteria is the presence of filamentous hyphae. In terms of overall shape, they look a lot like fungus. Most people believe they have a closer relationship to microbes. The Actinomycetales are an organized group of *Actinomycetota*. A member of

the order is often referred to as an *actinomycete*. Many *actinomycetales* are gram-positive, anaerobic, and characterized by mycelia that form in a filamentous and branching manner (Phongsopitanun et al., 2019; Rozirwan et al., 2020).

As mentioned before, *actinomycetes* play an essential role in the soil's decomposition of various organic substances. In the agricultural sector, this is crucial since they help recycle elements that plants can use (Mohan et al., 2013; Attimarad et al., 2012). Furthermore, they produce an extensive array of enzymes that have several practical applications. Beneficial bioactive compounds produced by these organisms are a key ingredient in many pharmaceuticals, including antibiotics, antifungals, and antiparasitic drugs (Basha et al., 2009; Ganesan et al., 2017).

Molecules like glucose are hydrolysed during fermentation, an anaerobic chemical reaction. Specifically, the foaming process that occurs during the production of alcoholic beverages (such as wine and beer) is known as fermentation, and it has been in use for at least 10,000 years. Usually, there are 3 phases of fermentation: primary, secondary, and conditioning, also called lagering (Janardhan et al., 2014; Acharyabhatta et al., 2013). All of the ethanol, flavour, and aroma molecules that are present in beer are produced during fermentation by yeast. Fermented food nutrients are simpler for the digestive system to process than non-fermented ones. As an example, the naturally occurring sugar in milk, lactose, is transformed into the simpler sugar's glucose and galactose by fermentation (Sharma et al. 2017).

Literature Review

Actinomycetes generated biosurfactant and other metabolites (George et al. 2012). This article suggests marine *actinomycetes* isolates may produce biosurfactants and are antibacterial and antifouling. *Actinomycetes* remain important due to their diversity and potential to develop novel bioactive chemicals. They appear to have an infinite supply of innovative compounds with medical potential.

The 10 mg/ml dosage caused inhibitory zones to be 9 mm–32 mm wide (Ogunmwonyi et al. 2010). Based on infrared spectra research, crude extracts may include terpenoid, long-chain fatty acid, and secondary amine derivative chemicals. "Suggest that marine *actinomycetes* on Nahoon beach might produce bioactive chemicals and novel drugs. To isolate *actinomycetes*, 101 marine samples were obtained from coastal and mangrove areas.

Objectives

- Isolation of marine *actinomycetes*.
- Screening of antibacterial activity
- Fermentation studies.

Research methodology

- Isolation of *actinomycetes*

Location and method of sampling

We took samples of marine silt from ten separate locations. The Visakhapatnam district in Andhra Pradesh is home to many beaches, including Tenneti and Rushikonda, where soil samples were taken. The collected samples were carefully transferred to the laboratory in sterile plastic bags and then stored at 4°C to ensure their integrity throughout travel (Tab.1).

Table 1: Location and method of sample collection.

Sample No.	Source	Place	Sediment texture and character
1	Shore (Depth-10 inches)	Tenneti Park area	Fine yellowish brown muddy sample
2	Shore (Depth- 5 inches)	Rushi Konda area	Fine brownish muddy sample
3	Shore (Depth- 10 inches)	Gangavaram area	Fine brownish muddy sample
4	Shore (Depth- 5 inches)	Jodugullapalem area	Grayish muddy sample
5	Shore (Depth- 10 inches)	Bheemili area	Dark blackish muddy sample

6	Shore (Depth- 15 inches)	Vuda Park area	Fine brownish muddy sample
7	Shore (Depth- 10 inches)	Appikonda area	Fine brownish muddy sample
8	Shore (Depth- 5 inches)	Yarada area	Grayish muddy sample
9	Shore (Depth- 10 inches)	R.K Beach area	Dark blackish muddy sample
10	Shore (Depth- 5 inches)	Sagarnagar Beach area	Dark blackish muddy sample

Actinomycetes' segregation

Prior to isolation, samples of marine and sediment materials were kept at 4°C. Isolation of *actinomycetes* was achieved by plating samples in appropriate dilutions. Unlike fungus and real bacteria, *actinomycetes* colonies stand out clearly on a petri dish. They have a dry surface, are compact, and have a leathery look that gives them a conical shape.

The samples were mixed in a 250 ml conical flask with 100 ml of sterile water, and then shaken on a rotary shaker for 30 minutes, with about 1g of each sample added. In order to reach the 10^{-6} threshold, the suspension in every flask was successively diluted. To prevent contamination from bacteria and fungi, respectively, the Starch Casein Agar plates were treated with rifampicin 2.5 µg/mL and cycloheximide 75 µg/mL. 50 milliliters of sterile molten starch casein agar medium was combined with 1 milliliter of each of the three samples' dilutions (10^{-1}, 10^{-3}, and 10^{-5}). The mixture was then put onto petri dishes and left to incubate at 28°C for 14 days. The *actinomycete* colonies that had developed on the SCA plates after 14 days were carefully removed and placed on SCA slants. They were then cultured at 28°C for another week. Isolates were chosen based only on their perceived differences from one another. To keep the isolates alive, they were subculturing them in SCA slants at regular intervals (Tab.2).

Table 2: Starch Casein Agar (SCA) medium composition.

Constituent	Concentration (g/L)
Soluble starch	10
Vitamin free casein	0.3
Potassium nitrate	2
Sodium chloride	2
Dipotassium haydrogen phosphate	2
Magnesium sulfate	0.05
Calcium carbonate	0.02
Ferrous sulfate	0.01
Agar	20
Ph	7.0 ± 0.2

We combined the isolates and discarded cultures that seemed visually similar in terms of aerial *mycelium* color, reverse color, soluble pigment, and colony texture. The samples yielded around four *actinomycete* isolates.

Antibacterial activity studies

We tested the collected isolates for their ability to kill germs. The following tab. 3 lists the test organisms used in investigations of antibacterial activity:

Table 3: An inventory of the creatures used as test subjects for the research.

S. No	Bacteria	Gram $^{-}$ve / $^{+}$ve
1	*Bacillus cereus*	Gram $^{+}$ve
2	*Bacillus subtilis*	Gram $^{+}$ve
3	*Staphylococcus aereus*	Gram $^{+}$ve

4	*Escherichia coli*	Gram ⁻ve
5	*Pseudomonas auroginosa*	Gram ⁻ve
6	*Klebsiella pneumonia*	Gram ⁻ve

Initial evaluation using the cross-streak technique

The antibacterial activity of the marine *actinomycete* isolates was tested using the cross-streak technique on agar plates that included equal parts nutrition agar and Starch Casein Agar (SCA). Inoculation was carried out at 28°C for 5 days after each plate had a central stripe of one isolate along the length of the plate." The test organisms were streaked in a direction perpendicular to the *actinomycete* culture's growth after 5 days. After 24 hours of incubation, the level of inhibition shown by each isolate against the test bacteria was recorded. A control plate was kept with the same media but without *actinomycete* streaking. This plate also had streaking from the test organisms.

Underwater fermentation

Studies on the generation of antibacterial metabolites were conducted using well sporulated slants of the isolates that were seven days old. 2 ml of sterile water was aseptically added to each slant. Then, using a sterile inoculating needle, the growth on the surface of the medium was scraped and mixed with 50 ml of production media. The mixture was then incubated at 28°C on a rotary shaker set at 180 rpm for duration of 7 days. The samples were then transferred to sterile centrifuge tubes and spun at 10,000 rpm for 20 minutes at 8°C to separate the culture filtrate, which was clear. The well diffusion technique was used to conduct an antimicrobial test using the transparent supernatant (Tab.4).

Table 4: Media composition for manufacturing.

Constituent	Concentration (g/L)
Sucrose	20
Malt extract	10
Yeast extract	4
Dipotassium hydrogen phosphate	5
Sodium chloride	2.5
Zinc sulfate	0.04
Calcium carbonate	0.4

Isolation and screening of *actinomycetes* sp.

Ten samples of marine dregs were collected from various depths and better spots along the Visakhapatnam coast in an effort to separate *actinomycetes* from the marine environment. The *actinomycetes* were isolated by plating the samples in suitable weakening on Starch Casein Agar (SCA) medium, which was supplemented with rifampicin 2.5 µg/ml and cycloheximide 75 µg/ml to prevent bacterial and parasite contamination, respectively. The samples were then incubated at 28°C for a duration of 14 days. After 14 days, SCA agar inclines were used to maintain *actinomycetes* states, which were carefully removed from the plates to avoid contamination by bacteria or parasites.

Provinces of actinomycetes are easily distinguishable on a microscopy plate from those of fungus and other real microbes. They tend to be conservative, harsh, and dry-skinned, giving them a cone-like look. The samples were combined, and populations that were visually comparable in terms of airborne mycelium colour, switch tone, soluble pigment, and state surface were eliminated. "A total of 125 actinomycete-containing segregates were isolated from 10 different marine silt tests (1, 2, 3, 4, 5, 6, 7, 8, 9, and 10). These segregates were then categorized into the following groups: A bunch (A1-A15), B bunch (B1-B15), C cluster (C1-C10), D batch (D1-D10), E group (E1-E10), F clump (F1-F10), G bunch (G1-G10), H clump (H1-H10), I batch (I1-I15), J cluster (J1-J5), K cluster (K1-K10), and L cluster (L1-L5)(Fig. 1).

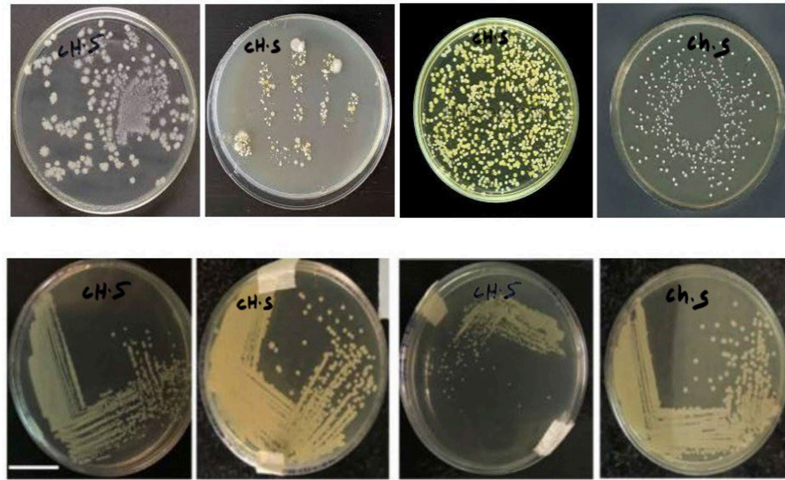

Figure 1: The SCA media is used to isolate *actinomycetes* from samples of sea sediment. These *actinomycetes* are characterized by their compact, leathery, dry-surfaced, and conical appearance.

Isolated marine *actinomycetes*' antimicrobial activity

Cross-streak method: Using the cross-streak method on supplement agar plates, the antibacterial mobility of each of the 125 marine *actinomycetes* species was assessed. For 5 days at 28°C, we striped each plate with an engagement in its center and then hatched the eggs. Actinomycete disengagements were followed by the streaking of bacterial test organisms in the opposite direction after 5 days (Mohseni et al. 2013). As a control, we retained plates with the same media but no *actinomycete* vaccination. However, we streaked the test organisms at the same time. After 24 hours of brooding, the test microorganisms of each *actinomycete* detached were examined for their binding strength. According to the results shown in tables, out of the 125 marine *actinomycetes* that were tested for antibacterial spectra using a cross-streak strategy, approximately 15 isolates were effective against all microorganisms, 52 isolates were effective against some, and 73 isolates showed no antibacterial activity at all.

All observations for the primary screening were done with the naked eye, and the following powers of impediment were noted: Each letter stands for a different zone: Normal, Good, and Excellent/Significant Inhibition. The following bacteria were used in the cross-streak antibacterial experiments: *Bacillus cereus (B. cereus), Staphylococcus aureus (S. aur), Bacillus subtilis (B. sub), Klebsiella pneumonia (K. pne), Pseudomonas auroginosa (P. aur), and Escherichia coli (E. coli)* (Tab. 5 and 6).

Table 5: Examination of a series of isolates' antimicrobial efficacy using the cross-streak technique.

A Series	*B. cereus*	*B. subtilis*	*S. aure*	*K. pne*	*P. aeru*	*E. coli*
			Inhibition Zone			
A1	N	N	G	N	N	G
A2	N	N	N	G	N	N
A3	G	N	N	N	N	N
A4	N	G	N	G	N	N
A5	G	N	N	N	N	G
A6	N	G	N	N	N	G
A7	N	N	N	N	G	G
A8	N	N	G	N	G	N
A9	G	E	E	E	G	E
A10	N	N	G	N	N	G

Table 6: The antimicrobial properties of the marine sediment isolates that were chosen using the cup-plate technique, measured in millimeters of inhibition zone.

Series	*B. cereus*	*B. subtilis*	*S. aure*	*K. pne*	*P. aeru*	*E. coli*
	Inhibition Zone Diameter (in mm)					
A9	20	24	27	32	20	29
B4	29	26	31	35	33	33
C6	18	22	26	30	27	20
D15	28	28	34	39	32	32
E7	16	15	25	18	22	22
E12	12	18	24	32	29	26
F1	19	22	22	30	27	24
F11	27	30	35	38	34	30
G3	22	26	21	31	23	28
H6	24	25	20	29	21	27
I2	26	28	16	22	20	22
J4	29	29	33	36	31	29
K7	28	26	28	26	29	26
L2	22	25	23	21	27	29

Antimicrobial activity of fermentation research using submerged fermentation

In order to determine the optimal bactericidal conditions, optimization experiments were conducted. Based on the extent of the zone of inhibition, we were able to determine how effective the optimized conditions were.

The trials were designed to ensure that the parameter optimization from one experiment could be transferred over to the next. The findings were presented as the mean of three independent experiments. Examining the study's findings in the context of previous research.

Implications of incubation term

Using four distinct B4, D15, F11, and J4 isolates, we tested the impact of fermentation duration on antibacterial activity during incubation durations ranging from 1 to 8 days. On the 6th day, bacteria Bacillus cereus was most effectively inhibited by isolate J4, with a 20 mm inhibition zone.

The organism's development rate may have slowed after an ideal incubation period because nutrients were quickly depleted. After 6 days of brooding in an environment with a 20 mm obstruction zone, the production of optional metabolites (antibacterial) will be consistent (Syarifuddin et al. 2021). Extensive study has shown that antibacterial activity increases with increasing incubation time from days 2 to 6 days, and then gradually decreases when incubation time is increased further (Tab.7).

Table 7: Incubation duration and its impact on antimicrobial efficacy.

Isolate	Incubation time							
	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day
B4	9	10	12	13	14	15	11	10
D15	8	9	10	11	12	14	10	8
F11	10	12	13	14	15	16	12	10
J4	12	13	14	15	16	20	16	12

Implications of incubation heat

To evaluate the influence of temperature on antibacterial metabolite synthesis by submerged fermentation, a range of temperatures ranging from 26°C to 34°C were used. At 30°C, isolate J4 exhibited the highest antibacterial activity, with an inhibition zone of 22 mm (Tab.8).

Table 8: Cup plate technique for determining antibacterial production temperature.

Isolate	Temperatures (°C)					
	24°C	**26°C**	**28°C**	**30°C**	**32°C**	**34°C**
B4	10	12	15	17	15	12
D15	9	11	16	18	14	10
F11	10	11	15	19	16	13
J4	11	14	17	22	18	12

Prompt pH has an impact

The ideal starting pH for antibacterial activity was determined by adjusting the pH of the production medium to various levels (3.0, 4.0, 5.0, 6.0, 7.0, and 8.0) using 0.1 N NaOH/0.1 N HCL. Antibacterial activity of 24 mm was seen in isolate J4 at a pH of 7.0, according to the data. The growth and antibacterial production during submerged fermentation are affected by the initial pH of the production medium, which is a critical component. Starting at a pH of 3.0 and rising to 7.0 enhanced antibacterial activity, while additional increases in pH values reduced this activity. The generation of the active component, which altered the inhibitory action, was reduced when the starting pH was too high or too low (Tab. 9).

Table 9: Cup plate technique for determining optimal pH for antibacterial synthesis.

Isolate	pH					
	3	**4**	**5**	**6**	**7**	**8**
B4	9	10	11	13	15	13
D15	10	11	13	15	18	14
F11	11	13	15	17	20	16
J4	12	15	18	20	24	20

Impact of inoculum concentration

We employed a range of inoculum volumes (6×10^6 spores/ml) to find the optimal volume for highest antibacterial production: V^{-2}, V^{-4}, V^{-6}, V^{-8}, V^{-10} (%v/v). They were fermented and tested for antibacterial activity according to the standard protocol. Table show that the antibacterial activity of the J4 isolate was maximally inhibited by an inoculum volume of 6.0% v/v (V6-28 mm), indicating that the volume concentration of the inoculum had a substantial impact on the antibacterial activity. If the inoculum is too high, the biomass will be too much, and the product quality will suffer, but if it's too low, the biomass will be just right (Tab. 10).

Table 10: Volume of inoculum required to produce antibacterial agents as determined by the cup plate technique.

Isolate	Inoculum volume (% v/v)				
	V^{-2}	V^{-4}	V^{-6}	V^{-8}	V^{-10}
B4	16	18	20	18	15
D15	17	19	22	19	17
F11	18	21	25	22	19
J4	20	24	28	25	21

The impact of supplemental carbon

A 1.0% w/v concentration of each carbon source (sucrose, glucose, cellulose, maltose, and lactose) was added to the Production Medium (PM1) to ascertain the impact of the carbon sources on antibacterial activity. In the case of cellulose, the highest inhibition zone measured 29 mm (Tab. 11).

Table 11: Use of the cup-plate technique to identify carbon sources for antimicrobial synthesis.

Isolate (1% w/v)	Sucrose	Cellulose	Maltose	Lactose	Glucose
B4	14	20	18	17	16
D15	16	22	20	16	14
F11	19	25	22	19	16
J4	21	**29**	26	24	21

Cellulose with a diameter of 29 mm exhibited the highest antibiotic efficacy for isolate J4, according to the results. Cells rely on carbohydrates for energy and structural support. On a mannitol-containing medium, almost half of the 125 isolates exhibited excellent growth. Contrarily, most instances of development were severely stunted once cellulose was introduced to the basal medium.

Determining the ideal Cellulose content

Researchers looked examined how different concentrations of cellulose (1.0% w/v, 2.0% w/v, 3.0% w/v, 4.0% w/v, 5.0% w/v, 6.0% w/v, 7.0% w/v, and 8.0 %w/v) affected antibacterial production as cellulose was shown to be the best carbon source. The baseline production medium was supplemented with each of the aforementioned concentrations and subjected to further optimal physical conditions for incubation. We used the same method as before to find out how effective the J4 isolate was against bacteria (Charlies Louis et al. 1952). The findings show that the optimal concentration for maximal antibacterial activity was 3.0% w/v Cellulose (Tab. 12).

Table 12: Optimizing cellulose concentration for antimicrobial production via the cup-plate method.

Cellulose (%w/v)	Antibacterial activity (mean inhibition zone in mm)
1	22
2	25
3	30
4	26
5	23
6	21
7	20
8	19

The impact of supplemental nitrogen

In lieu of malt extract, the production medium was supplemented with nitrogen sources at a concentration of 1.0% w/v to find out how different nitrogen sources affected antibiotic synthesis." According to the results, the antibacterial activity of isolate J4 was highest when ammonium nitrate was used as the nitrogen source, as shown in tab. 13 with a 31 mm zone.

Table 13: The cup-plate technique for determining the source of nitrogen for antibacterial synthesis.

Isolate 1% w/v	Malt Extract	Tryptone	$(NH_4)_2SO_4$	$(NH_4)(NO_3)$
B4	17	18	20	22

D15	19	21	23	25
F11	21	23	25	27
J4	26	27	29	31

There were 0.2% (w/v), 0.5% (w/v), and 1.0% (w/v) concentrations of inorganic nitrogen sources used. In comparison to existing inorganic nitrogen sources, the new soil actinomycete diammonium phosphate exhibited the highest activity at 0.2% (w/v).

Conclusion

Marine sediment samples were gathered from several coastal sites in Visakhapatnam, Andhra Pradesh, with the aim of identifying *actinomycetes*. 10 samples of maritime silt yielded 125 *actinomycete* isolates. Using the cross-streak technique, these isolates were tested for their antibacterial activity against 6 different test species. Out of the total number of isolates tested, 15 shown action against all test bacteria, 52 against some bacteria, and 73 against none. A9, B4, C6, D15, E7, E12, F1, F11, G3, H6, I2, J4, K7, and L2 were the isolates that showed the greatest promise after additional testing utilizing the well diffusion technique.

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