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RESEARCH ARTICLE

EVALUATION OF ANTIOXIDANT POTENTIAL OF BIOACTIVE COLORED METABOLITE ISOLATED FROM *EXIGUOBACTERIUM PROFUNDUM* BC2-11 AND IT'S BIOACTIVITIES

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ABSTRACT

The polluted area would be considered as stressed environment which induces the microorganisms to produce several secondary metabolites for their survival between their microbial communities. Prominently fourteen bacterial isolates were isolated from the terrestrial soil samples of steel mills near Coimbatore, among that 7 were pigmented bacteria. All the seven bacterial strains were determined for antagonist activity and BC2-11 strain was a potential strain. The strain was identified as *Exiguobacterium profundum* BC2-11 through morphological, biochemical, physiological and molecular characterization. The organism produced orange colored metabolite and the crude colored metabolite was extracted using methanol. The crude pigment extract was subjected to HPTLC and it exhibited six peaks, the 5th peak was observed to be highest area of 5973.3 (47.39%) which was the bioactive metabolite. The crude colored metabolite was assessed for antimicrobial profile against clinical pathogens and it revealed highest activity against maximum pathogens. The radical scavenging activity for the colored metabolite was determined through DPPH, metal chelation, reductive power and total antioxidant capacity. The colored metabolite disclosed 50% of inhibitory activity at lower concentrations. Further the colored metabolite will be taken for bioprospecting studies and pharmaceutical applications.

INTRODUCTION

Micro organisms are the prominent sources for natural products especially secondary metabolites and they have been used for a long period for production of molecules as diverse as antibiotics, enzymes, vitamins and texturizing agents. There is a growing interest in the food industry for the use of natural ingredients such as metabolites (Nelis H J *et al*., 1991). Among the microbes, bacteria were ranking the first life forms to appear on Earth, and are present in most of the habitats. Bacteria constitute a large domain of prokaryotic micro organisms. Bacteria inhabit soil, water, acidic hot springs, radioactive waste and the deep portions of Earth's crust. They also produce different secondary metabolites under stressed conditions such as quinones, flavonoids, carotenoids, phenols, melanins, xanthophylls, etc. Bacterial metabolism can be classified into three major categories: the kind of energy used for growth, the carbon source, and the electron donors used for growth. Through these pathways it produces different metabolites for their survival. Among different types of bacteria *Exiguobacterium* sp was found to produce various secondary metabolites such as melanin, carotenoids and xanthenes. These metabolites can be used in industrial microbiology to obtain amino acids, develop vaccines and antibiotics, and isolate chemicals necessary for organic synthesis. Secondary metabolites are typically organic compounds produced through the modification of primary metabolite synthases.

Several investigations were conducted for the comparison of antibiotic activities among bacteria especially *Exiguobacterium* sp. From the broad classification of bacteria the potential strains have been taken for further research. These metabolites will prevent the cardiovascular diseases, cell death, diabetes and cancer as they contain more antioxidants. This will aim to be bioactive colored metabolite for food and pharmaceutical industries without causing any side effects for the human system. In the present stu
dy an attempt was made to isolate a potential pigmented bacterium for colored metabolite production with antioxidant activities.

MATERIALS AND METHODS

Isolation of bacterial strains

Two different soil samples were collected from a Steel Rolling Mills, Coimbatore, TN, India (latitude: 11.0183° N & longitude: 76.9725° E) were serially diluted from 10^{-2} to 10^{-8} in sterile water using nutrient agar medium to isolate the bacterial by standard protocols for the growth of bacteria. Distinct colonies of bacteria were selected based on the pigmentation

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and sub cultured on fresh Nutrient agar media. Further, the isolates were maintained in 50% glycerol and incubated at -20 ^{o}C.

Screening of potential bacterial strains

The pigmented bacterial isolates were screened for antagonism by cross streak method against clinical pathogens of Gram positive and Gram negative bacteria such as *Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris* and *Shigella* sp which are facultative anaerobes and further the inhibition zone was measured.

Phenotypic characterization

The potential antagonistic bacterial strain was taken for morphological, biochemical and physiological characterization were determined by following the directions of **Bergey's Manual of Bacteriology**. The macro and micro morphological characteristics were identified by culturing the organism in Nutrient agar media and also by negative staining, Gram's staining and endospore staining respectively. Further, the biochemical characterization was done by following the method described by Shirling and Gottlieb, (1966). The physiological characterization was evaluated as the strain may be sustained to specific/ or stressed environments which may enhance their metabolic activities and this characterization was determined by growing the strain in Nutrient broth at varying pH (3, 4, 5, 6, 7, 8, 9), temperature (15^{o}C, 25^{o}C, 35^{o}C, 45^{o}C, 55^{o}C) and salt tolerance (NaCl concentrations – 0.1% to 10%). The carbohydrate fermentation was tested with reducing and non reducing sugars such as dextrose, lactose, maltose, mannose and sucrose. The maximal growth and pigment production characteristics were determined using different growth media Nutrient broth, Mueller Hinton broth, Mac Conkey broth, Soybean Casein Digest broth and King's broth media. The strain was identified as per the directions stated earlier.

Genotypic characterization

The isolated strain BC2-11 was characterized by Marmur (1961) using using PCR primers f(5'-GCCCACGACCAGTTCGAC-3') and r(5'-CATCCCCCTCCCTATGAC-3'. The amplified sample was taken for 16s rRNA sequencing. The 16s rRNA gene sequence obtained was trimmed and aligned using DNAStar software. The aliened sequence was used for database similarity search using BLAST in NCBI gene bank. The aligned 16s rRNA gene sequence of isolated strain BC2-11 and sequence of various type strains were taken in FASTA format and edited by multiple sequence alignment using Bio-edit software. Then the phylogenetic tree was constructed and the sequence was submitted in Genebank and accession number was obtained.

Production and extraction of colored metabolite

The bacterial strain BC2-11 inoculated in 100mL of Nutrient broth in 2x250mL Erlenmeyer flasks (seed medium) and incubated at 37^{0}C for 18 hrs at 120 rpm on a reciprocating shaker. After incubation, Nutrient broth enriched with 1% dextrose served to be production media and 250mL of this stated media was prepared in 10x500mL Erlenmeyer flasks which were inoculated with 2% v/v seed medium and incubated at 37^{0}C for 36 hrs. Culture broth was centrifuged at 10,000 rpm for 15 min at 4°C. The crude metabolite extract were maximum in biomass when compared to cell free culture filtrate. The yield of crude colored metabolite was measured using the formula 1 and it is expressed in Units litre per gram. Using different polar and non polar solvents the colored metabolite was extracted from biomass. About one gram of the biomass was added to 50 ml of solvent and homogenized. Formula 1: Yield of metabolite = $\frac{O.D \times V \times D}{x}$ (O.D – Optical Density units; V- Sample submitted for metabolite extraction; D - 50ml of the solvent taken for cell disruption).

Separation of Bioactive compounds

Thin Layer Chromatography (TLC) was carried out to find out the separation of compounds from colored metabolite extract efficiently using different solvent systems (Odakura *et al.*, 1984) and the R_f value of each compound separated was calculated. The crude metabolite extract was also subjected to HPTLC analysis using winCATS Planar Chromatography Manager using the plate size 10x10 cm. The solvent type is methanol and the spray gas was inert gas. The syringe size was 100µl and number of tracks was 6. The mobile phase was chloroform: methanol (45:55).The sample used for the analysis was 0.2µl. The plates were measured at 254 nm.

Biological activity of colored metabolite extract

Antimicrobial profile and MIC for extract

The antimicrobial activity and Minimum Inhibitiory Concentration (MIC) were done by well diffusion method (Cappuccino and Sherman, 2004) against the clinical pathogens on Mueller Hinton agar plates. Different concentrations (10µL to 40µL) of aliquots of the crude colored metabolite extract were added in respective wells. The diameter of inhibition was determined after 24 hrs of incubation at 37^{0}C for bacteria.

Antioxidant activity

DPPH radical scavenging assay

The effect of colored metabolite extract on DPPH radical was determined using the method of Szabo *et al.* (2007). Different concentrations of the colored metabolite extract (50 to 100 µg/ml) were prepared and subjected to antioxidant tests. To 1 ml of each concentration of the extract, 5 ml of 0.1mM methanol solution of DPPH was added, vortexed and followed by incubation at 27^{o}C for 20 min.

The control was prepared without any extract and absorbance of the sample was measured at 517 nm using UV/VIS Spectrophotometer using methanol to set 0. The ability to scavenge DPPH radical was calculated by the following equation: DPPH scavenging effect (%) = $[(A_0-A_1)/A_0] \times 100$; Where A_0 was the absorbance of the control reaction and A_1

was the absorbance in the presence of the standard sample or fraction.

Reductive ability

This was carried out as described by Yildirim *et al*., 2001. Colored metabolite extract was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml 1% potassium ferricyanide. The reaction mixture was incubated at 50ºC for 30min. Afterward 2.5 ml of 10% trichloroacidic acid was added to the mixture and centrifuged for10 min at 3000 rpm. 2.5 ml of upper layer solution was mixed with 2.5ml of distilled water and 0.5 ml of 0.1%ferric chloride. Absorbance was measured at 700nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Chelating effects on ferrous ions

The method of Dinis *et al*., 1994 has been used to estimate the chelating effect on ferrous ions with some modifications. 0.5 ml of various concentrations of colored metabolite extract was mixed with 0.5 ml of $FeSO_4$ (0.12 mM) and 0.5 ml of Ferrozine (0.6 mM). The mixtures were allowed to stand for 10 min at room temperature. After incubation, the absorbance was measured at 562 nm. Ultra-pure water with ferrozine solution was used as a control without crude extract and sterile distilled water without ferrozine solution was used as a blank. EDTA-Na_2 was used as reference standard. All measurements were performed in triplicate. The ferrozine solution (3-[2-Pyridyl]-5, 6-diphenyl- 1, 2, 4-triazine-4,4´-disulfonic acid Na- salt) (0.6 mM) was prepared in Ultra-pure water and stored in the dark at room temperature. The ability of the sample to chelate ferrous ion was calculated relative to the control (consisting of iron and Ferrozine only) using the formula: Ferrous ion - chelating ability (%) = [(Abs control − Abs sample) / Abs control] ×100.

Total Antioxidant activity

The total antioxidant activity of the colored metabolite pigment extract was evaluated by the phospho molybdenum method - Prieto *et al*., 1999. The assay is based on the reduction of Mo (IV) - Mo (V) by the extract and subsequent formation of green phosphate Mo (V) complex at acid pH, 2ml of the sample was combined with 1ml of reagent solution (0.6M of sulphuric acid, 28mM sodium phosphate, 4mM ammonium molybdate). In case of the blank methanol are used in place of sample. The tubes containing the reaction solution were capped and incubated in water bath at 95ºc for 90min. The samples were cooled to room temperature and the absorbance of the solution was measured at 635nm using spectrophotometer against blank. The antioxidant activity was expressed as an equivalent of Ascorbic acid. All the measurements were measured in triplicate.

RESULTS

Isolation of bacteria

The research aimed to isolate the colored bacteria from stressed conditions of terrestrial environment. Two soil samples were taken from Steel plant, Coimbatore. About 14 bacterial isolates (BC1-1, BC1-2, BC1-3, BC2-1, BC2-2, BC2-3, BC2-4, BC2-5, BC2-6, BC2-7, BC2-8, BC2-9, BC2-10 and BC2-11) were isolated from 2 soil samples. From that 7 were pigmented bacterial strains (BC1-2, BC1-5, BC2-2, BC2-3, BC2-4, BC2-7, and BC2-11).

Screening for antagonist activity

All the seven colored strains were taken for antagonist activity and among the seven one strain BC2-11 showed highest activity against 5 clinical pathogens such as *E. coli*, *S. typhi*, *S. aureus*, *P. aureginosa*, *P. vulgaris* and *K. pneumoniae* with maximum zone of inhibition. BC2-11 showed maximum inhibition of 4.3 cm and 3.1 cm against *P. vulgaris* and *S. typhi* respectively. *E.coli* and *P. aureginosa* was susceptible to BC2-11 forming zone of inhibition at 2.6 cm and 2.4 cm respectively.

Phenotypic characterization

The morphological characterization for the strain BC2-11 was observed microscopically under 400x and 1000x light microscopy was Gram positive, non sporulating rods occurring singly, in pairs or in short chains and motile by means of its peritrichous flagella. The macroscopic observation of the isolate was creamy, circular and convex colonies were appeared after 36 hours of incubation.

The colonies were creamy orange in colour. The biochemical characterization for BC2-11 bacteria was observed to be negative for IMVC tests, nitrate reduction, TSI, oxidase, starch hydrolysis and casein hydrolysis test and positive for catalase and gelatin hydrolysis test. The strain BC2-11 was tolerable to acidic and alkaline pH from 3 to 9 and relatively good growth and metabolite production was observed from pH 5 to pH 9. The maximum growth and colored metabolite production was exhibited in pH 8 which was $7.3gL^{-1}$ and 1.64 UL^{-1} respectively and this is the optimum pH for the strain growth.

The organism BC2-11 was adaptive to a wide range of temperature and the maximum biomass and colored metabolite production was evaluated at 37ºC (Ambient temperature) of $6.8gL^{-1}$ and 1.25 UL^{-1} respectively.

The isolated strain was tolerable to 6% of NaCl concentration and this meant that the strain was sustainable to different stressed environments. The reducing sugars such as dextrose, fructose and maltose were fermented by the strain BC2-11. The strain produced maximum biomass and colored metabolites in Nutrient media and this media was found to be efficient for mass production. From the results of morphological, biochemical and physiological characterization the strain was confirmed that it belonged to *Exiguobacterium* sp.

Genotypic characterization

16S rRNA gene sequence was amplified for the strain BC2-11. A BLAST search of the GenBank database (available at NCBI) with this sequence showed its similarity to that of many species of the genus *Exiguobacterium profundum* (Genebank accession No.: KP966110). A phylogenetic tree based on 16S rRNA gene

sequences of members of the genus *Exiguobacterium* was constructed using PHYLIP package.

Production and extraction of colored metabolites

The colored biomass (biomass contained maximum colored metabolites than culture filtrate) was harvested by centrifugation process at 10,000 rpm for 10 minutes. The biomass was processed with sterile distilled water. The colored metabolite was extracted using polar solvent methanol. From the mass cultivation in production media, the biomass and colored metabolite produced were 6.8g/L and 1.53U/L respectively. Further the colored metabolite extract was assessed for biological studies.

Separation of bioactive compounds

TLC was evaluated using 3 different solvent systems such as methanol: chloroform, ethyl acetate: petroleum ether and hexane: methanol and maximum separation of four compounds was found to be with methanol: chloroform (45%). Six peaks were obtained from HPTLC in which 5^{th} peak was observed to be highest peak, the total area was 5973.3 and the percentage of area 47.39%. The R_f value was 0.98 (Fig 1).

Biological activity of colored metabolite extract

Antimicrobial activity

The antimicrobial profile was determined for the colored metabolite extract. *S. typhi* was found to be highly susceptible to the methanolic extract with the zone of inhibition of 2.4 cm. The colored metabolite extract inhibited *P. aeruginosa* with the inhibition zone of 1.7 cm. The maximum zone of inhibition of 2.7 cm and 1.5 cm were also observed against *E. coli* and *K. pneumoniae* respectively. The least concentration which showed maximum activity was considered to be MIC (Table 1). The profile was assessed with methanol as a control.

Antioxidant activity

DPPH antioxidant assay

DPPH free radical determines the free radical scavenging capacity or antioxidant potential (AOD) of the pigment sample, which shows its effectiveness by prevention, interception and repair mechanisms against injury in a biological system (lee *et al.*, 2001). In this investigation, the IC_{50} value of colored metabolite extract was found to be 870µg/mL concentration. It means that the colored metabolite had good scavenging activity at least concentration which is equivalent to standard ascorbic acid (Fig 2).

Reductive ability

The reducing capacity of the compound may serve as a significant indicator of potential antioxidant activity. For the measurement of the reducing ability, the Fe^{3+}, Fe^{2+} transformation was investigated using colored metabolite extract. The result showed that colored metabolite extract consists of hydrophilic poly phenolic compounds that cause the greater reducing power and this was estimated by obtaining the Optical Density reading from UV/VIS Spectrophotometer (Table 2).

Ion chelating ability

Ferrous ions chelating activity was employed as another indicator to assess the quantity of the antioxidant (Lianhe *et al.*, 2011). It has been recognized that chelating agents may inhibit lipid oxidation by stabilizing transition metals. In the Fe^{2+} chelating assay, the chelating activities of the antioxidants are assayed by inhibiting the formation of red-colored ferrozine–Fe^{2+} complex. The chelating activity of colored metabolite extract of *Exiguobacterium profundum* BC2-11 was observed at 951 µg/mL which exhibited the IC_{50} activity (Fig 2).

Total antioxidant activity

Total Antioxidant activities of the colored metabolite extract was evaluated by phosphomolybdate method. The phosphomolybdate method has been routinely used to evaluate the total Antioxidant capacity of the extract in the presence of the colored metabolite extract then MO (IV) is reduced to MO (V) and forms a green coloured phosphomolybdate V complex which showed maximum absorbance at 695nm. The colored metabolite extract showed the total antioxidant capacity at 980µg which is equivalent to the standard ascorbic acid.

winCATS Planar Chromatography Manager

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.00	0.1	0.01	17.3	3.27	0.02	3.3	124.4	0.99	unknown *
2	0.02	4.5	0.03	192.8	36.39	0.08	13.0	2820.1	22.37	unknown *
3	0.62	1.8	0.64	20.9	3.95	0.66	3.5	201.8	1.60	unknown *
4	0.78	3.9	0.85	50.4	9.51	0.89	21.4	1870.7	14.84	unknown *
5	0.90	23.8	0.98	143.7	27.13	1.01	90.1	5973.3	47.39	unknown *
6	1.01	90.1	1.02	104.6	19.75	1.04	0.4	1613.6	12.80	unknown *

Fig 1 TLC & HPTLC of colored metabolite extract

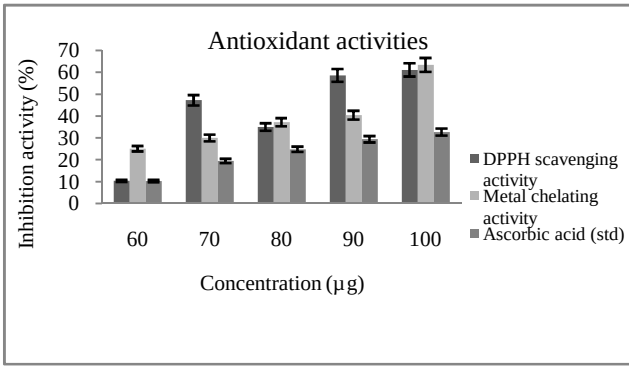

Fig 2 Antioxidant activities – DPPH and Metal chelation activity

Table 1 Antimicrobial activity of colored metabolite extract

S.No	Pathogens	Zone of inhibition(in cm)			
		10µl	20µl	30µl	40µl
1.	*Klebsiella pneumoniae*	0.5	0.7	1.2	1.5
2.	*Salmonella typhi*	1.3	1.7	2.1	2.4
3.	*Staphylococcus aureus*	-	-	0.6	0.7
4.	*Escherichia coli*	1.2	1.9	2.5	2.7
5.	*Pseudomonas aeruginosa*	0.7	1.0	1.3	1.7
6.	*Proteus vulgaris*	-	-	-	-
7.	*Shigella* sp	-	-	-	-

Table 2 Reductive ability

Sl.No.	Concentration of BC2-11 (µl)	OD Value
1.	20	0.022±0.06
2.	40	0.04±0.012
3.	60	0.26±0.072
4.	80	0.54±0.06
5.	100	0.78±0.1

DISCUSSION

The bacterial strains were found to inhabitant on soil environment. The iron and steel industry causes significant effects on environmental media such as air emissions, soil pollution by sedimentation and water pollution of settling up of organic matter, oil, grease, suspended solids, benzene, phenols, acids and ammonia. These interact with the soil community and the community includes plants, microbes and water source. These conditions drastically affect the microbe which leads to produce different molecules and metabolites for their survival between the microbial communities. Because of these serious problems the micro organisms were able to develop different pathways for synthesizing different metabolites.

In this study, the colored metabolite producing bacterial strain BC2-11 was isolated from the steel mills containing stressed conditions at Coimbatore and the isolated strain was a potent one when compared to other strains through antagonist activity against 9 clinical pathogens. Based on the morphological, biochemical, physiological and molecular characterization, the strain was identified as *Exiguobacterium profundum* BC2-11. The strain does not produce endospore and it is a Gram positive rod shaped bacteria and also the biochemical tests determined that the strain belongs to *Exiguobacterium* species through the reference of Bergey's manual with morphological, biochemical and physiological characterization. As the organism does not produce endodspore, it is considered to be non virulent organism. The strain was tolerable to varying pH range so this proves that it was adaptive to stressed environment with acidic/ or alkaline conditions. The metabolite production was maximum when temperature was set to 37±°C. *Exiguobacterium* sp. grew maximally when incubation temperature was set to 30 °C (Arora *et al*., 2012), while maximum colored metabolite production at 30 °C was recorded by *Serratia* sp. and *Micrcococcus* sp. (Hardijito *et al*., 2002 and Ibrahim, 2008). The 16s rRNA sequencing confirmed the strain BC2-11 is *Exiguobacterium profundum.* This strain was reported to produce the carotenoids (Fatima *et al*., 2013). The colored metabolite was extracted using methanol when compared to other solvents. The same was identified in a research that the methanol was very efficient in the extraction and quantification of metabolite from the autotrophic cell cultures Britton (1985) and Fikselova *et al.* (2008) recommended water miscible organic solvents, such as ethanol, methanol and acetone, to extract orange colored metabolite such as carotenoids. The colored metabolite was taken for the determination of antimicrobial activity and from that it is evident that this colored metabolite has the ability to inhibit the growth of *S. typhi*, *E. coli* and *P. aureginosa* with the inhibition zone of 2.1 cm, 2.5 cm and 1.3 cm respectively. Tao *et al*., (2010) mentioned that carotenoids extracted from *Shatian pumello* had antifungal activity. Ma *et al*,. (2004) also mentioned that the soluble pigment in polar solvents extracted from citrus peel had strong antimicrobial activity. This proves that the strain produces different metabolites and this is the reason for showing maximum inhibition activity through antimicrobial assay. The extracted colored metabolite was subjected to evaluate antioxidant properties through DPPH assay, reducing power assay, iron chelating assay and total antioxidant activity. The IC_{50} concentration for the stated assays was found in a range of 860µg/ml to 1000µg/ml. For the sample concentration which is less than standard may be due to the other compounds which interferes the bioactive compound. If the crude is purified, the IC_{50} value will be greater than the standard ascorbic acid. The colored metabolite was further taken for partial purification by HPTLC and from that six bands were obtained. The strain *Exiguobacterium profundum* BC2-11 was found to produce orange colored metabolite which may contain carotenoids. Concluding the investigation, the stressed environmental soil had majority of micro organisms which were prevalent in producing different metabolites for their survival between microbial communities. The isolated *Exiguobacterium profundum* BC2-11 produced orangish metabolite which possessed the antimicrobial as well as possesses the antioxidant property. So we suggest that this orangish metabolite can be taken as antibiotic in medical fields. Further the colored metabolite extract has to be purified and studied for characterization of the colored compound also to be done to make it a potential drug.

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