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Process optimization for the production of antimicrobial compounds from marine sponge associated bacteria *Rhodopseudomonas palustris MSB 55*

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Abstract

Antibiotic resistance in bacteria has become a serious problem. Therefore, the search for new antibiotics is an important endeavour and is very much needed. Around 57 morphologically different bacteria isolated from marine sponge *Axinella donani* were screened for antimicrobial activity and enzyme producing ability. Among the 57 isolates, only two strains were more efficient to produce valuable primary and secondary metabolites. The present study is preceded with only one isolate to determine process optimization procedures for the production of bioactive compounds using sponge associated bacteria *Rhodopseudomonas palustris MSB 55*. The conditions were incubation period 72 hours, pH 8.5 and temperature 25ºC. Aeration also played a significant role in terms of product formation. Agitation speed of 150 rpm showed maximum yield. Secondary metabolites had showed broad-spectrum activity against human pathogenic bacteria and fungi. Hence, the new strain associated with sponge *R.palustris MSB 55* can serve as a source of antimicrobials.

Keywords: Bacteria; *Rhodopseudomonas palustris* MSB 55; Anti-microbial; Marine-sponge.

Introduction

Despite the great promise and the wide spectrum of biological activities of marine natural products, their subsequent development as therapeutic products has been slowed down due to several factors including the low availability of biologically active compounds, their high chemical complexity and, in some cases, their high toxicity at therapeutic doses (Rajasimman and Subathra, 2010). Some compounds are produced by organisms, which comprise only a minority component within the diverse population in the sample. In fact, many promising natural products associated with marine sponges and corals have been found not to be made by the invertebrate itself, but rather by symbiotic bacteria which are difficult to isolate, characterize and cultivate (Kennedy *et al*., 2009).

The biosynthetic pathways leading to the production of secondary metabolites such as antibiotics are often connected to and influenced by the pathways of primary metabolism (Atta, 2010). For example, an end product or an intermediate of a primary pathway frequently serves as a precursor for the antibiotic molecule. Such intermediates or end products could be part of a biosynthetic, amphibolic, or catabolic pathway (Nermeen and Gehan, 2006). Catabolic pathways involving energy metabolism of the organism are under strict control in microorganisms. When a favored carbon/energy source is used, the cell is usually prevented from producing enzymes, which catabolize other carbon compounds. It is possible that such a catabolic regulatory mechanism could also control the production of secondary metabolites (Iwai and Omura, 1982). The strong influence of carbon sources on the production of antibiotics has been reported in several cases (Konig *et al*., 2005).

Materials and methods

Media optimization

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Zobell marine agar was used to isolate the bacteria; modified starch agar, modified carboxy cellulose agar, and tryptic soy agar were used for the screening of primary metabolites. Zobell Marine Broth, Tryptic digest broth and Brain Heart infusion broth were used for the production of bioactive compounds.

Primary and secondary metabolite producing ability was studied in both wild strain and UV treated strains. The isolate *Rhodopseudomonas palustris* MSB 55 was inoculated on tryptic soy agar and exposed to UV for 10 minutes. Plates were incubated at 37ºC for 24 hours. Treated isolate was inoculated on Casein milk agar and incubated at 37ºC for 24 hours. Zone of proteolysis on both the strains were compared with a wild strains proteolytic pattern.

Process optimization

The optimum conditions such as incubation period, pH, temperature, agitation rate and substrate concentration for the production of secondary metabolites were studied. Tryptic digest broth was prepared. Four flasks were taken and labeled as 48hrs, 72hrs, 96hrs and 120hrs. Five flasks were taken and labeled as pH 6.5, 7.0, 7.5, 8.0 and 8.5. Five flasks were taken and labeled as 50rpm, 75rpm, 100rpm, 150rpm and 200 rpm. Bacterial isolate *R. palustris* MSB 55 was inoculated and incubated at 37ºC in a shaking incubator at 150 rpm. Crude extract of secondary metabolites were used for antibiotic sensitivity assay using Muller Hinton agar (Well diffusion method). *Escherichia coli and Klebsiella pneumoniae* were used as test organisms. Zone of inhibition was determined to find out the optimum incubation period.

Substrate concentration

Soybean meal concentration was optimized for the production of secondary metabolites. Various concentration such us 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9% and 1.0% were used.

Product recovery

Purification of bioactive compounds

Tryptic digest broth was prepared and optimum conditions were maintained to produce maximum yield and wide activity. Following inoculation with strains *R. palustris* MSB 55 was incubated in a shaking incubator at 25 ºC for 72 hours. It was centrifuged at 10,000rpm for 10 minutes, the supernatant was filter-sterilized (0.2µm pore-size filter), heated at 85 ºC for 10minutes, and stored at 4ºC until use.

Solvent extraction

Cell free extract was acidified using 0.1N HCL and various solvents such as n-Butanol, Methanol, Hexane, Chloroform: Methanol (2:1) and Ethyl acetate were used to extract the bioactive compounds and their antibacterial and antifungal activities were studied using the test organisms *Bacillus cereus, Micrococcus luteus, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Aspergillus niger and Aspergillus fumigatus*. Each solvent system was studied separately and the suitable solvent was identified. Solvent and cell free extract was taken in 1:1 ratio in a separating flask and which was shaken vigorously for 30 minutes and kept in a burette stand for 1hour. The organic phase was separated and their ability to inhibit the growth of test isolates was studied.

Antibiotic sensitivity test (Kirby Bauer method)

Antibacterial assay

Muller Hinton agar plates were prepared. Test organisms *B.cereus, M.luteus, S.aureus E. coli, and K.pneumoniae,* were inoculated on brain heart infusion broth and incubated at 37ºC for 3 hours. Log phase fresh cultures were used to perform lawn culture. Various concentrations such as 50µl, 100µl and 150µl of crude ethyl acetate extract were prepared and which was impregnated in to a sterile 10mm diameter Whatman no.1 filter paper. The bioactive compounds impregnated discs were placed on plates inoculated with a test pathogens. The plates were incubated at 37ºC for 24 hours and

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zone of inhibition was determined. Standard antibiotic ciprofloxacin was used to compare the efficiency.

Antifungal assay

Fungal representatives *A.niger* and *A.fumigatus* spores were used. One ml of spore suspension was added along with the sterile, molten state Sabourads dextrose agar in a tolerable heat. It was poured on a sterile Petri plates. The plates were allowed to solidify and bioactive compounds incorporated discs were placed on the plates. The plates were incubated at 25ºC for 7 days. Zone of inhibition was determined. Ketakonazole was used as a standard antibiotics.

Results and Discussion

Media optimization

Rhodopseudomonas palustris MSB 55 had showed higher yield in Tryptic digest broth. It was 68.4% higher than Zobell marine broth and 81.6% higher than Brain heart infusion broth.

Strain improvement

Muhammd *et al*. (2010), focused on the improvement of *Bacillus licheniformis* through random mutagenesis to obtain mutant having enhanced production of bacitracin. There were no yield improvement after UV mutation in terms of secondary metabolites but their proteolytic activity was drastically increased. There was a 57.6% reduction in the bioactive compound yield by *R. palustris* after mutation.

Process optimization

Bushra Uzair *et al*. (2009), analyzed that *Pseudomonas aeruginosa* established antibacterial activity could be detected in an ethyl acetate fraction of the crude extract, and this suggests that the substance was not bound to cell surface. Growing CMG1066 on King B medium resulted in increase in degree of production of antibacterial compound with increasing culture age as evident from maximum zone size after 72h of growth. Similar kind of result was found in the present study also. Maximum zone size was achieved after 72 hours of growth.

Yield of secondary metabolite was high after 72 hrs of incubation period. pH optima was studied stating from 6.5 to 9.0 with 0.5 difference among them pH 8.5 showed maximum activity. Temperature optima was determined with ranges from 15ºC to 40ºC among them 25ºC was found to an optimum temperature. Influence of agitation during incubation for the production of secondary metabolite was studied with an agitation speed ranging from 50 rpm to 200 rpm. The maximum product production was noticed at 150 rpm. The substrate soybean meal concentration was optimized and which was found to be 0.5% with a maximum yield.

Solvent extraction method for the recovery of bioactive compounds

The study done by Selvin *et al.* (2009) has also stated a similar kind of report. The extraction of the cell free supernatant with ethyl acetate yielded bioactive crude extract that displayed activity against a panel of pathogens tested (Selvin *et al.,* 2004). In this present study, also solvent extraction procedure was carried out with ethyl acetate (1:1). Solvents such as n-Butanol, Methanol, Hexane, Chloroform: Methanol (2:1) and Ethyl acetate were used to extract bioactive compounds from the selected bacteria. Among the five solvents, studied ethyl acetate was found to be a suitable solvent.

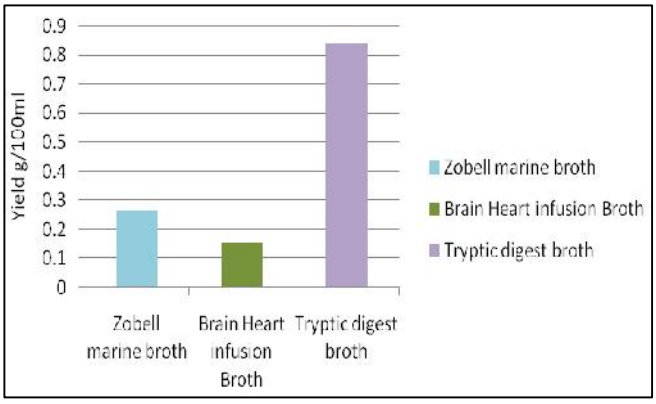

Fig. 1. Yield of secondary metabolites by Rhodopseudomonas Palustris MSB in Zobel marine broth, Brain heart infusion broth and Tryptic digest broth

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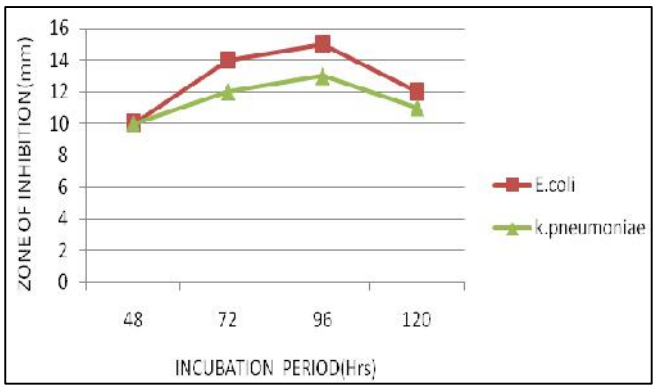

Fig. 2. Effect of Incubation period on the activity of bioactive compounds: Rhodopseudomonas Palustris MSB-55 against E.coli and K.pneumoniae

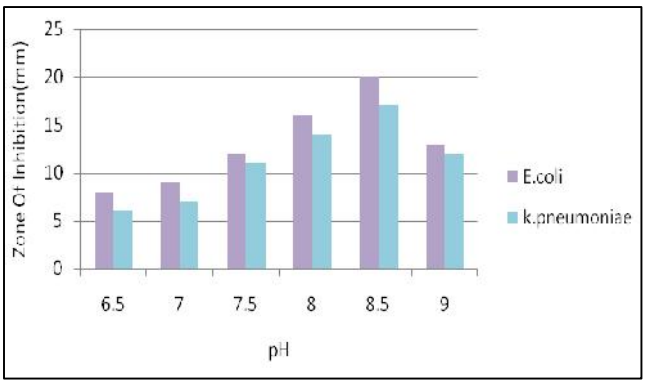

Fig. 3. Effect of pH on the activity of bioactive compounds; Rhodopseudomonas Palustris MSB 55 against E.coli and K.pneumoniae

Minimum inhibitory concentration of ethyl acetate extracts of *R. palustris MSB 55 & Rhodobacter sphaeroides MSB 57*

Minimum inhibitory concentration of ethyl acetate extract of bioactive compound produced by *R. palustris MSB 55 was* studied. Three difference concentrations such as 50 µl, 100 µl and 150 µl were used. There was a gradual increase in the zone diameter when the concentration increased. Ciprofloxacin was used as standard antibiotic for bacteria and ketakonazole was used as a standard antibiotic for fungi. The minimum inhibitory concentrations of the bioactive compounds were determined as 50 µl (Fig.1). The compound has broad-spectrum activity against *Bacillus cereus, Micrococcus luteus, Staphylococcus aureus, E.coli, K. pneumoniae, A. niger and A. fumigatus.* The correlation of *R.palustris* MSB 55 against *E.coli* and *K.pneumoniae* were 0.110657 and 0.361478, respectively (Fig.2). Maximum activity was found in the bioactive compound separated after 72 hrs of incubation. Incubation period and zone diameters were positively correlated for both the strains.

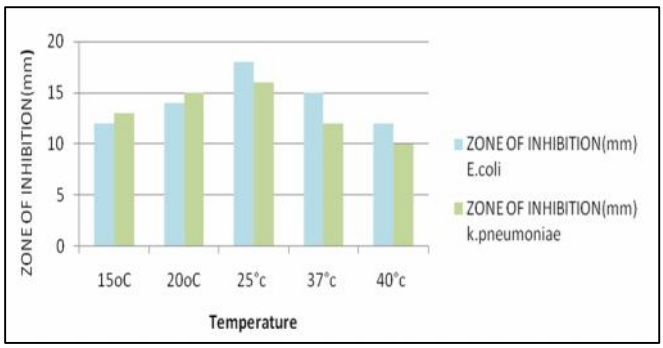

Fig. 4. Effect of temprature on the activity of bioactive compounds; Rhodopseudomonas Palustris MSB 55 against E.coli and K.pneumoniae

Optimum pH was found to be pH 8.5. The pH of the media and zone of inhibition were positively correlated (Fig.3). The correlation of *R.sphaeroides* MSB 57 55 against *E.coli* and *K.pneumoniae* for effect of Ph were 0.741042 and 0.808069, respectively. The selected strains preferred alkaline pH range rather than acidic. Though they had grown in the media nearly neutral, the antibacterial activity was very poor when compared with alkaline range.

The mesophylic range of temperature was determined as an optimum temperature for the bioactive compounds. The temperature and zone of inhibition were negatively correlated. Because of the increases in temperature there was a decrease in zone diameter. Agitation of the media led to increased product formation. Agitation speed of 150 rpm was found to be an optimum speed. The rate of agitation and the amount of yield were positively correlated. The substrate soy bean meal concetration had the ability to influence the product formation. Optimum concentration was found to be 0.5%. Both the attributes were positvely correlated with each other. The correlation of *R. palustris* MSB 55 against *E.coli* and *K.pneumoniae* were -0.00372404 and -0.673863904 respectively (Fig.4).

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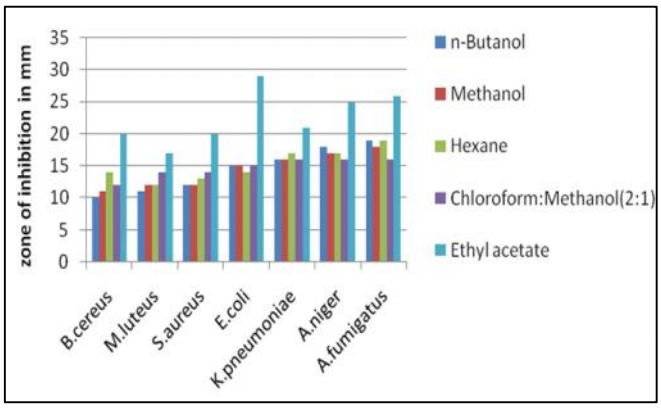

Fig.5. Efficiency of various solvents for the extraction of secondary metabolites produced by Rhodopseudomonas Palustris MSB 55

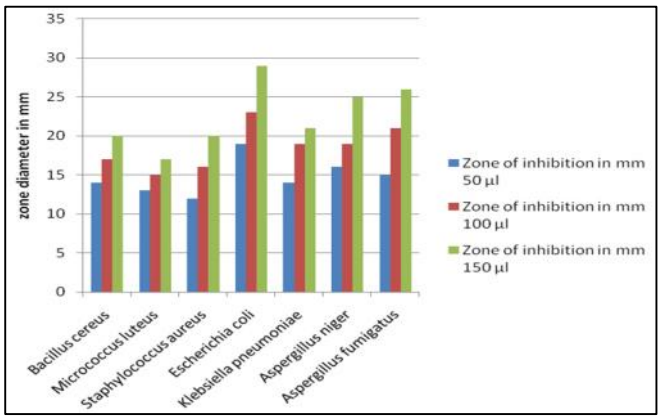

Fig.6. Minimum inhibitory concentration of Ethyl acetate extract of Rhodopseudomonas Palustris MSB 55

Based on the previous experiment, the best solvent for the extraction of secondary metabolites produced by the sponge-associated bacteria were found to be ethyl acetate (Fig.5 and 6). Hence, the same solvent was used to find out minimum inhibitory concentration. Minimum inhibitory concentration of crude extract was 50 µl. The compound has broad-spectrum activity against *B. cereus, M. luteus, S. aureus, E. coli, K. pneumoniae, A. niger and A. fumigates* (Fig.7).

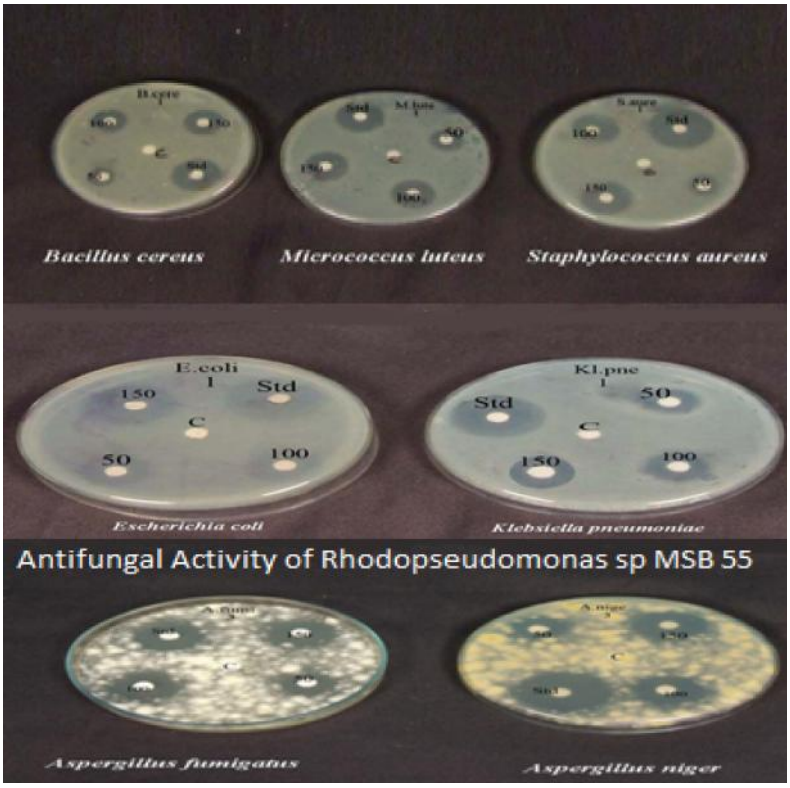

Fig.7. Antibacterial activity of Rhodopseudomonas Palustris MSB 55 against Bacillus cereus, Micrococcus luteus, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, and anti-fungal activity against Aspergillus niger and Aspergillus fumigatus

Conclusion

Process optimization revealed that, the optimum incubation period for the production of bioactive compounds using sponge associated bacteria *R. palustris MSB 55* was, incubation period 72 hours, pH 8.5 and temperature 25ºC. Aeration also played a significant role in terms of product formation. Agitation speed of 150rpm showed maximum yield. Secondary metabolites had showed broad-spectrum activity against bacteria as well as fungi. Hence, the new strain associated with sponge, *R. palustris MSB 55* can serve as a source of antimicrobials.

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