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Invitro Antibacterial Activity of Coral Reef Associated Bacteria and Optimization of Bioactive Metabolite Production

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ABSTRACT

This study reports the antibacterial activity of coral reef associated bacteria against bacterial pathogens and optimization of metabolite production. Twelve morphologically different bacterial were isolated from stony coral reef collected from Rameshwaram coastal area, Tamil Nadu. Cell free supernatant of all the bacterial isolates were tested against *Staphylococcus aureus, Bacillus cereus, Salmonella typhi, Escherichia coli,* and *Klebsiella pneumoniae* by agar well diffusion method. Of the 12 isolates tested, seven isolates showed antibacterial activity again at least one of the test organisms with the zone of inhibition in the range of 9-14 mm. The antibacterial compounds were extracted from the cell free supernatant using ethyl acetate and tested for antibacterial activity. In diffusion method, crude extract from strain RC12 showed 11-17 mm inhibition against all the test pathogens and the same was selected for optimization studies. Effect of culture conditions and medium components such as different carbon, nitrogen, mineral, pH, temperature on antibacterial metabolite production was studied by adopting one-factor-at-a- time method. The metabolite production was influenced by lactose, ammonium sulphate, K_2HPO_4 while the optimal cultivation conditions were pH 7 and 37°C. The potent strain RC12 was identified as *Pseudomonas sp* based on their phenotypic and biochemical characteristics. The finding of the present study concludes that coral reef bacteria found to be the best source for bioactive metabolites with broad spectrum activity.

INTRODUCTION

Marine ecosystems harboring coral reefs are unique, biologically rich and promising resource for various bioactive principles. Reefs cover 0.2% of the ocean's area and yet they provide home to one-third of marine fishes and to tens of thousands of other species. There is increasing evidence that coral microbiota are crucial to at least two aspects of the host's physiology: biogeochemical cycling and pathogen resistance. It has also been hypothesized that coral-associated bacteria play a role in resistance to disease (Reshef *et al.,* 2006) via competition for nutrients and/or space, and/or production of antibiotics (Rohwer and Kelley, 2004). Several studies have demonstrated the antibacterial activity of isolates of coral mucus against human bacterial pathogens like *Escherichia coli*, *Staphylococcus aureus* and others) as well as potentially invasive microbes and putative pathogens of coral such as *Vibrio shiloi, V. coralliilyticus*, and *Serratia marsecens*) (Ritchie, 2006). The search for bio-active compounds extracted from coral reef invertebrates which is emerging as an area of increasing interest among biotechnological companies. Increasing observations suggest that a number of bio-active metabolites obtained from invertebrates are in fact produced by associated microorganisms: this has prompted research into the rapidly expanding field of study of metabolites derived from microorganisms associated with reef invertebrates. It is also expected that there is a still number of unexplored culturable coral associated microorganisms in the reef environment. Such information might be desirable, as some of these bacteria may serve beneficial purposes as a source of secondary metabolites including novel natural products such as antibiotics, lipids, pigments, and pharmaceuticals (Ritchie, 2006). Isolating bioactive compound producing bacteria is obviously offers a much better approach than cultivation and harvest invertebrates, which are in most cases extremely difficult (Radjasa *et al.,* 2009). Designing an appropriate fermentation medium and conditions is of crucial importance to

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improve the efficiency and productivity of bioactive microbial metabolites fermentation process, because it can significantly affect product concentration, yield, and the ease and cost of downstream product separation (Wang and Lu, 2004). Hence an attempt is made to exploit coral reef bacteria for bioactive metabolites and to optimise the medium and cultural conditions for production.

MATERIALS AND METHODS

Collection of sample

Hard corals samples were collected in Rameshwaram coasts, Tamil Nadu (9° 17' N Latitude 79° 22 ' E Longitude) Precautionary measures were strictly followed to minimize contamination through sampling and handling. After collection, all the samples were transferred to the laboratory in an ice container at 4°C and bacteriological analysis were made immediately.

Isolation of coral reef bacteria

The coral sample was washed three times with sterile seawater to remove loosely attached bacteria. The sample was then placed in another 100 ml of sterile seawater and homogenized by shaking at 200 rpm for 15 minutes. Hundred microlitres of aliquot from serially diluted sample was transferred on nutrient agar plates supplemented with 2% NaCl. All the plates were incubated at 28°C for 5 days. After incubation morphologically different bacterial colonies were selected, purified and subcultured on nutrient agar medium supplemented with 2% NaCl (Uzair *et al.*, 2009). All the medium used to grow coral associated bacteria were supplemented with 2 % NaCl.

Primary screening for antibacterial activity

For the production of bioactive metabolites, 5% of bacterial inoculum prepared in nutrient broth was aseptically transferred into fermentation medium consists of (gm/l) starch-20.0; sucrose-15.0; soyabean meal-20.0; yeast extract-5.0; calcium carbonate- 3.2; magnesium sulphate- 2.5; dipotassium hydrogen phosphate-5.0; manganese chloride- 0.2; sodium chloride- 20.0; ferrous sulphate- 0.3, pH-7. All the flasks were incubated at 28°C for 4 days. The cell free supernatant from all the flasks was prepared by centrifugation at 10,000 rpm for 10 minutes. Antibacterial activity was determined by loading 50 µl of cell free supernatant into 5 mm diameter well on Muller Hinton Agar (MHA) plates (Chellaram *et al.*, 2011). Test organisms used in this study include *Staphylococcus aureus* NCIM 2079, *Salmonella typhi* NCIM 2263, *Bacillus cereus* NCIM 2016, *Escherichia coli* NCIM 2256¸ *Klebsiella pneumonia* NCIM 2706 were obtained from National collection of industrial microorganisms (NCIM), NCI, Pune.

Production and extraction of bioactive metabolite from promising isolates

Based on the results of antagonistic activity, four isolates were selected for further investigation. Bioactive substances from selected bacterial isolates were produced through submerged fermentation by adopting shake flask method. About 5% of bacterial inoculums were transferred into each 100 ml of fermentation broth and incubated in rotary shaker with 95 rpm at 28^0C for 4 days. Totally 1000ml of fermentation medium was used for the production of bioactive metabolites from each isolates. After fermentation, the cell free supernatant was separated by centrifugation at 10,000 rpm for 10 minutes (Awais *et al.*,2007). Crude extract from cell free supernatant was prepared by liquid-liquid extraction method using equal volume of ethyl acetate (1:1) in the separating funnel for 24 hours. The organic layer was collected and evaporated to dryness in vaccum evaporator at 40°C.

Secondary screening for antibacterial activity

The crude extracts were quantified using preweighed eppendorf tubes and dissolved in ethyl acetate in order to get 1mg/ml concentration for antimicrobial studies. Sterile filter paper discs with 5 mm in diameter were impregnated with 50µl (50µg/disc concentration) of crude extract and allowed to dry under aseptic conditions. The discs were placed over the MHA plates previously inoculated with test organisms. Zone of inhibition was measured after 24 hours of incubation at 37^0C. One out of four isolates, which showed maximum zone of inhibition was selected for further studies (Hosny *et al.*, 2011).

Optimization of antimicrobial compound

Effect of critical medium components on antimicrobial compound production was studied by adopting classical one factor at a time method. Factors which are studied include pH, carbon source, nitrogen source, minerals, temperature, and incubation period (Kelman *et al.*, 2006).

Identification of potential strain

Phenotypic characteristics such as micromorphology (gram staining, capsule staining endospore staining and motility), cultural characteristics (on basal media, differential media and selective media), and biochemical characteristics (catalase, oxidase, IMViC) of the selected isolates was studied by adopting standard procedures. Based on the results of studied phenotypic characteristics the potential bacterial strain was identified with the help of Bergey's Manual of Determinative Bacteriology.

RESULT AND DISCUSSION

The need for discovery of novel antibiotics is imperative because evidence suggests that development and spreads of resistance to any new antimicrobial agents is inevitable. Many of the reports on antimicrobial activity of extracts of marine organisms and the subsequent purified antibiotics isolated from these organisms were tested against human pathogens as potential novel clinically useful drugs. Little is known on the antimicrobial activity of other corals, especially reef-building stony corals. This is rather surprising, considering that these organisms are the most dominant and conspicuous members of many reefs (Uzair *et al.*,

2009). Orland and Kushmaro, 2009 stated that stony corals produced the highest percentage of active bacterial isolates (25%) when compared with soft corals (13%). Hence in the present stony corals was selected for the exploitation of antibacterial compound.

Isolation and primary screening of coral reef bacteria for antibacterial activity

In our study totally 12 morphologically different bacterial colonies were selected from isolation agar. Similar to this, Hardner *et al.,* 2003 isolated eleven bacterial morphotypes from the coral surface and identified as species of the genus *Flexibacter, Pseudoalteromonas, Ruegeria, Tenacibaculum* and *Vibrio*. Orland and Kushmaro, 2009 stated that over 20% of cultivable bacteria isolated from mucus demonstrated activity against indicator bacteria. In our study, of the 12 isolates tested 7 isolates showed antibacterial activity again at least one of the test organism with the zone of inhibition ranged between 9 and 14 mm in diameter (Table 1). Based on the primary screening results, four coral reef bacterial strains viz., RC1, RC3, RC6 and RC12 were selected for further studies. Marine microbes have a higher possibility of yielding natural products with unprecedented and interesting bioactivity. The antagonistic marine bacteria isolated from the corals may produce antibacterial potential compounds with novel structures which can be explored to generate pronounced biological activity in the future (Awais *et al.*,2007).

Table. 1: Primary screening of antimicrobial activity.

Strain	*S. aureus*	*S. typhi*	*B.cereus*	*E. coli*	*K. pneumoniae*
RC1	13	14	-	-	-
RC2	-	-	-	-	
RC3	12	-	-	11	-
RC4	-	-	-	-	-
RC5	-	-	-	-	-
RC6	12	-	-	10	-
RC7	-	-	-	11	-
RC8	-	-	-	-	-
RC9	-	-	-	-	-
RC10	-	-	13	-	-
RC11	-	-	10	-	-
RC12	12	14	11	10	9

*zone of inhibition expressed in millimeter in diameter

Table. 2: Antibacterial activity of crude extracts prepared from coral reef associated bacteria.

Strain	*S.aureus*	*S.typhi*	*B.cereus*	*E. coli*	*K. pneumoniae*
RC_1	14	12	--	--	--
RC_3	15	--	--	14	--
RC_6	14	--	--	14	--
RC_{12}	16	17	13	12	11

Production and extraction of bioactive metabolites

The secondary screening for the antibacterial activity was studied by disc diffusion method. Goh and Chou, 1998 showed that the average inhibition diameters of gorgonian coral extracts against *B.subtilis* , *E.coli* and the yeast *Sacchromyces cerevisiae* was found to be 9.8 mm, 10.4 mm and 14.4 mm respectively. In the present study the extracts of selected four coral reef bacterial strains showed activity against *Staphylococcus aureus* NCIM2079, *Salmonella typhi* NCIM 2263, *Bacillus cereus* NCIM 2016, *Escherichia coli* NCIM2256¸ *Klebsiella pneumonia* NCIM2706 with the zone of inhibition ranged between11 and 17 mm (Table 2). Among the four strains, RC12 showed maximum activity against all the test pathogens and hence it was selected for optimization studies.

Effect of carbon source

The synthesis of antibiotics can be influenced by manipulating the type and concentration of nutrients formulating the culture media. Among them, the effect of the carbon source has been the subject of continuous studies for both industry and research groups (Sanchez *et al.,* 2010). Of the various carbon sources tested, medium supplemented with 1% lactose showed maximum of 19 mm zone of inhibition. In contrast to our studies the results indicated that glucose influenced the antimicrobial substance production of *Bacillus licheniformis* NRC-18 (Kelman *et al.,* 2006).

Effect of nitrogen source

As like carbon sources, nitrogen source was also an important factor for the biosynthesis of antibiotics by any microorganism. In some of the previous studies, it was found that the inorganic nitrogen sources were weakly supported both antimicrobial production and cell growth. On the other hand, the addition of some complex nitrogen sources resulted in a significant increase in antimicrobial production (Sole *et al.,* 1997). In the present study, of the different nitrogen sources such as ammonium sulphate, potassium nitrate, ammonium chloride and peptone were evaluated, medium supplemented with inorganic nitrogen source ammonium sulphate showed maximum zone of inhibition of 17 mm. In contrast to our studies Pavl and Banerjee, 1983 concluded that the organic nitrogen sources produce fair amounts, of antibiotic showing very little variation in quantity but the biomass production showed a wide variability.

Effect of mineral source

Among the minerals tested, K_2HPO4 showed positive effect on antibiotic production followed by NaCl, $MgSO_4.7H_2O$, and KCl. K_2HPO_4 enhanced the production of bioactive metabolites with the zone of inhibition of about 17mm. In contrast, the production of bioactive metabolites was very low with $MgSO_4.7H_2O$, and KCl. Majumdar and Majumdar, 1965 reported maximum yield of neomycin by *Streptomyces fradiae* with K_2HPO_4 and least with $ZnSO_4$. Similar results have been recorded by Ripa *et al*., 2009 and Narayana and Vijayalakshmi, 2008.

Effect of pH

Changes in external pH affect many cellular processes like the regulation of the biosynthesis of secondary metabolites (Sole *et al.,* 1997) . In the present study, highest inhibition zone (17 mm) was exhibited by the medium adjusted with the initial pH 7.0. Similar results were obtained by Chang *et al.,* 1991 and Yousaf, 1997 who reported that the optimum for bacitracin yield from *B. licheniformis* was obtained with initial pH of 7.0.

Effect of temperature

The biosynthesis of antimicrobials was strongly affected by different incubation temperatures. In the present study, the fermentation carried out at 37°C was found to produce maximum of 16 mm zone of inhibition. Early studies by Berdy, 1974 have shown that maximum titers of bacitracin were obtained at incubation temperature of 37°C. Also, Awais *et al*., 2007 reported that maximum inhibition was observed by *Bacillus subtilis* and *Bacillus pumilus* strains at 30ºC against *S. aureus* and *M. luteus*.

Identification and characterization of potential strain

Based on the studied phenotypic characteristics, strain RC12 was identified as *Pseudomonas sp* RC 12. Radjasa *et al.*,2009 isolated a total of 13 bacterial isolates belonging to the genera *Arthrobacter* (1 strain), *Bacillus* (7 strains), *Micrococcus* (1 strain), *Pseudoalteromonas* (1 strain), *Pseudovibrio* (1 strain), and *Vibrio* (2 strain), were successfully for their inhibitorial effect against at least 1 test strain. Nair *et al.*, 2011 reported that the most dominant group of antibacterial strains consisted of the *Pseudomonas/Alteromonas* isolates (81.0%) and *Vibrio* isolates contributed 16.3%. Gokulkrishnan *et al.*, 2011 isolated 21 isolates that was subjected to secondary screening, 10 isolates were active against *Bacillus subtilis*, 12 against *Staphylococcus aureus*, 6 against *Escherichia coli*, 3 against *Proteus vulgaris* and 4 against *Salmonella typhi*. In that study *Pseudomonas spp.* No.MN05 proved to have promising antimicrobial activity. Though there are marine *Pseudomonas sp* are reported to produce bioactive metabolites, it is a strain specific process rather than a species or genus specific process.Coral reef associated bacterial strain RC12 isolated in this study will be a potential source for bioactive metabolites. Isolation, characterization and biological evaluation of the coral reef bacterial metabolite from RC12 is needed to strengthen its potential further.

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