Research Article

Optimization of Culture Conditions for Production of Antibacterial Metabolite by Marine Bacteria

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ABSTRACT

The aim of the present study was to isolate and screen the antibiotic producing bacteria from marine soil samples and to optimize the various culture conditions to get high yield of the antibacterial metabolites against test pathogen. The most active strain which showed significant growth inhibition of test organism was identified as *Micrococcus* sp. AM7. The effect of various parameters viz., incubation time, temperature, pH, carbon and nitrogen sources, and sodium chloride concentration on antibacterial metabolite production were studied by varying single parameter at a time. It was found that metabolite production by this isolate was greatly influenced by various cultural conditions. The optimum carbon source sucrose, nitrogen source tryptone, pH 8.0, temperature at 40 °C, 1 % NaCl concentration and incubation period of 72 h were found for the maximum antibiotic production. Findings from this investigation revealed that the antagonistic bacterial strain, *Micrococcus* sp. AM7 control the growth of pathogenic bacteria, which was further enhanced by the optimization of culture conditions.

Keywords: Marine bacteria, Metabolite, Antibacterial activity, Optimization.

INTRODUCTION

Antibiotics are produced by bacteria as secondary metabolites and employed in wide range. This antibacterial metabolite includes a chemically heterogeneous group of small organic molecules of microbial origin that, at low concentrations, are deleterious to the growth or metabolic activities of other microorganisms[1]. Originally, an antibiotic is defined as a substance, produced by one microorganism[2], or of biological origin[3] which at low concentrations can inhibit the growth of other microorganisms or infectious organisms[4]. The isolation of antibiotics from bacteria is relatively safe as compared to chemical synthesis of antibacterial agents.

Research in finding newer antibiotics and increasing productivity of such agents has been a very important activity[5,6]. There are some parameters which affect the productivity of antibiotics, the nutritional factors such as carbon sources, nitrogen sources, inorganic salts with various environmental factors, temperature, pH, incubation period. Even small changes in the culture medium may not only impact the quantity of certain compounds but also the general metabolic profile of microorganisms[7]. In particular, in the field of antibiotics, much effort was directed toward optimizing production rates and directing the product spectrum. Manipulating nutritional or environmental factors can promote the biosynthesis of secondary metabolites and thus facilitate the discovery of new natural products[8].

The marine environment is becoming increasingly appreciated as an exceptional reservoir of bioactive natural compounds, which exhibit structural/chemical features not found in terrestrial natural products[9]. Marine bacteria are a rich source of potentially useful antibacterial molecules. It was known to produce antimicrobial metabolites have been reported like macrolactin F, 7-*O*-succinylmacrolactin F and 7-*O*-succinylmacrolactin A, (*Bacillus sp.* Sc026)[10], new thiopeptide compounds (*Bacillus cereus* QN03323)[11], and three bacteriocin-like peptides namely Lichenin, Bacillocin 490 and P40[12]. This investigation was undertaken to isolate antibiotic-producing bacteria from marine environment and making an assessment of its potentials by validating the effects of several factors for maximum antibacterial metabolite production.

MATERIALS AND METHODS

Collection of soil sample and isolation of bacteria

Soil samples were randomly collected from coastal area of Kanyakumari, Tamil Nadu. Samples were taken from 2-3 cm depth and collected in sterile polythene bag. The samples stored at 4˚C. The soil samples were serially diluted up to 10^{-9} dilutions, in distilled water and 1ml sample from 10^{-8} to 10^{-9} were pour plated in Nutrient Agar (NA) (Hi-Media, Mumbai, India) plates. The plates were kept for incubation at 37°C for 24 h in an inverted position. The bacterial isolates were purified by pure culture techniques and refrigerated in agar slants for further studies.

Test microorganisms and Inoculums preparation

To evaluate the antibacterial potential Gram-positive bacteria *Staphylococcus aureus* was procured from local hospitals and maintained on NA slant at 4°C. Test bacterial inoculum was prepared by growing cells in Nutrient Broth (NB) (Hi-Media, Mumbai, India) for 24 h at 37°C in a rotary shaker (200 rpm). This cell suspension

was diluted with sterile NB to provide cell counts of about 10^8 CFU/ml and used for antagonism study.

Screening of active bacterial isolates

Scratching method was used to screen the antibacterial producing active isolate *S. aureus* was used as test organisms. The test bacterial suspension was spread on solid Mueller Hinton Agar (MHA) (Hi-Media, Mumbai, India) plate using sterile swab aseptically and allowed to dry. Then pure isolated cultures were scratched on it and incubated for 24-48 h at 37°C. The isolates showed maximum positive potential antimicrobials, was screened and used for further test.

Identification of active isolates

The potent isolate selected was characterized by morphological and biochemical methods. The results of microscopic examination were compared with Bergey's manual of systematic bacteriology[13,14] and the organism was identified upto genus level. Various biochemical tests were performed for the identification of the potent isolates are as follows; Fermentation of sugars, Hydrolysis of starch, Indole production, Methyl red, Vogues-Prauskauer, Citrate utilization, Nitrate reduction test, Catalase test, Oxidase test.

Extraction of antibacterial metabolites

The antibacterial metabolites were obtained from the crude culture filtrate by solvent extraction method. The active strains were batch cultured in a rotary shaker at 200 rpm for 3 days at 37°C containing 50 ml of fermentation medium (pH 7) in 250 ml Erlenmeyer flasks. The production medium contained (g/l): glucose 10.0; NH_4NO_3 5.0; yeast extract 5.0; NaCl 5.0; K_2HPO_4 1.0; KH_2PO_4 1.0 and $MgSO_4$, $7H_2O$ 1.0. After the incubation, cultures were centrifuged and supernatant was used for extraction process. The crude metabolite was extracted using equal volume of ethyl acetate (1:1) using a magnetic stirrer for 1 h. The ethyl acetate containing active metabolite was separated in a separatory funnel and the ethyl acetate phase was removed and concentrated by evaporation in water bath at 80-90°C. The concentrate (crude extract) was used for the determination of antibacterial study against test organism.

Antibacterial assay

To determine the potentiality of the selected isolate, the antibacterial assay was done by agar well method[15]. The wells (6 mm diameter) were cut using a sterile cork on MHA plates. 24 h young fresh culture of test microorganism was swabbed with sterilized cotton swab on the surface of plates. 60 µl crude extract was loaded into each well and left for 30 min until the metabolite was diffused.

Then the plates were incubated for 24 h at 37 °C. After incubation, antibacterial efficacy of the identified strain was determined under varying conditions. The antagonistic activity of the antibiotic was evaluated by measuring the resulting diameters of zone of inhibition in millimeters.

Effect of different parameters on metabolite production

The effect of various culture conditions (both nutritional and environmental) on production of antibacterial metabolites by the antagonistic isolate was evaluated. The parameters studied included incubation period, pH, temperature, carbon source, nitrogen source, and sodium chloride (NaCl). All results presented are mean ± SD of triplicate analysis.

Effect of incubation period

Shake flask fermentations were run in 500 ml flasks containing 100 ml nutrient broth inoculated with active isolates. The flasks were incubated at 37 °C for optimum yields on a rotary shaker at 200 rpm for different time interval (12 h, 24 h, 36 h, 48 h, 72 h). At every interval, the flasks were harvested and the crude product was extracted. The antibacterial metabolites production determined in terms of their antibacterial spectrum against *S. aureus.*

Effect of pH and temperature

Influence of pH on antibacterial metabolite production of the strain was determined by adjusting the pH of production medium ranging from 5-10. The pH exhibiting highest antibacterial metabolite production was selected as optimum pH for further studies. Optimum temperature for antibacterial metabolite yield was determined by incubating the production medium at different temperatures varying from 25-45 °C maintaining all other conditions at optimum levels. The maximum inhibition zone exhibited by the isolate against test organism was taken as optimum.

Effect of carbon and nitrogen sources

To determine the influence of carbon sources on bioactive metabolites production, different carbon sources such as sucrose, D-fructose, D-sucrose, D-lactose, D-maltose, mannitol were used. The carbon source inducing the maximum yield of antibacterial compounds in terms of inhibition zone was selected for further studies. Similarly, the influence of nitrogen sources on antibiotic yield was investigated by different nitrogen sources like peptone, casein, beef extract, tryptone, potassium nitrate (KNO_3).

Further, the nitrogen source supporting optimal yield of antibacterial metabolites was selected for next studies. The concentration of carbon (1 %) and nitrogen sources (0.03 %) were taken in production medium.

Effect of sodium chloride

Salt concentration has a profound effect on the production of antibiotic from microorganism due to its effect on the osmotic pressure to the medium[16]. Optimum NaCl concentration for antibacterial metabolite

yield was determined by incubating the active bacteria the production medium at different concentration varying from 0.5-5.0 % with rest all other parameters at optimized.

RESULTS AND DISCUSSION

A variety of parameters influence the production of antibacterial metabolites by bacteria. Both nutritional and environmental parameters like carbon sources, nitrogen sources, inorganic salt concentration, fermentation time, temperature, pH etc have significant effect on production of active metabolites. Together with these parameters, the combination of media components also influences growth and metabolite production[17,18]. Therefore, to achieve high product level of yields, one prerequisite is to design and optimize the fermentation process which also helps to reduce fermentation costs.

Screening and identification of active isolates

Table 1: Morphological, physiological and biochemical characteristics of the isolated strain AM7.

Characters	Isolate AM7
Morphology	Cocci shaped, gram +ve, obligate aerobic and without endospore
Motility	-ve
Catalase	+ve
Oxidase	+ve
Methy red	+ve
Voges- Proskauer	-ve
Indole production	-ve
Citrate utilization	+ve
Nitrate reduction	-ve
Hydrolysis of starch	-ve
Fermentation with glucose, lactose, sucrose, mannitol	-ve

Screening of antibacterial metabolite producers were carried out by scratching method. In which the isolate showing maximum zone of growth inhibition of *Staphylococcus aureus* was selected and used for production study.

The bacterial isolated designated as AM7 was selected as good candidate for secondary metabolite production, based on its ability to form clear and large zone of growth inhibition of pathogen.

Finally the strain was identified at genus level with the help of Bergey"s manual of systematic Bacteriology. Table 1 revealed the morphological, biochemical characters of strain AM7 and it was identified as *Micrococcus* sp. AM7 which was used for rest of studies.

Optimization of culture conditions

The production of antibacterial metabolites by *Micrococcus* sp. AM7 was determined at different conditions and nutrients by well diffusion assay method measuring the zone of inhibition against *S. aureus*.

Effect of incubation period on metabolite production

Fermentation time determination for specific strain is of high importance for maximum harvesting of the metabolite because the incubation period required for growth and metabolite production varies significantly by different bacteria.

Antibacterial metabolites production by the strain was determined after 24 h of incubation and till 5th day of fermentation was analyzed.

The highest level was obtained on 3rd day of incubation and then production was declined gradually (Figure 1). Thus the organism was allowed to incubate for 72 h for the maximum production of antibacterial metabolites.

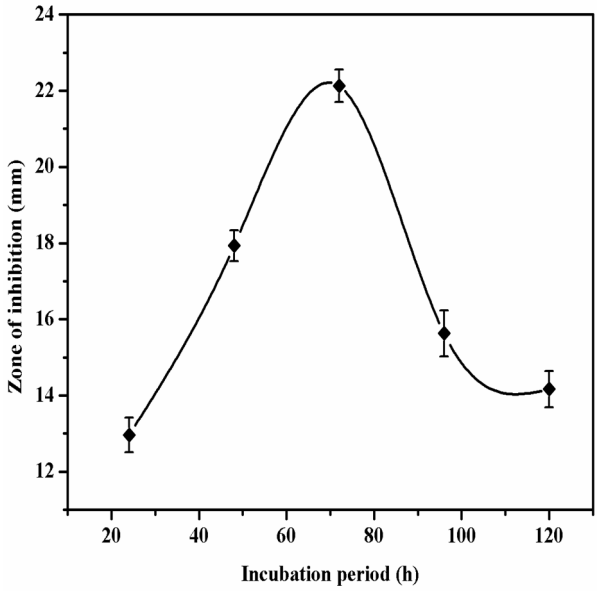

Figure 1: Optimization of Incubation period

Effect of pH and temperature on metabolite production

The pH of a culture medium is usually not constant throughout fermentation and the changes that occur are highly affecting the metabolite synthesis.

In order to determine the optimum pH for maximum metabolite production the test strain was grown with different hydrogen ion concentrations.

The optimum pH for antibacterial metabolite production by *Micrococcus* sp. AM7 is highly varied and the production was optimal at pH 8.0 (Figure 2).

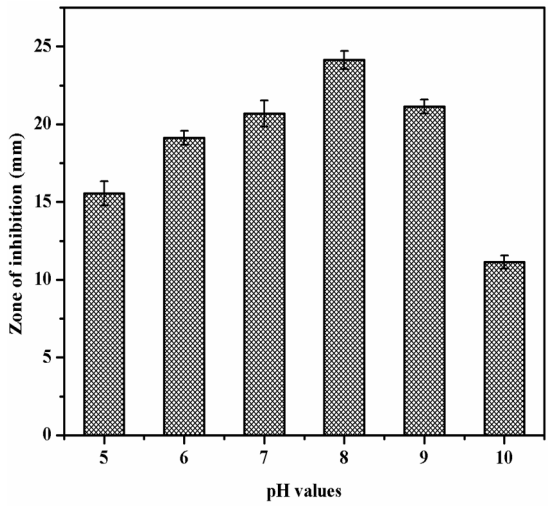

Figure 2: Optimization of pH for antibacterial metabolite production

For optimization of temperature, a wide range of temperature was used in this study because temperature is the parameter which influences directly the overall growth and development of any organism and it affects the physiology and subsequently the synthesis of various metabolites[19,20]. The incubation temperature 40 °C was found for maximum bioactive metabolite production by *Micrococcus* sp. AM7, but the antibacterial activity was reduced at higher temperature (Figure 3).

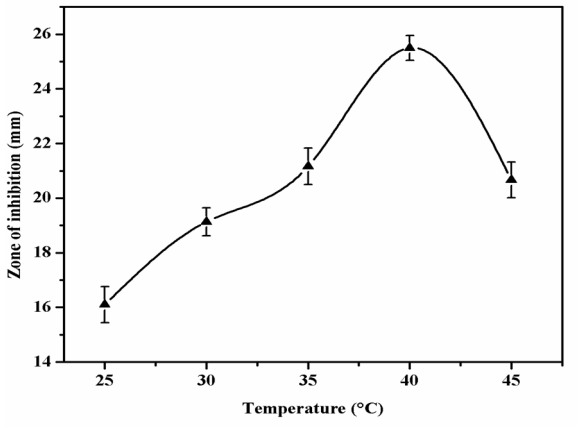

Figure 3: Optimization of Incubation temperature

Effect of carbon and nitrogen sources on metabolite production

Formation of antibiotics is also regulated by nutrients (such as carbon, phosphorous and nitrogen source), metals, growth rate, feedback control and enzyme inactivation[21]. Among these nutrients, the effect of carbon and nitrogen source on antibiotic production has been the subject of continuous study for both industry and research groups, not only from fermentation but also from biochemical and molecular biological stand points. The carbon and nitrogen sources are the important constituents to be considered which are reported to have highly influenced on the antibiotic production by nematode associated bacteria[22,23]. In the present study, isolate AM7 produced maximum antibacterial metabolites when grown in broth containing sucrose which profoundly high level compared to other carbohydrates (Figure 4).

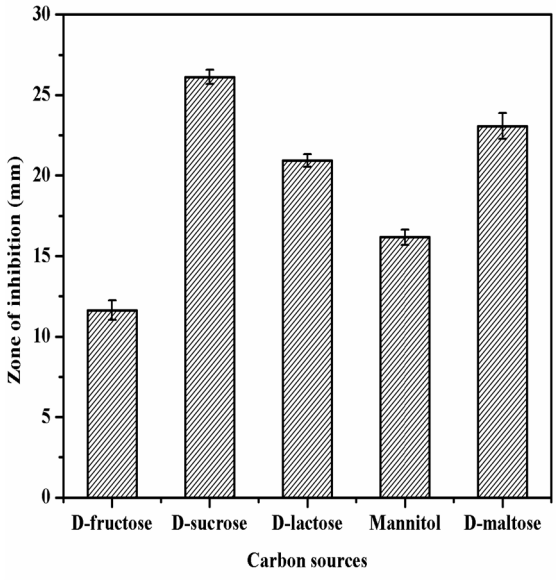

Figure 4: Effect of different carbon sources on the production of antibacterial metabolites

Bacterial growth and biosynthesis of antibacterial compound are influenced by several nitrogen sources. Fermentation medium with different nitrogen sources was found to produce significant amounts of antimicrobial metabolite. The results of present study clearly indicate that the highest level of antibacterial activity was found with tryptone which is much higher than other nitrogen sources (Figure 5).

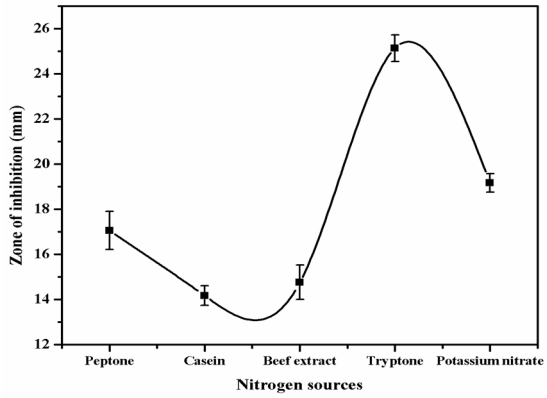

Figure 5: Effect of different nitrogen sources on the production of antibacterial metabolites

Effect of NaCl concentration on metabolite production

Growth and metabolite production by bacteria especially, marine bacteria are largely affected by NaCl concentration utilized by them**.** The antibiogram (Figure 6) showed that different concentration of NaCl had varied effect on metabolite production. The appreciable production of antibacterial compound was observed in presence of 1% NaCl with other optimized conditions. The production by the organism gradually decreased with the increase of NaCl concentration.

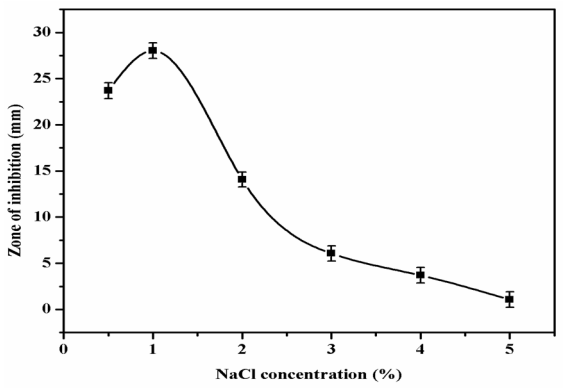

Figure 6: Effect of NaCl concentration (%) on antibacterial metabolites production

CONCLUSION

Finally from this present study it was concluded that, the isolate *Micrococcus* sp. AM7 showed good antibacterial metabolite production which was significantly increased by optimizing the different nutritional and environmental parameters. The antibacterial metabolite produced by these antagonistic bacteria could be further purified, characterized and used as an antibacterial substance for disease management.

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