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Original Article

Antibacterial and anti-inflammatory activities of marine *Brevibacterium sp.*

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Abstract

The marine environment covers three quarters of the surface of the planet and is estimated to be home to more than 80% of life but yet it remains largely unexplored. It harbours a number of macro and microorganisms that have developed unique metabolic abilities to ensure their survival in diverse and hostile habitats, resulting in the biosynthesis of an array of secondary metabolites with specific activities. In this study, pigment forming bacterial strains were isolated from the sea surface inter tidal zones at different sampling sites along the Visakhapatnam coastal region. The bacterial isolates showed various types of colour pigments like pink, yellow, orange, and brown. Out of 26 pigmented isolates obtained, the bacterial isolate with bright yellow pigmentation was selected for further study. This strain was identified as *Brevibacterium sp* by using morphological, physiological, biochemical and 16s rRNA sequencing methods. The pigment was extracted in methanol solvent and antibacterial activity of the pigments extracted from the bacteria was determined and it was found active against pathogenic bacteria. The pigment extract was tested *In vivo* for anti-inflammatory activity and was effective.

Keywords: Pigments; Antibacterial; *Brevibacterium sp.;* Anti-inflammatory activity

INTRODUCTION

Historically, nature has provided the source for the majority of the drugs in use today. The use of traditional medicine is widespread and natural compounds still represent a large source of natural secondary metabolites that might serve as leads for the development of novel drugs. More than 70% of our planet's surface is covered by oceans, which is inhabited by 80% of all the life forms and consequently aquatic life has a greater diversity than their terrestrial counterparts. Ocean has been considered as a rich source of compounds with novel structures and biological activities. The ocean also offers the potential for the production of metabolites, which may be different from the terrestrial microorganisms (1,2). Due to the complex nature and dynamic system in the ocean, the marine microorganisms have developed unique metabolic and physiologic capabilities that ensure their survival in extreme variations of pressure, salinity and temperature. Marine microorganisms have proven to produce a variety of chemically interesting and biologically significant secondary metabolites and some of them are expected to serve as lead compounds for drug development or pharmacological tools for basic studies of life sciences (3). Marine microorganisms as a whole represent a vast essentially untapped source of new and potential biologically active natural products (4).

The utilization of natural pigments in foodstuff, dyestuff, cosmetic and pharmaceutical manufacturing processes have been mounting in recent years (5-7). Natural colorants or dyes derived from flora and fauna are believed to be safe because of non-toxic, non-carcinogenic and biodegradable in nature.

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There is growing interest in microbial pigments due to their natural character and safety to use, medicinal properties, nutrients like vitamins, production being independent of season and geographical conditions, and controllable and predictable yield (8). Hence, microbial pigment production is now one of the promising and emerging fields of research to reveal its potential for various industrial applications (9-11).

Inflammation and oxidative stress play an important role in various diseases. Inflammation is an immunological defence mechanism elicited in response to mechanical injuries, burns, microbial infections, allergens and other noxious stimulus (12-16).

In the human body, inflammation is considered to be part of the complex biological response to remove injury or harmful stimuli such as pathogens, damaged cells, or irritation. This response leads to many physical symptoms such as pain, fever, and swelling, as a result of many associated changes such as vasodilation, increased vascular permeability, and plasma extravasation.

There has been a great interest for the researchers to explore marine microorganisms as new source of antibacterial compounds as increasing resistance of pathogen to present antibiotics.

Hence, the present study was focused to isolate the pigmented bacteria from Visakhapatnam coastal areas and assess the pigment for the antimicrobial and anti-inflammatory activities.

MATERIALS AND METHODS

Sample collection and isolation of marine bacteria

The surface sea water samples were collected from the intertidal zones of Vishakapatanam coastal region of India. Water samples were collected in sterile containers at 20 meters off the shore line at a depth of about 40 cm from the top and were brought to the laboratory in clean, sanitized and autoclaved bottles.

About 8 µL sample water was spread on the entire surface of marine agar (g/L) consisting of peptone, 5.0; yeast extract, 1.0; ferric citrate, 0.1; sodium chloride, 19.45; magnesium chloride, 8.8; sodium sulphate, 3.24; calcium chloride, 1.8; potassium chloride, 0.55; sodium bicarbonate, 0.16; potassium bromide, 0.08; strontium chloride, 0.034; boric acid, 0.022; sodium silicate, 0.004; sodium fluoride, 0.0024; ammonium nitrate, 0.0016; disodium phosphate, 0.008; agar, 15.0.

After incubation at 37 ^{o}C, morphological characteristics of the colonies were recorded. The plates exhibiting discrete pigmented colonies were selected and the morphologically different pigmented colonies were sub cultured in the respective agar slants and stored for further use.

Selection of potential pigmented strain

Primary screening of the bacterial isolates was done based on the pigmentation exhibited on Zobell marine agar plates. The potential strain was selected based on the pigmentation of the isolate.

In the first phase, all chromogenic cultures that showed bright pigmentation were short listed. During the second phase, the bacteria that showed intense yellow pigmentation on Zobell marine agar medium were selected for further studies.

Biochemical characterization

Different tests were performed to characterize the biochemical properties of the isolated strain, using specific tests for Gram staining, spore staining, motility, indole production, MR-VP test, gelatin hydrolysis, citrate utilization, catalase production, nitrate reduction, urease production and starch hydrolysis according to standard methodology Table 1.

The ability to ferment the sugars such as sucrose, glucose, fructose, and mannitol as sole carbon sources was also evaluated for the isolated strain.

Molecular identification of the most promising bacterial isolate

The bacterial isolate which showed intense yellow pigmentation was identified to genus level by PCR amplification of the 16S rRNA gene, BLAST analysis, and comparison with sequences in the GenBank nucleotide database.

Table 1. Morphological, physiological and biochemical properties of the colonies.

Sample Number	Characteristic	Nature
1	Colony morphology	Yellow pigmented, shiny, translucent
2	Cell shape	Coccus
3	Motility	-ve
4	Gram staining	+ve
5	Starch hydrolysis	+ve
6	Indole test	-ve
7	Methyl red	+ve
8	Voges proskauer	+ve
9	Citrate utilization	-ve
10	Catalase	+ve
11	Urease test	-ve
12	Nitrate reduction test	+ve
13	Arabinose	+ve
14	Mannitol	-ve
15	H_2S production	-ve
16	Gelatin hydrolysis	+ve

+VE, present; -VE, absent.

Table 2. The experimental design of anti-inflammatory activity.

Groups	Group name	Drug administered
Group 1	Normal	Vehicle (0.9% normal saline) by oral route
Group 2	Standard	Diclofenac (5 mg/kg) + 0.1 mL of 1% carrageenan
Group 3	Treatment	(100 mg/kg) by oral route + 0.1 mL of 1% carrageenan

Extraction of the pigment

The selected pigmented bacterial isolate was cultured in Zobell marine broth and incubated in rotary shaker for 3 days at 28 ± 2 °C. After incubation, the cells were harvested by centrifugation at 10,000 rpm for 10 min at 4 °C.

The collected pellets were extracted with methanol, and then separated from the cells by centrifugation at 10,000 rpm for 10 min at 4 °C. The methanolic extract of pigment was concentrated in vacuo.

Pathogenic microorganisms

The bacterial strains employed in the study were obtained from IMTECH, Chandigarh. Microbial cultures of four pathogenic strains of bacteria viz. *Staphylococcus aureus* (ATCC-29737), *Bacillus subtilis* (ATCC-6633), *Escherichia coli* (ATCC-2343) and *Pseudomonas aeroginosa* were maintained on nutrient agar slants. The bacterial cultures are sub cultured in nutrient broth and incubated for 24 h to be used as inoculum.

Antibacterial activity of crude pigment

The antimicrobial activity of the pigment dissolved in methanol was tested by agar well diffusion method (17). Streptomycin and methanol was used as positive and negative control. The 6 mm diameter well was made using sterile cork borer. The pigment extract, methanol and streptomycin was poured into the wells separately and incubated at 37 ºC for 24 h after which the activity was confirmed by the presence of zone of inhibition surrounding the well.

Anti-inflammatory activity (In vivo)

Animals

Wistar Albino rats of male sex weighing 150 – 200 g were procured from Mahaveer Enterprises, Reg No. 177/99/CPCSEA, Hyderabad, were used in the present study. They had free access to food and water and were maintained under standard laboratory conditions with alternating light and dark cycles of 12 h each. Animals were acclimatized to laboratory conditions for

2 days before behavioral studies. All the observations were recorded during the same time i.e. between 10 a.m. and 5 p.m. every day.

The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) and care of animals was taken as per guidelines of CPCSEA, Department of Animal Welfare, and Government of India with a registration No. 2011/10/1/12.

Experimental design

Anti-inflammatory activity was assessed by carrageenan induced paw oedema method suggested by Xu Z, *et al*. (18). Rats were divided into 3 groups (6 animals in each group). Animals of all the groups were injected with 0.1 mL of 1% carrageenan, under the plantar aponeurosis of the right hind paw. Group I animals, served as control and received vehicle (0.9% normal saline) by oral route; group II, the standard reference group was given p.o., an aqueous solution of diclofenac (5 mg/kg), 30 min prior to carrageenan injection; group III received p.o., 100 mg/kg of prepared methanol extract suspension, 30 min prior to carrageenan injection, respectively (Table 2). The paw volume of the rats was measured plethismographycally just before and up to 5 h after carrageenan injection.

The anti- inflammatory activity was determined as the percentage of inhibition of inflammation after it was induced by carrageenan by taking volume of inflammation in control group as 100%. The percentage of inhibition was calculated by using following formula.

$$Inhibition\ (\%) = \frac{Test\ mean\ paw\ inflamation}{Control\ mean\ paw\ inflamation} \times 100$$

RESULTS

In the present study, pigmented marine bacteria were isolated from water samples collected from Visakhapatnam sea coast, India and a total of 26 bacterial colonies were isolated.

The isolated colonies exhibited different colours like bright yellow, dark orange, yellowish orange, peach and brown.

Among pigmented isolates the yellow pigmented bacteria were the most dominant. In preliminary screening the isolate showing bright yellow pigmentation was selected and tested for antibacterial activity.

The pigment was extracted from the potential marine bacterial strain by using methanol as solvent and the antimicrobial activity of obtained pigment extract was checked against human pathogens using well diffusion method. Crude extracts of the pigment from bacterial isolate cell pellet was tested against the test organisms and the results are presented in Table 3. Gram staining of the isolate revealed that it was Gram positive cocci.

The morphological characterization for the strain *Brevibacterium sp*. was observed microscopically under 400× light microscopy was Gram positive, non sporulating coccus occurring in pairs or in short chains and nonmotile. The macroscopic observation of the isolate was yellow, shiny, translucent and smooth colonies on Zobell marine agar medium.

The antibacterial activity was studied on several organisms like *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa* that is shown in Fig 1. Jayanth, *et al.* (19) studied antagonistic activity of marine bacteria and they tested their ability to inhibit the growth of test organisms.

After the incubation period the antimicrobial activity of pigment was determined by measuring the diameter of the zone of inhibition (ZOI) in millimeters around the wells.

The pigment extract was tested for the *in vivo* anti-inflammatory activity and is summarized clearly in Table 4. In the present investigation bioactive pigmented extract showed inhibitory effect on carrageenan induced paw oedema assay of inflammation. This effect may be due to inhibition of COX enzyme.

The results showed a significant decrease in paw volume when compared to control animals. Pigment showed anti-inflammatory action with (42.18 % inhibition) after 5 h in comparison with control and their activity was comparable to diclofenac.

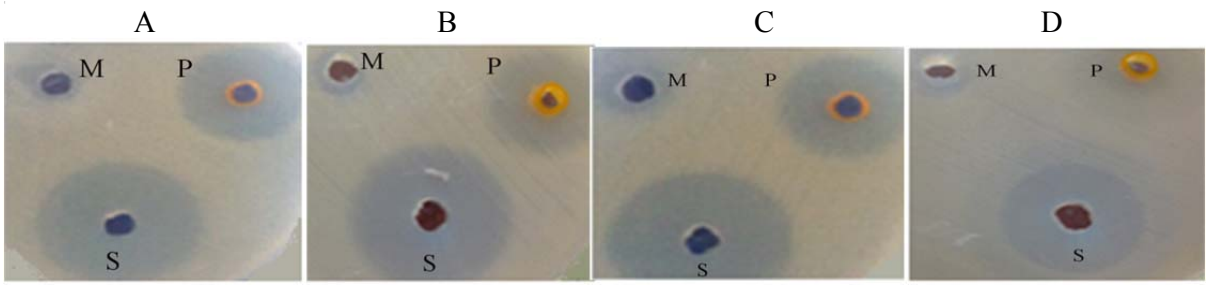

Fig. 1. Antibacterial activity of pigment extract against bacterial pathogens (A) *Staphylococcus aureus,* (B) *Bacillus subtilis,* (D) *Escherichia coli* and (C) *Pseudomonas aeruginosa*. (M) methanol, (S) streptomycin, (P) pigment extract.

Table 3. Antibacterial activity of pigment extracted from marine isolate against bacterial pathogens.

S. No	Test organism	Diameter of zone of inhibition in mm		
		Pigment extract	Standard (PC)	Methanol (NC)
1	*Staphylococcus aureus*	29	38	11
2	*Escherichia coli*	17	25	10
3	*Pseudomonas aeroginosa*	27	35	11
4	*Bacillus subtilis*	28	35	8

PC, positive control (streptomycin); NC, negative control (methanol).

Table 4. Anti-inflammatory activity of crude pigment extract of the pigmented isolate.

Compound	Time (h)	Volume variation (µL)	% Inhibition
Vehicle control	1	1.53 ± 0.12	0
	2	1.84 ± 0.17	0
	3	2.22 ± 0.24	0
	4	2.44 ± 0.26	0
	5	2.11 ± 0.21	0
Standard	1	1.17 ± 0.01	23.53
	2	1.35 ± 0.01	26.63
	3	1.14 ± 0.02	48.6**
	4	1.03 ± 0.02	57.79***
	5	0.89 ± 0.01	57.82***
Yellow pigment	1	1.27 ± 0.04	16.99
	2	1.44 ± 0.06	21.74
	3	1.49 ± 0.04	32.88*
	4	1.45 ± 0.07	40.57*
	5	1.22 ± 0.08	42.18*

Vehicle control, control group; standard, diclofenac; yellow pigment, treated group with yellow pigment.

Statistical analysis

The values were calculated as mean ± S.E.M. The significance of the difference of the mean value with respect to control group was analyzed by one way ANOVA followed by Dunnet's t- test using statistica 8.0. Statistically significant at a level of $P < 0.05$ or above was considered to be significant.

DISCUSSION

The changing pattern of diseases and the emergence of resistant bacterial strains to currently used antibiotics continuously put demand on the drug discovery scientists to search for novel antibiotics such as bacterial pigments (20). In an attempt to isolate the potential pigment producing bacteria, sea water samples were collected from intertidal zones of Visakhapatnam coastal region, India. Microorganisms from intertidal zones must be able to tolerate rapid and repeated fluctuations in environmental conditions including temperature, light and salinity, and are exposed to wave action, ultraviolet radiation, as well as periods of drought (21). Pigment

producing microorganisms are yeast, fungi, bacteria, micro algae and are quite common in nature (22). The isolates exhibited varied pigmentation on Zobell marine agar medium. The intense yellow pigmented bacterium was selected for the study because it was stated in earlier studies that most of the antibiotic producing marine bacteria were pigmented (23). Many studies have concentrated on the screening of marine bacteria for antibacterial activity against human pathogenic bacteria (24). The pigment producing isolate was identified as gram positive cocci. Gram positive bacteria collected from coastal sand dune vegetation showed a predominance of pigmented isolates (25).

The crude extract exhibited a better antibacterial activity which is expressed in terms of diameter of zone inhibition. Marine bacteria showing antibacterial activities have been described for more than 50 years (26). Inflammation is a complex process; various mediators like prostaglandins, leukotrienes and kinins, platelet activating factor are involved in the development of inflammation. Carrageenan shows its effects by two phases i.e., biphasic response.

In phase-I, inflammatory mediator serotonine is released and cause inflammation. This first phase occurs within the first h after the injection of carrageenan. In phase-2, inflammatory mediator prostaglandins are released and show their inflammatory effect around third h of inflammatory process (27). NSAIDS (non-steroidal anti-inflammatory drugs) are more effective in treating inflammation. As these drugs decreases the prostaglandins synthesis by inhibiting the cyclooxygenase enzyme, which is involved in prostaglandin synthesis (28).

Oxidative stress plays an important role in endothelial dysfunction (29), lung disease (30), gastrointestinal dysfunction (31), and atherosclerosis (32), all of which involve inflammatory reactions.

The crude pigment extract was subjected to anti-inflammatory activity using carrageenan induced paw oedema on Wistar male rats. To assess the anti-inflammatory profile of a specific compound, it is necessary to select a reliable approach.

Effective compounds in analgesic test were screened for their anti-inflammatory activities using carrageenan induced paw oedema on Wistar male rats (33). Marine bacteria considered highly valuable as they produce various antibiotics and other therapeutically useful compounds with diverse biological activities (34).

CONCLUSIONS

The result of this study provides further evidence for the implication of the bioactive pigment extract which showed antibacterial and anti-inflammatory responses also represents a potential therapeutic target for novel anti-inflammatory agents.

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