RESEARCH PAPER | Biotechnology | Volume : 4 | Issue : 10 | October 2014 | ISSN - 2249-555X

Antibacterial Activity by Pigmented Psychrotrophic Bacterial Isolates

KEYWORDS: Antimicrobial, Carotenoids, Psychtrophic, Growth inhibition, Minimum inhibitory concentration (MIC)

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ABSTRACT Four pigment producing psychrotrophic bacteria were isolated from the water and soil samples collected from Leh and Ladakh which are situated at an altitude of 11,562 and 19,700 ft., respectively in Himalayas, India. Isolates were characterized as *Sanguibacter antarcticus* KK13 (yellow), *Kocuria turfanensis* KK7 and K. *rosea* KK12 (pinkish orange) and *Planococcus maritimus* KK21 (orange). Pigment extracts from all the isolates gave absorption spectra in 400-500 nm which validated the pigments as carotenoids. Pigment extracts from isolates were screened for antibacterial activity against Gram (+) *Bacillus cereus* (ATCC 14579), *Staphylococcus aureus* (ATCC 25923) and Gram (-) *Vibrio cholera* (ATCC 39315) and *Shigella dysenteriae* ATCC 13313) pathogenic bacterial cultures obtained from ATCC. Pigment extract from any of the isolate did not inhibit the growth of *S. dysenteriae,* while growth of *B. cereus* was not inhibited by pigment extracts from *S. antarcticus* `and *P. maritimus*. Pigment extracts in concentration range of 40-80 µg ml^{-1} successfully inhibited growth of selected pathogenic bacteria. The present study thus suggested that *carotenoids* from *psychrotrophic* isolates may be used as a new potential source of natural *nutraceutical* components.

Introduction

Currently emerging and reemerging infectious diseases caused by different drug resistant strains are a major problem in public health and global economies. Such pathogens are increasing significantly over time because they are becoming progressively more resistant to conventional antibiotic compounds [Hawkey and Jones, 2009].

Psychrophilic and psychrotolerant bacterial species are relatively poorly studied as compared to their mesophilic or thermophilic counterparts. Exploration of Arctic and Antarctic microorganisms as a source of new biologically active compounds has started in last 15-20 years *[*Lyutskanova et al., 2009*]*. Survival of microorganisms in these cold and extreme conditions requires a special kind of adaptation and hardiness against stress factors such as substratum limitation, UV irradiations, all-year low temperatures and short-time intensive heating during the Arctic summer. Strategies to cope up extreme low temperatures include increase in membrane fluidity and increased content of large lipid head group, proteins and non-polar carotenoid pigments [Chintalpati et al., 2004]. Hence, the strains isolated from the regions with temperatures under 0°C attains a special significance regarding isolation and identification of secondary metabolites useful in pharmacy [***Lyutskanova et al., 2009***].

The search for new antimicrobial agents is a field of utmost importance. Antimicrobial substances, such as antibiotics, have been successfully used for preventing and treating microbial diseases for many decades. To date, thousands of antimicrobial substances have been identified and applied in clinical therapy. Due to the wide application of antibiotics, more and more bacterial strains have developed antimicrobial resistance or even multidrug-resistance in last few decades [Sipali, 2008] resulting in increase of antimicrobial resistance among key microbial pathogens at an alarming rate worldwide. Pigments make the nature colourful. Many pigment producing microorganisms such as fungi, yeast, bacteria are abundant in nature. The pigments produced by microorganisms viz. carotenoids, melanins, flavins, quinones, and more specifically monascins, violacein and indigo showed distinct antibacterial effect against many pathogenic bacteria [Molnar and Farkase, 2010].

The present study deals with determination of antimicrobial activity against pathogenic strains of *Vibrio cholerae* G (-) (ATCC 39315), *Shigella dysenteriae* G (-) (ATCC 13313), *Bacillus cereus* G (+) (ATCC 14579) and *Staphylococcus aureus* G (+) (ATCC 25923). *Carotenoid* extracts from *psychrotrophic* isolates belonging to the genera Kocuria, Planococcus and Sanguibacter were used as a new source of antimicrobial substance. Thus, cold-adapted microorganisms might provide valuable novel antimicrobials.

Materials and Methods

Culture of psychrotrophic bacteria:

Psychrotrophic bacteria were isolated from the water and soil samples collected different parts of Siachen, India and nearby areas situated at 35.5°N 77.0°E The isolates were cultured in 250 ml flask of Antarctic Bacteria Medium (ABM) with continuous shaking in an incubator at 10°C up to two weeks as described by Shivaji et al. [2005]. Bacterial cells were harvested; pigment was extracted in methanol and quantified. The molecular characterization of the isolates was carried out using 16S rDNA approach and sequences were submitted to Genebank.

Pathogenic microorganisms

Microbial cultures of four pathogenic strains of bacteria viz. *Vibrio cholerae* G (-) (ATCC 39315), *Shigella dysenteriae* G (-) (ATCC 13313), *Bacillus cereus* G (+) (ATCC 14579), *Staphylococcus aureus* G (+) (ATCC 25923) obtained from American Type Culture Collection (ATCC), USA were used for determination of antibacterial activity. All the bacterial strains were stored frozen in medium containing 10% glycerol and sub cultured on plates at 37°C on Mueller Hilton agar (Himedia) only as needed.

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Extraction of bacterial carotenoids

The pigment extraction steps were carried out rapidly on ice under minimal light conditions to avoid degradation of pigments. Bacterial isolates were thawed and centrifuged (5000 rpm for 5 min at 4°C). The resulting cell pellets were transferred to 1.5 ml methanol (HPLC grade) in sterile Eppendorf tubes and gently sonicated in an ice water bath (two 30 sec. bursts at 17 W). Samples were allowed to freeze for 12 h at -20°C. After extraction the supernatant was collected by centrifugation (5000 rpm for 5 min at 4°C). If the sample still appeared to be cloudy the centrifugation step was repeated. Absorbance scans (300 to 700 nm) were measured using a spectrophotometer (Nanodrop ND 1000) with a UV-VIS absorbance module. Extracted pigment was evaporated till dryness by spinning in Centri fan PE evaporator and then again dissolved in 1 ml sterile Dimethyl sulfoxide.

Quantification of total extracted carotenoids

Total volume of pigmented methanolic extract was used to quantify total carotenoid/ pigment content in bacteria according to the equation provided by [Rodriguez and Kimura, 2004].

$$\text{Total carotenoid content in bacteria} = \frac{\text{ATotal x volume (ml)x10000"}}{\text{A1\%cm x sample wt. (g)"}}$$

Where, ATotal= Absorbance at 474 nm

Volume= Total volume of extract (ml)

A1%= Absorption coefficient of carotenoids mixture in methanol

Antimicrobial activity assay:

Antibacterial activity of pigment extract from isolates was determined by agar well diffusion method. 100 µl of standardized inoculum (0.5 MacFarland) of each test bacterium was inoculated on 90 mm disposable Petri dishes containing Mueller- Hilton medium under aseptic conditions. Standard cork borer of 5 mm in diameter was used to make uniform wells into which 30 µl of pigment extract dissolved in DMSO was added. DMSO alone was used as negative control. The plates were then incubated at 37°C for 24 hours. The zone of inhibition was measured with the help of standard scale (Himedia). The experiments were carried out in triplicate and results were calculated as mean ± SD.

Determination of minimum inhibitory concentration (MIC): The MIC of pigment extracts was determined by macro dilution method. Dilution ranges of pigment extracts from 100 to 0.010 µg/ml were prepared from stock solution by serial dilution technique. Each sample dilution was mixed properly with 20 ml of sterile molten Muller Hinton agar and poured into 90 mm Petri plates and allowed to cool under laminar air flow before streaking with 10 µl of (0.5 McFarland) standard inoculate of tested bacterial strains. Plates were incubated at 37°C for 24 hours. The lowest concentration of the extract at which there was no visible growth of microorganisms was considered as minimum inhibitory concentration (MIC).

Results and Discussion

Our approach during the study was to isolate and analyze the applicability of psychrotrophic bacterial strains from high altitude region of India as antimicrobial sources. The isolates were validated as *Sanguibacter antarcticus* KK13 (Genebank accession no.JN638048) (yellow colony), *Kocuria turfanensis* KK7 (Genebank accession no.JN255748) and K. *rosea* KK12 (Genebank accession no.JN638047) (pinkish orange colony) and *Planococcus maritimus* KK21 (Genebank accession no.JN638058) (orange colony). No diffusible pigment was observed in any of the isolate. UV-Vis spectroscopic scans of pigment extract in 300-700 nm, show maximum absorption peaks between 400-500 nm suggesting extracted pigments as carotenoids. 100 ml of culture broth yielded 2.089 g of cells in S. *antarcticus*, 2.443 and 2.460 g respectively in K. *turfanensis* and K. *rosea*, while P. *maritimus* yielded 2.287 g cells. The pigment concentration for S. *antarcticus* was 982 µgg^{-1}, 644 and 675 µg g^{-1} respectively for K. *turfanensis* and K. *rosea*. In P. *maritimus* pigmentation quantified was maximum i.e. 1061 µg g^{-1}.

Table 1 shows that the mean zone of inhibition varies between 0.00±0.00 and 24±0.2 mm and 40-80 µg ml^{-1} MIC of yellow/ orange pigments from various bacteria. MIC is defined as the lowest concentration of antibacterial material that can inhibit cell growth completely. 40-80 µg ml^{-1} yellow/ orange pigments showed antibacterial activity against gram-positive bacteria such as *B. cereus* and *S. aureus* and that for gram-negative bacteria, *V. cholera*. However, extracted pigment from all isolates could not inhibit the growth of gram-negative bacteria such as *S. dysenteriae* and pigment extracts from strains *S. antarcticus* and *P. maritimus* did not inhibit growth of gram-positive bacteria Bacillus cereus even when more than 10 µg/ml yellow/ orange pigment was added (Fig.1). Since the antibacterial activity of a material depends on the destruction of the physical structure or the inhibition of the necessary metabolic reaction in a microorganism, it seems that the presence and the level of the antibacterial activity of the yellow/ orange pigment varied significantly with the type/ strain of microorganism used.

Pigmentation is widespread in bacteria and consists of carotenoids and many other pigments [Yehia et al., 2013]. Microorganisms have become an important point of study in search of novel microbial products showing antimicrobial activities. Antimicrobial activities of pigments from microbial sources have been reported by many workers. Yellow pigmented *Pseudoalteromonas* sp. produced pigment which inhibited growth of S *aureus* and Pseudomonas *aeruginosa* [Cetina et al., 2010].

As an adaptive strategy some extremophilic group of bacteria are reported to be producing pigments [Mueller, 2005]. Sufficient number of reports are not available on antimicrobial activity of carotenoids of psychrophilic/ psychrotrophic bacteria. Antimicrobial activity of violet pigment from psychrotrophic strain of *Janithinobacterium lividum* has been reported by Nakamura et al. [2003], which inhibited growth of *Bacillus licheniformis, B. subtilis, B. megaterium, Staphylococcus aureus and Pseudomonas aeruginisa*, high concentrations even caused the cell death. Recently, carotenoid extract from halophilic archaea *Halobacterium halobium* showed significant antiproliferative activity against human cancer cell lines [Abbes et al., 2013].

Natural sources of carotenoids are wide and considered as main and most plentiful pigment groups, which appear yellow, orange or red [Marit et al., 2010]. Carotenoids from non microbial sources are also reported as antimicrobial sources by many researchers as carotenoid extract from medicinal plant *Guiera senegalensis* gave growth inhibitory activity against *Escherichia coli, Salmonella typhimurium,*

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Bacillus cereus, Staphylococcus aureus and *Proteus mirabilis* [Dijifaby et al., 2012]. Antimicrobial activity of pigment extract from yellow/ orange flower of *Calendula officinalis* also inhibited growth of fungus and G (+) and G (-) bacterial clinical pathogens [Efstratiou et al., 2012], whereas carotenoid extract from fruit peels of Shatian pummelo inhibited growth of *Bacillus subtilis, Staphylococcus aureus, E. coli* and fungal strains of *Aspergillus niger, A. flavus, Penicillium chrysogenum, Rhizopus oryzae* and *Saccharomyces cerevisiae* [Tao et al., 2010]. Carotenoid fractions from red paprika, Valencia orange peel and the peel of Golden delicious apple showed anti- *Helocibacter pylori* activity, *cytotoxic* activity against human tumor cell lines [Molnar et al., 2005].

The metabolic and physiological capacities of microorganisms allow them to survive across all environmental conditions on Earth. The adaptation of bacteria to diverse habitats can be attributed to their special skills for the production of unique secondary metabolites [Bernan et al., 2004]. Microorganisms isolated from marine/ cold environment can even show properties different from known terrestrial species.

Conclusions

The extracted pigments/ carotenoids from bacterial isolates could be an important therapeutic alternative particularly for those directed against multidrug resistant strains. In conclusion, high altitude region of Indian subcontinent may offer an opportunity of biological resource for antimicrobial therapeutic agents in the form of carotenoids.

List of tables:

Table1. Antibacterial activity of the pigment extracts from psychrptrophic bacterial isolate.

Source of pigment	Test Organism	Inhibition Zone (mm)	MIC of pigment (µg ml^{-1})
Sanguibacter antarcticus (KK13) (JN638048)	*Vibrio cholerae* (ATCC 39315)	22±0.2	50
	Shigella dysenteriae (ATCC 13313)	-	-
	Bacillus cereus (ATCC 14579)	-	-
	Staphylococcus aureus (ATCC 25923)	18±0.2	40
Kocuria turfanensis (KK7) (JN255748)	*Vibrio cholerae* (ATCC 39315)	24±0.2	60
	Shigella dysenteriae (ATCC 13313)	-	-
	Bacillus cereus (ATCC 14579)	18±0.2	60
	Staphylococcus aureus (ATCC 25923)	20±0.2	60
Kocuria rosea (KK12) (JN638047)	*Vibrio cholerae* (ATCC 39315)	21±0.2	40
	Shigella dysenteriae (ATCC 13313)	-	-
	Bacillus cereus (ATCC 14579)	24±0.2	70
	Staphylococcus aureus (ATCC 25923)	19±0.2	70
Planococcus maritimus KK21 (JN638058)	*Vibrio cholerae* (ATCC 39315)	24±0.2	60
	Shigella dysenteriae (ATCC 13313)	-	-
	Bacillus cereus (ATCC 14579)	-	-
	Staphylococcus aureus (ATCC 25923)	20±0.2	80

(Zone of inhibition values are mean ± SD of 3 replications)

List of figures:

figure 1 (a)

figure 1 (b)

figure 1 (c)

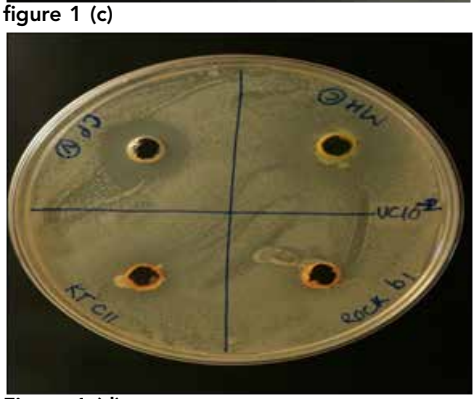

Figure 1 (d)

Fig. 1 Zone of inhibition in pathogenic bacterial strains (a) *Staphylococcus aureus, (b) Bacillus cereus, (c) Vibrio cholera (d) Shigella dysenteriae.*

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