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Isolation and Production of Antimicrobial Metabolite by Actinomycetes from Marine Sediments

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ABSTRACT: *Marine actinomycetes produce secondary metabolites potentially. Though many antibiotics are discovered till now, still some of the pathogenic organisms are showing resistance to the existing antibiotics. Actinomycetes are present in dry land and aquatic habitat. Present study deals with the collection of samples from 4 different places along the coast of Bay of Bengal near Visakhapatnam and Chirala, India. 15 isolates were obtained from the samples and all the isolates were screened for antimicrobial activity, out of which 7 isolates showed activity against 6 bacterial test organisms. Among 7 isolates, 4 isolates showed activity in secondary screening. The isolate C2 that showed broad antibacterial activity against test organisms was selected for further study. Four different types of production media were screened for optimum antibacterial production. The maximum antibacterial activity was obtained with PM1 (production medium 1) medium containing maltose, 2.0% w/v; casein, 2.0% w/v with inoculum age 7th day at pH 9.0, incubation temperature 32°C, 180rpm and 7th day of incubation time.*
Keywords: *Actinomycetes, Antimicrobial metabolite, Optimization and Screening.*

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I. INTRODUCTION

Actinomycetes are Gram positive aerobic bacteria, filamentous, spore forming with maximum G+C (57-75%) in their DNA [1]. Actinomycetes are well known to produce bioactive compounds with innovative structures [2]. They are present in dry land and aquatic habitat [3]. Marine actinomycetes have attracted nice attention because they have advanced distinctive substance and physiological properties for bioactive compounds production and fascinating pharmacologic properties which may not be noticed from dry land actinomycetes [4]. Almost all the marine bioactive compounds have victorious screened, isolated and morphologically explained which are started mainly from bacteria [5].

Antimicrobial agent is an agent that kills or inhibits the growth of microorganism. These are evolved from the microbes which are living in the stressful environment develop antibiotics to protect themselves from the predators [6]. Till now nearly 70% antimicrobial agents were produced from marine actinomycetes [7].

To increase the production of antimicrobial agents, not only cultural conditions but also fermentation medium would effect for the formation of the product may be directly or indirectly. There is an essential nutritional requirement for the antibiotic production because lesser nutrients lead to improper yields where as higher concentration of nutrients leads to catabolic suppression. In order to overcome this situation, medium must possess optimum conditions of different nutrients that support the formation of product.

The present study focused on isolation and screening of actinomycetes from marine sediment samples from Bay of Bengal at Visakhapatnam and Chirala, India and to optimize the process parameters for the maximum production of antimicrobial metabolite under submerged fermentation.

II. MATERIALS AND METHODS

2.1 Samples Collection

A total of four marine sediment samples from Bay of Bengal (Bheemili, Visakhapatnam and Ramapuram, Chirala) were collected and set aside in sterile containers for the systematic screening of actinomycetes. About 50g of each sample was collected from different regions at a depth of 20cm.

2.2 Isolation of Actinomycetes

Marine sediment samples were set aside at 4°C until isolation. Actinomycetes are isolated by planting out the samples in proper dilutions. About 5g of each of above sample was taken in a 250ml conical flask

containing 100ml of sterile distilled water kept on an orbital shaker for 24 hours. The sample suspension was diluted serially up to 10^{-10} folds. Isolation was carried out on starch casein agar (SCA) plates by pour plate method seeded with sample suspension of 1.0ml each and incubated at 28°C for 14days [8]. After 14days, actinomycetes colonies were isolated from different plates. The actinomycetes colonies, which appeared were transferred and incubated at 28°C for 14days and maintained on SCA slants and pure cultures at 4°C [9].

2.3 Screening of Actinomycetes

All the obtained isolates were screened for antimicrobial activity against Bacillus cereus NCIM 2155, Bacillus subtils NCIM 2010, Bacillus megaterium NCIM 2051, Staphylococcus aureus NCIM 5021, Escherichia coli NCIM 2067 and Pseudomonas aeruginosa NCIM 2143 were procured from NCIM, Pune.

The marine actinomycetes isolates were screened preliminarily by cross-streak method for antimicrobial activity on 1:1 ratio of SCA and nutrient agar plates. Isolates that exhibited a broad spectrum of antimicrobial activity were selected for secondary screening by well-diffusion method. Well sporulated isolates (7-10 days old) were used for the antibiotic production studies. 5ml sterile distilled water was transferred aspectically into each slant and the growth of the isolate on the surface of the medium was scrapped with sterile inoculating loop and transferred each into 45ml of production medium PM1 and incubated at 28°C on a rotary shaker at 180rpm for 5days. Then the samples were collected into sterile centrifuge tubes and centrifuged at 10,000rpm for 20min, at 4°C and clear culture filtrate was separated. The clear supernatant was used for antibiotic assay using well-diffusion method. The antimicrobial activity against the bacterial organisms was tested on nutrient agar medium. The sterile nutrient agar medium was cooled to 40-45°C, inoculated with test organisms, mixed thoroughly then pour-plated and allowed them to solidify for 2h. Wells were made using sterile cork borer. The clear supernatant fermentation broth was added to each well (50µl) by using micropipette. The plates were kept in the refrigerator for about 2h for antibiotic diffusion and then incubated at 37°C. After 24h the inhibition zones were recorded [10].

2.4 Submerged Fermentation Studies

From screening, isolate which shows broad antimicrobial activity was selected to study further. These studies were carried out by selecting 4 types of production medium (PM1, YPG, MNGA and MHA). The spore suspension of the selected isolate was prepared by scraping with 5ml of sterile distilled water and transferred to 45ml of each medium contained in 250ml conical flasks and incubated on an orbital shaker at 28°C and 180rpm for 2days. The fermentations were carried out at 28°C for 5days on an orbital shaker at 180rpm and the antimicrobial activity was studied with the clear centrifuged samples by well-diffusion method. The productivity of the selected isolate was confirmed and the best production medium was selected and used further.

2.5 Growth Profile

Take 10 conical flasks containing 45ml suitable production medium is autoclaved and allowed to cool to room temperature. 5ml seed culture was transferred to the conical flasks. Incubate the conical flasks at 28°C in an orbital shaker at 180rpm for 1-10days. Daily measure the biomass dry weight of secondary metabolite from 1st day to 10th day.

2.6 Optimization Studies

Optimization is a process of determination of ideal conditions for the growth of the organism and formation of metabolites by the organism. The fermentation product yield is not only dependent on the nature of the strain and composition of the medium but also on cultural conditions [11-22]. To improve the yield of antibiotic in production various parameters were studied and optimized. The effect of incubation time was determined by incubating the inoculated flasks for 1-10 days and the antibacterial activity was estimated by well-diffusion method. The effect of temperature was studied by incubating from 28°C, 30°C, 32°C and 34°C. Influence of initial pH was studied by adjusting pH from 5 to 10 by varying one unit. Influence of inoculums age studied from 1st day to 9th day. To determine the effect of carbon and nitrogen sources on antibiotic production, different carbon and nitrogen sources were tested which include sucrose, glucose, lactose, starch, maltose, fructose, mannose and mannitol; organic nitrogen (malt extract, casein, peptone, tryptone, soya bean meal) and inorganic nitrogen (NH_4Cl, NH_4NO_3, urea) respectively.

III. RESULTS AND DISCUSSION

3.1 Isolation of Actinomycetes

A total of 15 actinomycetes were isolated from the three marine sediment samples and were designated as B, R and C. The no. of isolates obtained was shown in Table.1. The isolation plates were shown in Fig.1.

Table No.-1: No. of isolates obtained from marine sediment samples

SAMPLE	ISOLATE
B	8
R	5
C	2

Individual colonies from the isolation plates were picked up and streaked on SCA medium. Mixed colonies containing more than one culture were distinguished on the pure culture plates and incubated for 14days at 28°C and stored in refrigerator which can be further used as master cultures (Fig.2). The isolated strains were shown in slants (Fig.3).

3.2 Antimicrobial Activity Studies

All the 15 isolates were preliminary screened for antimicrobial activity. Among these 15 isolates, 7 isolates namely B3, B6, B8, R3, R5, C1 and C2 showed antimicrobial activity against B.cereus, B.subtils, S.aureus and E.coli (Table.2). These 7 isolates were sent to secondary screening by well-diffusion method. Out of these 7 isolates, 4 isolates namely B3, B8, R5 and C2 showed zone of inhibition against test organisms. Among these isolates, C2 showed broad antimicrobial activity (Fig.4) and maximum zone of inhibition of 16mm, 14mm, 10mm and 12mm against B.cereus, B.subtils, S.aureus and E.coli respectively (Table.3) and (Fig.5).

Table No.-2: Primary screening of isolates showing antimicrobial activity

Isolate	B.cereus	B.subtils	S.aureus	E.coli	B.megaterium	P.aeruginosa
B1	-	++	++	-	-	-
B2	-	-	-	-	-	-
B3	++	+	+	-	+	-
B4	-	-	-	-	-	-
B5	-	-	-	-	-	-
B6	+++	++	++	+	+	-
B7	+	+	-	-	-	-
B8	++	++	-	+	++	-
R1	+	+	-	+	-	-
R2	-	-	-	-	-	-
R3	+	++	-	-	+	-
R4	-	-	-	-	-	-
R5	++	++	+	+	-	-
C1	++	+	++	+	+	-
C2	+++	+++	++	++	+	+

Table No.-3: Secondary screening of isolates showing antimicrobial activity in terms of zone of inhibition

Isolate	B.cereus	B.subtils	S.aureus	E.coli	B.megaterium
	Zone of inhibition in mm				
B3	6	7	4	5	-
B6	-	-	-	-	-
B8	5	4	-	-	-
R3	-	-	-	-	-
R5	4	5	4	3	-
C1	-	-	-	-	-
C2	**16**	**14**	**10**	**12**	**-**

3.3 Submerged Fermentation Studies

From screening, isolate C2 which shows broad antimicrobial activity was selected to study further. These studies were carried out by selecting 4 types of production medium (PM1, YPG, MNGA and MHA). Among these four production media, PM1 medium showed a maximum zone of inhibition 16mm against B.cereus (Fig.6). Hence, PM1 medium was selected as the best production medium to study further.

3.4 Growth Profile

Take 10 conical flasks containing 45ml suitable production medium PM1 is autoclaved and allowed to cool to room temperature. 5ml seed culture of C2 was transferred to the conical flasks. Incubate the conical flasks at 28°C in an orbital shaker at 180rpm for 1-10days. Daily, measure the biomass dry weight of secondary metabolite from 1st day to 10th day. The growth curve of biomass concentration of C2 is shown in Fig.7. The maximum biomass concentration of C2 (dry weight) is 2.567g/l obtained on 7th day.

3.5 Optimization of Process Parameters

1. Effect of Incubation Time

The effect of incubation time on antibacterial activity was determined by carrying out the fermentation at different incubation time till 10th day. The fermentation was carried out at 28°C, pH 7 at 180rpm. C2 showed optimum incubation time on 7th day with the zone of inhibition of 18mm against B.cereus (Fig.8). The result indicate that a gradual increase in antibacterial activity was observed with increase in the incubation time from day 2 to day 7 and further increase in incubation time resulted in gradual decrease of antibacterial activity (Table.4). The decrease of incubation time was might be due to the depletion of nutrients available [23]. Similar result was reported by Gaurav V. Sanghvi [24], that optimum incubation time was obtained on 7th day for the antimicrobial production.

Table No.-4: Effect of Incubation Time on Antimicrobial Activity

Incubation time (days)	**B.cereus**	**B.subtils**	**S.aureus**	**E.coli**
	Zone of inhibition in mm			
1	-	-	-	-
2	12	6	9	7
3	-	-	-	-
4	-	-	-	-
5	15	8	12	15
6	-	-	-	-
7	**18**	**16**	**15**	**17**
8	17	15	11	7
9	-	-	-	-
10	-	-	-	-

2. Effect of Incubation Temperature

With the optimized incubation time, the selected C2 isolate was subjected to fermentation with the temperatures from 28°C to 34°C with 2°C temperature variation. Maximum antibacterial activity was obtained at 32°C against B.cereus with a zone of inhibition of 19mm (Fig.9) and (Table.5). The results were good agreement with Mangamuri [25] they stated that actinomycetes appear to be mesophilic. Similar results were depicted in Siva kumar [26], reported that maximum antibiotic yield was obtained at 30ºC with biomass of 3.6mg/ml.

Table No.-5: Effect of Incubation Temperature on Antimicrobial Activity

Temperature (°C)	**B.cereus**	**B.subtils**	**S.aureus**	**E.coli**
	Zone of inhibition in mm			
28	17	13	9	11
30	17	15	11	14
32	**19**	**18**	**14**	**17**
34	14	16	10	13

3. Effect of Initial pH

The initial pH of the production medium is an important factor which affects the growth and antibacterial production during submerged fermentation. The hydrogen or hydroxyl ion concentration may have a direct effect on cell or it may act indirectly by varying the degree of dissociation of substances in the medium [26]. The effect of initial pH of the medium was studied varying the pH range of 5.0 to 10.0. The results indicate that the antibacterial activity increased and attained maximum with increase in the initial pH of the medium from 5.0 to 9.0; and further increase in pH decrease the antibacterial activity (Table.6). The C2 isolate showed optimum pH 9.0 with the zone of inhibition of 19.5mm against B.cereus (Fig.10). Similar result of 9.0 as optimum was reported by Gaurav V. Sanghvi [24] for the production of antimicrobial metabolite.

Table No.-6: Effect of Initial pH on Antimicrobial Activity

Incubation pH	**B.cereus**	**B.subtils**	**S.aureus**	**E.coli**
	Zone of inhibition in mm			
5.0	-	12	10	11.5
6.0	-	13	11.5	-
7.0	16	14.5	13	14
8.0	18	16	14.5	15
9.0	**19.5**	**19**	**16**	**18**
10.0	17.5	17	14	16

4. Effect of Inoculum Age

The effect of inoculums age on antimicrobial metabolite production was studied by varying age of inoculums from 1st day to 9th day. All the above optimized conditions were maintained during fermentation process. Antibacterial production was obtained from 2nd day old culture to 7th day old culture but the antibacterial production was discontinuous. Maximum antimicrobial production was obtained on 7th day with 7 days old culture (Table.7) with a zone of inhibition of 20mm against B.cereus (Fig.11).

Table No.-7: Effect of Inoculum Age on Antimicrobial Activity

Inoculums Age (Days)	B.cereus	B.subtils	S.aureus	E.coli
	Zone of inhibition in mm			
1	-	-		
2	18	16	13	9
3	-	-	-	-
4	15	13	15	11
5	19	14	13	15
6	-	-	-	-
7	**20**	**19**	**17**	**18**
8	-	-	-	-
9	-	-	-	-

5. Effect of Carbon sources

The exogenous addition of various carbon sources to media may improve cell growth and antibiotic production [5, 27, 28]. To determine the effect of carbon sources on antibiotic production, different carbon sources were tested which include sucrose, glucose, lactose, starch, maltose, fructose, mannose and mannitol. Each carbon source was incorporated at 0.1% w/v level into production medium (PM1) in place of sucrose. Maltose has commonly been observed to repress the synthesis of enzymes that are required for antibiotic production. But that does not appear to happen in this case. The results indicate that C2 showed highest antimicrobial activity with the zone of inhibition of 22mm against B.cereus (Fig.12) when maltose was supplemented in the medium. Addition of other sources to the medium also favoured the antibacterial production but the activity was less when compared with maltose (Table.8). The result was similar with the Sunitha [29], reported that monosaccharide were suitable sources for the growth of actinomycetes and for production of bioactive metabolite.

Table No.-9: Effect of Carbon Sources on Antimicrobial Activity

Isolate	B.cereus	B.subtils	S.aureus	E.coli
	Zone of inhibition in mm			
Sucrose	20	17.5	18	17
Glucose	20	21	18	19
Lactose	21	18	17.5	17
Starch	19	19	18	17
Maltose	**22**	**20**	**19**	**19.5**
Fructose	20	18	17	19
Mannose	20	17	18	17.5
Mannitol	19	16	17	18

6. Optimization of selected carbon source (maltose) concentration

As maltose was found to be the suitable carbon source for the antibacterial activity, the effect of various concentrations (ranges 0.05%, 0.1%, 0.5%, 1%, 2%, 3% and 4%) of maltose on antibacterial production was studied. Each of the above concentration was incorporated into the PM1 production medium and incubated at 32ºC for 7days at 180rpm. The results indicated that the medium containing 2.0% of maltose was found to be maximum (Table.10) with a zone of inhibition of 25mm against B.subtils (Fig.13). Similar to my results, Venkata [10] got maximum antibacterial production with 2% D-glucose for Amycolatopsis alba var. nov. DVR D4 strain and further stated that, the increase or decrease of concentration of glucose showed reduced activity.

Table No.-10: Optimum of selected carbon source (maltose) concentration

Isolate	B.cereus	B.subtils	S.aureus	E.coli
	Zone of inhibition in mm			
0.05%	19	18	17	18
0.1%	22	20	19	19.5
0.5%	21	19	20	18
1%	23	22	20	18
2%	**22**	**25**	**21**	**20**
3%	21	22	19	18
4%	19	20	18	16

7. Effect of Nitrogen supplements

The following organic and inorganic nitrogen supplements were tested: malt extract, casein, peptone, tryptone, soya bean meal, and ammonium chloride, ammonium nitrate, urea respectively. Each nitrogen supplement was incorporated at 0.1%w/v level into the production medium PM1. The fermentation and evaluation of their antibacterial activities were carried out as per the general procedure. The maximum antibacterial activity was observed with casein as a supplement against B.subtils with a zone of inhibition of 29mm (Fig.14) and (Table.11). Usha [25] stated that nitrogen sources are important for the production of bioactive metabolite by microorganisms. Changes in the nature and concentration of nitrogen sources seem to affect antibiotic biosynthesis in different organisms. Streptomyces gulbargeneis DAS 131 produced maximum secondary metabolite production in the culture medium containing soya bean meal, it also enhanced the biomass and bioactive metabolite production.

Table No.-11: Effect of Nitrogen supplements on Antimicrobial Activity

Nitrogen supplements	B.cereus	B.subtils	S.aureus	E.coli
	Zone of inhibition in mm			
Malt extract	21	25	22	20
Casein	**27**	**29**	**25**	**20**
Peptone	18	23	20	21
Tryptone	20	22	21	22
Soya bean meal	23	24	20	21
NH4Cl	24	20	19	20
NH4NO3	26	27	21	23
Urea	20	21	23	22

8. Optimization of selected Nitrogen supplement (casein) concentration

Casein as a nitrogen source when supplemented in PM1 medium produced maximum antibacterial activity. The following concentrations of casein were investigated to determine the optimum concentration for maximum antibiotic production (%w/v) 0.1, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0% (Table.12). The results indicated that antibacterial activity was increased at 2.0% w/v concentration of casein and further increase in concentration decreased the antibacterial activity. Maximum antibacterial production was obtained against B.cereus and the zone of inhibition was found to be 35mm diameter (Fig.15). Yu [30] and Vahidi [31] reported that out of both organic and inorganic nitrogen sources, maximum antibiotic production was found in the medium containing yeast extract (1.5%) as nitrogen source.

Table No.-12: Optimization of Nitrogen supplement (casein) concentration

Casein concentration (%w/v)	B.cereus	B.subtils	S.aureus	E.coli
	Zone of inhibition in mm			
0.1%	27	29	25	21
1%	28	31	27	20
2%	**35**	**23**	**29**	**25**
3%	21	23	20	19
4%	18	19	16	15
5%	16	15	13	11

IV. FIGURES

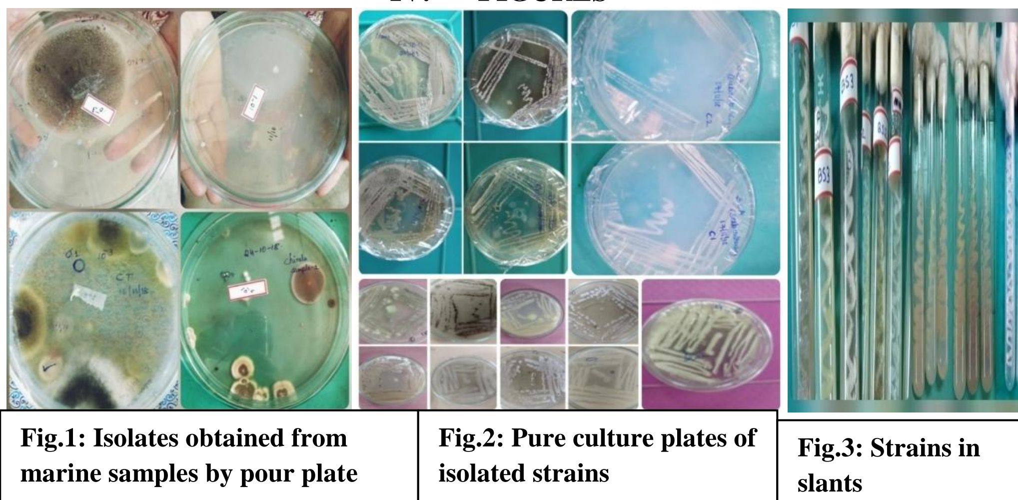


Fig.1: Isolates obtained from marine samples by pour plate

Fig.2: Pure culture plates of isolated strains

Fig.3: Strains in slants

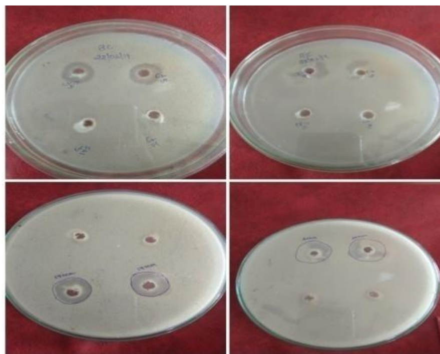
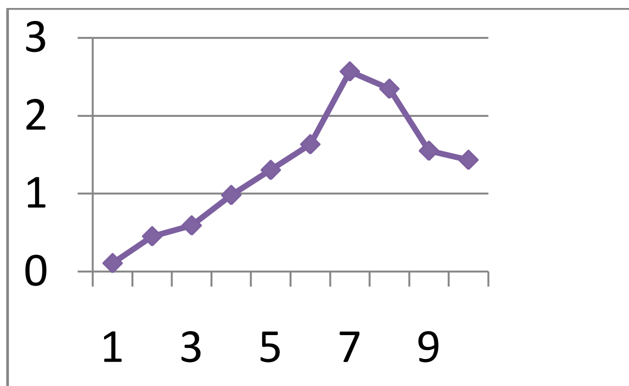


Fig.4: Cross-streak method of C2	**Fig.5: Well-diffusion method of C2**

Fig.6: Fermentation studies of C2 & showing zone of inhibition



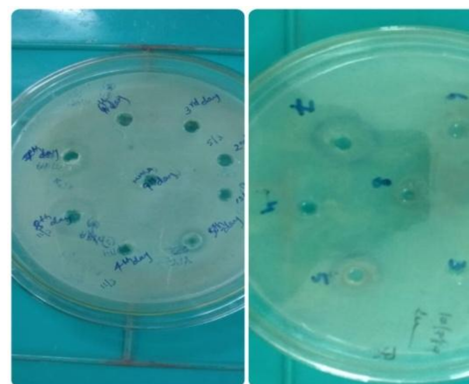


Fig.7: Growth Profile of C2	**Fig.8: Incubation Time**

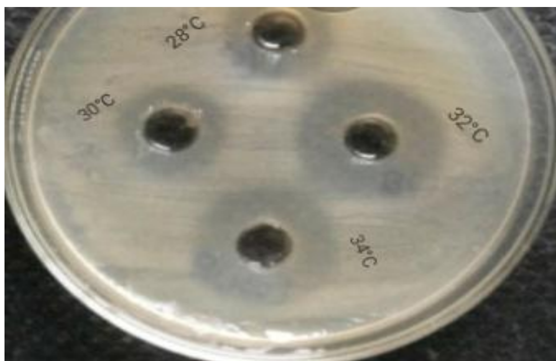


Fig.9: Incubation Temperature

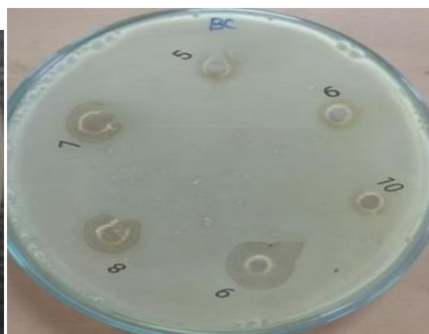


Fig.10: Initial pH

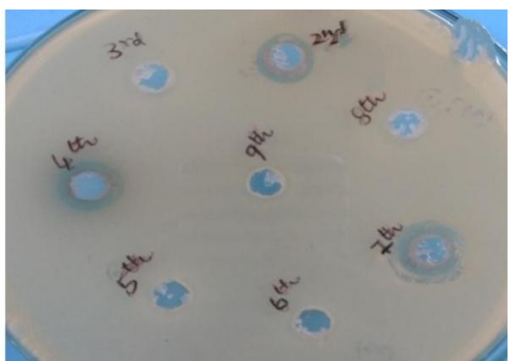


Fig.11: Inoculum Age

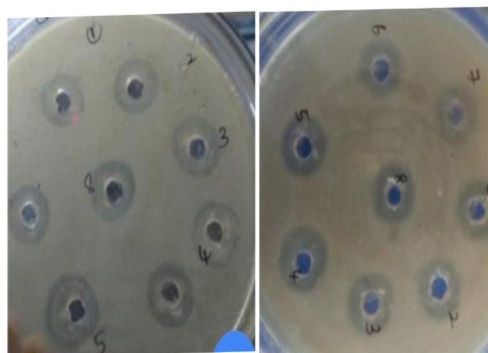


Fig.12: Effect of Carbon sources

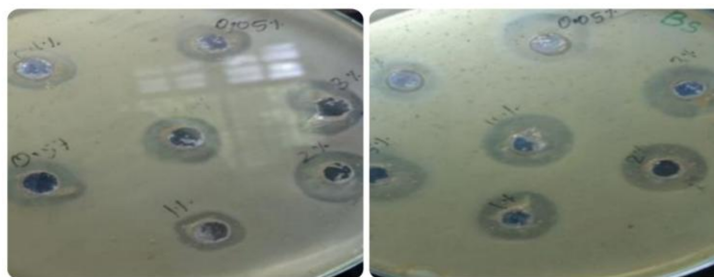


Fig.13: Maltose concentration

Fig.14: Effect of Nitrogen supplements

Fig.15: Optimized Casein concentration

V. CONCLUSION

Marine sediment samples from Visakhapatnam and Chirala coast of Bay of Bengal, India were investigated to isolate 15 actinomycetes. After preliminary screening, 7 isolates that showed activity against Gram-positive and Gram-negative bacteria by cross-streak method were selected. Of these, 4 isolates showing significant intensity of inhibition were selected for extracellular antibiotic production studies by submerged fermentation using well-diffusion method. Among the 4 active isolates, isolate C2 showed potential antibacterial activity against Gram-positive and Gram-negative bacteria. The maximum activity of the antibiotic was achieved by the optimized production medium containing maltose 2.0% w/v; casein 2.0% w/v for 7th day inoculum age at pH 9.0 for 7 days at 32°C. This study indicates that the marine environment is a good source for the isolation of actinomycetes and C2 is a potential candidate for exploitations after a comprehensive study.

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