Advances in Bioresearch
Adv. Biores., Vol 13 (4) July 2022: 62-75
©2022 Society of Education, India
Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
DOI: 10.15515/abr.0976-4585.13.4.6275

Advances in Bioresearch

ORIGINAL ARTICLE

Isolation and Characterization of Marine fungi for their Bioactive pigments

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ABSTRACT
Microorganisms are known resource for the pigment production due to their stability, availability, cost efficacy, downstream processing and yield. The elevated biological activities of natural pigments are incited to evaluate their biological importance against various pathological indices. In this regards, the study aim to evaluate the antibacterial and antioxidant activity of pigments extracted from marine fungi. The marine fungi PC, CL and PP isolated from marine sediments, were identified as Rhodotorula evergladensis, Aspergillus sydowii and Talaromyces coalescens, respectively through ITS sequence similarity analysis. Then, the pigments were extracted from the fungal isolates and their purity was verified using HPLC chromatogram. Further, the pigments from PC and CL found to possess promising antibacterial activity against human pathogens like Escherichia coli, Bacillus cereus, Shigella sp, Salmonella typhi and Klebsiella pneumoniae. But, the pigment from PP did not show such conspicuous antibacterial activity. Further, DPPH and peroxide ion scavenging activity showed 65% and 70% of antioxidant potential for PC and PP pigment extracts respectively. When the extracts were subjected to reducing power assay, CL pigment extract showed 50% reduction to ferrous ions, but PP and PC showed only 30% reducing activity. Thus, the study revealed that the pigments of R. evergladensis (PC)and T. coalescens (PP) are found to have promising antioxidants activity besides antibacterial activity. Among the fungal strains, the pigment from A. sydowii (CL) also exhibited a better metal ion reducing property. Therefore, these fungal pigments can be harnessed in textile, food processing and other beverage producing industries.
Keywords: *Marine fungi, Natural pigments, Antibacterial activity, Antioxidants*

Received 24.06.2022 Revised 20.06.2021 Accepted 20.07.2022

How to cite this article:
S Lingappa, S Ganapathi, S Awasthi, S T Somasundaram. Isolation and Characterization of Marine fungi for their Bioactive pigments. Adv. Biores. Vol 13 [4] July 2022. 62-75

INTRODUCTION

The marine ecosystem is a rich source of diverse micro and macroorganisms. Marine microbes are highly associated with an evaluation of the ecosystem and evolve themselves to survive in all kinds of ecosystems. These microbes can be synthesis an array of unique and potential functional biomolecules and are highly present in silty, muddy and soil sediments[1]. Marine fungi are found in various regions such as coastal, estuarine, and offshore regions of marine environment and are also present in all marine environment, including marine plants and animals [2]. These marine microbes can secrete various intra and extracellular biomolecules to sustain in different environmental stress factors [3]. It has been reported that the marine yeasts are capable to producing many bio-active substances, such as amino acids, glucans, glutathione, toxins, enzymes, phytase and vitamins. These molecules could be used in biomedical purposes, food processing, as well as chemical industry like metal detoxification, textile industries and cosmetics [3-4].

Fungi are a good resource of microbial pigments and most of these species can be identified by their distinct colour patterns with pink to coral and also orange to red. Biotech industries are interested in microbes as a source for production of different high value pigment components, such as β- carotene, torulene, and torularhodin [5]. Different species of fungi such as *Rhodotorula glutinis*, *Cryptococus sp.*, *Phaffia rhodozyma,* and *Yarrowia lipolytica* are found to produce different types of pigments [6]. Due

tothe growing evidence of health benefits, fungi are harnessed for one of the high value products like carotenoids. Because of the immunopharmacological and anti-inflammatory properties of carotenoids, they have also been used as anticancer and free radical scavenging agents[7].
The microbial pigments were studied for wound healing property on excision wound models. The ointment preparation of pigment extract has found to show significant wound healing activity in albino rats[8]. In addition, the bacterial pigments xanthomonadin and staphyloxanthin were found to protect photodamage by inhibiting the photodynamic lipid peroxidation and prevent the carbon tetrachloride induced oxidative stress in swiss albino mice, respectively[9].
In recent times, the treatment of various disease causing pathogenic microorganisms were found to be ineffective because of their multidrug resistance (MDR)properties. Therefore, it becomes inevitable to develop better antimicrobial agents against MDR pathogens [10].The investigation of such agents from natural resources is a valid one to tackle such emerging MDR pathogenic microbes [11].Several studies have been demonstrated that the microbial pigments can be considered as potential agents in treating different infectious diseases including antifungal, antiviral and antiprotozoal activities[12-13].
Moreover, the microbial pigments showed anticancer activity through apoptosis cascade activation and cell cycle inhibition in different cell strains [14]. Prodigiosin from *Serratia marcescens* was significantly effective against standard 60 cancer cell lines derived from lung, colon, renal, ovarian, brain, melanoma and leukemia [11]. In this context, this study was carried out to extract the pigments from marine fungi to evaluate their antioxidant and antibacterial properties. Thus, the isolated pigments showed potential antibacterial activity against five different human pathogens and also showed antioxidant and ferrous reducing properties. Hence, the fungal pigments are potential source for their application in the field of pharmacology, textiles, food processing and other beverage producing industries.

MATERIAL AND METHODS

Sample collection

Sediment samples were collected from Parangipettai coast (11°30'20.4" N, 79°46'37.5" E), at the South-East coast of India. The samples were collected in sterile containers from the coast and stored in an ice-box. Samples were brought to the laboratory for microbial analysis.

Isolation of marine fungi

Ten grams of each sample was diluted in 50ml of distilled water in conical flask. Serial dilution was carried out up to 10^{-7} dilution. An aliquot of 0.1ml of each dilution was spread plated on Wickerham's agar medium prepared with malt extract 3g, yeast extract 3g, peptone 5g, glucose 10g, Chloramphenicol 200mg and agar 20g in 1 litre of 100% seawater (35ppt). The inoculated plates were incubated for 14 days at 28±2°C. Chloramphenicol at the rate of 200mg/L was added as an antimicrobial agent to inhibit all bacteria growth. Isolates were sub-cultured on Wickerham's agar to check for purity and incubated at 28±2°C for a week. Purified cultures were routinely maintained on yeast malt agar medium.

Biochemical characterization of Marine fungi

Biochemical analysis for the selected fungi isolates labelled as PP, PC and CL was carried out using the method of Barnett et al. [15].

Assessing the ability to utilize Nitrogen for their growth: Nitrogen is a valuable nutrient and aid for yeasts cell growth in a fermentative condition. A mineral basal medium supplemented (KH_2PO - 2g, $MgSO_4.7H_2O$ - 0.5g, Ca_2HPO_4 - 0.5g, glucose - 20g, KNO_3 - 1g, NaCl - 20g/ 1lit.) with glucose as a carbon source and KNO_3 as the sole nitrogen source were employed for the test. The cultures were inoculated in the medium and incubated at 28±2°C for a week. The positive result was indicated by growth and turbidity in the tube.

Assessing the ability to utilize sugar under aerobic/ anaerobic conditions: The yeast cultures were inoculated in marine oxidation fermentation (MOF) medium to identify them either as aerobic (oxidative) or anaerobic (fermentative) in nature. The colour change from pink to yellow in the medium showed that dextrose was utilized and the medium became acidic. Yellow colouration at the slope region indicated an oxidative reaction, whereas the whole tube turning yellow revealed a fermentative reaction. Phenol red acted as an indicator.

Urea Hydrolysis: Urea hydrolytic ability of isolated yeast was also analysed. The cultures were grown on yeast malt agar and were transferred to fresh slants before testing. Fresh cultures from the slants were transferred to the surface of urea containing a medium with the following ingredients: Yeast extract - 0.1g, KH_2PO-9.1g, Na_2HPO_4 - 9.5g, NaCl - 20g, urea - 20g, agar-20g and 0.25% phenol red (4ml). All of the above mentioned ingredients were dissolved in 950ml of distilled sea water and autoclaved at 15lbs for 15min. Urea was dissolved in 50ml of sterile distilled water, which was added with the basal medium

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after autoclaving. The contents of the tubes were allowed to solidify with a long slant and a deep butt. The inoculated tubes were incubated at the optimal temperature (28 to 30°C) for one week. Urea hydrolysis was indicated by a distinct colour change.

Hydrolytic Enzyme production: The cultures were tested for the production of extracellular enzymes, i.e., amylase, lipase, protease, ligninase, cellulase, pectinase and chitinase.

Amylase activity: Nutrient agar plates were prepared with 1% starch. The isolates were spot inoculated and incubated at room temp (28±2°C) for one week. After incubation the plates were flooded with grams iodine solution (Iodine 1g and potassium iodide 2g in 300ml distilled water) and the presence of clear zone was noted as positive and the diameter of the zone was recorded.

Cellulase activity: Cellulose agar (Casein hydrolysate - 0.05g, yeast extract - 0.05g, $NaNO_3$-0.01g, cellulose powder - 0.5g, agar - 2g, sea water - 100ml, pH–7.0) was prepared and used for testing cellulase production. The plates were spot inoculated and incubated at room temperature (28±2°C) for 7 to 10 days. The zone of clearance around the colonies was observed and the result was confirmed as positive.

Pectinase activity: Pectin agar (pectin - 0.5g, $CaCl_2.2H_2O$ - 0.02g, NaCl - 2g, $FeCl_3.6H_2O$ - 0.001g, yeast extract - 0.1g, agar - 2g, distilled water - 100ml, pH 7.0) was prepared and used for testing the production of pectinase. The plates were spot inoculated and incubated at room temperature (28±2°C) for 2 weeks. After incubation the plates were flooded with 1% cetavlon and the zone of clearance was noted as positive result. 1% aqueous cetavlon was allowed to stand for 20-30 min.

Ligninase activity: Crawford's agar (glucose - 0.1g, yeast extract - 0.15g, Na_2HPO_4- 0.45g, KH_2PO_4 - 0.1g, $MgSO_4$ - 0.002g, $CaCl_2$ - 0.05g, agar - 2g, sea water - 100ml, pH 0.7) was prepared and used as the basal medium for testing lignin degradation. The basal medium was supplemented with 0.5% tannic acid and the plates were spot inoculated and incubated at 28±2°C for 7 to 14 days. Formation of a halo zone or brown colour around the colonies was considered as positive.

Molecular Identification of marine fungus

Molecular identification is known to provide generic level specificity to identify microorganisms at species level, therefore the cultures were subjected for molecular screening with ITS sequences. Genomic DNA was isolated from yeast cells as shown by Hanna et al. [16]. Individual colonies were segregated and purified for DNA isolation. Pure colonies were inoculated into YM media containing 1% yeast extract, 2% peptone and 2% dextrose (in seawater, 35 ppt salinity) and incubated for 32 hrs. Cells from 1.5ml of the culture medium were pelleted in a micro centrifuge tube at 3000 rpm for 3 minutes and the cell pellets were resuspended in 200µl of lysis buffer [2% Triton X-100, 1% SDS, 100m NaCl, 10mM Tris – HCl (pH 8.0), 1mM EDTA (pH 8.0)]. The tubes were vortexed at high speed for 3 minutes with 0.4 g of acid-washed glass beads and 200 µl phenol: chloroform: isoamyl alcohol (25:24:1). The tubes were centrifuged at 15,000 rpm for 10 minutes at room temperature. The aqueous layer was transferred to a tube containing 400 µl of ice cold 100% ethanol. The samples were allowed to precipitate DNA for 5 min at room temperature and then centrifuged at 15,000 rpm for 10 minutes. The supernatant was discarded and DNA pellet was washed with 500µl of 70% ethanol followed by vacuum drying for 5 minutes at 60°C. DNA was resuspended in 20µl of TE buffer (10mM Tris and 1mM EDTA, pH 8.0) and stored at 4°C for future use. DNA concentration as well as purity was assessed spectrophotometrically at 260nm followed by 1 % agarose gel electrophoresis, and finally, concentration of the DNA was calculated (Conc. of DNA (µg/ml) = OD at 260nm x 50 x dilution factor).

Amplification of ITS sequence: The ITS region was amplified by the universal primer pair V9G and LS266R designed by Mannarelli and Kurzman [17].The PCR amplification was performed using Master cycler gradient (Eppendorf). The PCR reaction was performed in a 25µl reaction mixture containing 2.5µl of 10x PCR buffer, 1.0µl of 10µM of each nucleotide primers V9G – 5'TTACGTCCCTGCCCTTTGTA3' (forward primer) and LS266 – 5'GCATTCCCAAACAACTCGACTC3' (Reverse Primer), 1µl of 200mM each deoxyribonucleoside triphosphate, 1µl of template DNA (540ng/µl), 1µl of 1U of Taq DNA polymerase and 14.5µl of autoclaved Milli-Q water. After an initial denaturation at 94°C for 4 minutes, amplification was made through 35 cycles, each consisting with the denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 2 minutes and a final extension was made at 72°C for 5 min. The PCR products were observed by electrophoresis on 1.5 % agarose gel prepared with ethidium bromide in 1x TAE buffer.

Cultivation, extraction and purification of fungal pigments

The isolated fungal species were cultured individually in 500 mL of yeast malt broth medium prepared in seawater. All the flasks were inoculated and incubated at 28±2°C in the dark for 10 days in a rotary shaking incubator at 200 rpm. Then, the yeast cells were harvested, and the supernatant was filtered in a sterilized muslin cloth. Later, two volumes of 95% (v/v) ethanol were added to exhausted culture broth according to the following procedure: (i) after dilution with 60% of the solvent, the resulting mixture was

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kept on the rotary shaker at 180 rpm at 30°C for 30 min; (ii) the ethanolic mixture was centrifuged at 3500g for 15 min; (iii) the supernatant had been recovered, the residue was dispersed in the remaining volume of ethanol and centrifuged again at 3500g for 5 min; and (iv) the supernatants were then collected and filtered through a pre-weighed Whatman GF/C filter paper (47 mm).The filtered pigments were concentrated in a rotary evaporator and lyophilized to obtain powder.

Determination of total protein: The protein concentration in the pigment extracts was estimated according to Lowry et al. [18]. against bovine serum albumin standard (BSA). Lowry's solution was prepared by mixing 2% alkaline sodium carbonate solution in 0.1 N NaOH, 1.56% copper sulphate solution and 2.37% sodium potassium tartrate in the ratio of 48:1:1. BSA test concentration (200-1000 µg/mL) was prepared from the stock BSA solution (1mg/ml) and 0.5 ml of unknown pigment samples taken in the test tubes and diluted to 1 mL final volume with distilled water. Then, 2ml of Lowry's reagent was added in the test tubes and incubated for 10 minutes at room temperature. 0.2 ml of 2N folin-phenol solution was mixed and incubated at room temperature for 30 minutes and the absorbance was measured at 660nm.A standard graph was plotted and the concentration of the pigment extracts was estimated using the standard graphs.

Estimation of total phenol: The amount of phenol in the crude pigment extract was determined with Folin-Ciocalteu reagent [19]. 2.5 ml of 10% Folin-Ciocalteu reagent and 2 ml of Na_2CO3 (2% w/v) were added to 0.5 ml of each sample (3 replicates). The resulting mixture was incubated at 45°C with shaking for 15 min. The absorbance of the samples was measured at 765 nm using UV spectrophotometers. Results were expressed as milligrams of Gallic acid (0-0.5 mg/ml) equivalent.

Estimation of total flavonoid: Total flavonoid content (TF) was evaluated according to a colorimetric assay [20].1 ml of diluted pigment extract was added in a 10 ml volumetric flask containing 4 ml of distilled water, followed with addition of 0.3 ml $NaNO_2$solution (0.5 g/l). After 5 minutes of incubation, 0.3 ml of $AlCl_3$ solution (1g/l) was added followedby further addition of 2ml of NaOH solution (1mol/ l)into the mixture. The total volume was made up to 10 ml with distilled water. The absorbance was measured against the blank at 510 nm. Gallic acid was used as the standard and the concentration of TF was expressed in mg/l as gallic acid equivalents.

Characterization of marine pigments

Thin Layer Chromatography (TLC)

The crude pigment extracts were subjected to thin layer chromatography (TLC) on silica gel 60 F254 coated aluminium plates (Merck, Germany). This was carried out to find out the separation of pigments from the crude methanolic extract using different solvents as mobile phase such as methanol: water (1:1) and water: methanol: acetonitrile (5:75:20). The solvent system was used to separate bioactive compounds from the crude extract. The solvent and solute front were marked to find the Rf values of the individual bioactive pigments.

High Performance Liquid Chromatography (HPLC)

The HPLC analysis was performed for the extracted pigments. The lyophilized pigments were diluted in methanol and filtered through a 0.45 µm syringe-driven filter. Then 20 µL samples were injected to reverse phase-high-performance liquid chromatography (RP-HPLC) on a C18 column (250 × 4.6 mm, 5 mm; SHIMADZU). The column was pre-equilibrated with 0.1 % TFA in water and then the samples were eluted with 75% methanol, 5% acetonitrile solvents in water with a flow rate of 1 mL/min for 60 minutes. The chromatograms were monitored and recorded at 254 and 370nm in UV detector.

Biological activities of pigment extracts

Antimicrobial activity

The antimicrobial activity was determined by the disc diffusion assay using Muller Hinton agar plates [21]. The plates were swabbed with human pathogens *E coli*, *Bacillus cereus*, *Shigella sp.*, *Salmonella typhi* and *Klebsiella pneumoniae* procured from Rajah Muthiah Medical college, Annamalai University, Chidambaram, Tamilnadu, India. The discs were prepared with 10mg/ml concentration of pigment extracts and placed on the plates. The plates were incubated for 24 hours at 37°C and the zone of inhibition around each disc was measured for sensitivity and resistance. Diameters of the inhibition zones were measured. The antibacterial activity was expressed as the mean zone of inhibition diameters (mm) produced by pigments. The antibacterial activity of the pigments was compared with standard amoxicillin (Amx.).

Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity for the pigment extract was determined by the method of Ruch et al. [22]. A plant extract was prepared in distilled water mixed with 0.6 ml of 4 mM H_2O_2 at various concentration. Solutions were phosphate buffered (0.1 M pH 7.4) and incubated for 10 min. Their

absorbance was read at 230 nm against blank solution containing the extract without H_2O_2. Ascorbic acid was used as a reference positive control.

DPPH radical scavenging activity

The crude pigment extracts were aliquoted into 5, 10 and 15 mg/ml concentrations for determining their ability to scavenge 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals [23]. 800µl of DPPH solution (1mM DPPH radical solution in 95% methanol) was mixed with 200µl of crude extract, vortexed and incubated for 30 min at room temperature in dark. After 30 min incubation, the samples were centrifuged for 5 min at 13,500 rpm. Then, the absorbance of each supernatant was measured at517 nm against 95% methanol as a control. Ascorbic acid was used as a reference positive control. The antioxidant activity was given as percent (%) DPPH scavenging, calculated by the following formula:

$$\text{DPPH radical scavenging activity (\%)} = \frac{[(\text{Abs control} - \text{Abs sample})]}{(\text{Abs control})} \times 100$$

Determination of reducing power

The reducing power of the ethanolic extract was evaluated according to the method of Oyaizu et al. [24]. The presence of antioxidants in the pigments would result in the reduction of Fe3+ to Fe2+ by donating an electron and measuring the formation of Perl's blue at 700 nm. The reaction mixture containing 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of $K_3Fe(CN)_6$ (1%) with 1.0 ml of the extract dissolved in distilled water. The resulting mixture was incubated at 50°C for 20 min, followed by the addition of 2.5 ml of TCA (10% w/v). The mixture was centrifuged at 3000 rpm for 10 min to collect the upper layer of the solution (2.5 ml), mixed with distilled water (2.5 ml) and 0.5 ml of $FeCl_3$ (0.1%, w/v). Then, the absorbance was measured at 700 nm against the blank. Butylated hydroxytoluene (BHT) was used as a reference positive control.

RESULTS

Marine isolates from different coastal region

A totalof 15 different types of marine fungal colonies were isolated from sediment sample from Parangipettai coastal region of Tamilnadu. The pigment producing coloured colonies were separated from the isolates and studied for their pigment producing physicochemical properties. Among the isolates, three colonies were found to show intense colour pattern in high salt stress condition and were sub-cultured for pigment production. These colonies produced red, green and greenish black coloured pigments. Further, they were subjected to pigment extraction, characterization and evaluation of their biological activities.

Biochemical characterization of marine isolates

The marine isolates were subjected to biochemical analysis in different media. PC, CL and PP cultures utilized sugar and nitrogen as a sole nutrient for their growth. Besides, PC and CL cultures also assimilated urea as a nutrient for their growth, but pp culture did not show any growth in urea supplemented medium.

Then, the isolates were analysed for their hydrolytic enzyme production. The cultures were found to utilize different polysaccharides such as carboxy methyl cellulose (CMC), starch, tannic acid and pectin for their growth by producing extra cellular enzymes like cellulase, amylase, xylanase and pectinase. Moreover, the ability of extracellular enzyme production and catalase activity of these cultures were varied between the strains (Table 1). Based on the colonies colour, texture, size, shape and their biochemical activities of the cultures PP, PC and CL were identified as *Rhodotorulasp.*, *Aspergillus* sp. and *Talaromyces* sp., respectively. Then, the species level identification was also carried out through molecular tools.

Molecular identification of marine isolates

The fungal cultures were subjected to DNA isolation and PCR amplification of internal transcribed spacer(ITS) regions with specific nucleotide primers for their species level identification. Amplicons were confirmed by agarose gel (1%) electrophoresis. The genomic DNA bands were observed at positions close to the wells of agarose gel because of their higher molecular weight, approximately >3 kb in size. Extracted DNA was used as a template for ITS sequence amplification with the specific universal primers V9G and LS266. The PCR amplicons were found at 600 base pairs. ITS sequences were retrieved through the gene sequencer and identification of the species in the NCBI database was done by sequence comparative analysis. PC, CL and PP isolates were unambiguously identified in NCBI-nBLAST at species level with more than 99% sequence similarity with *Rhodotorula evergladensis, Aspergillus sydowii* and *Talaromyces coalescens,* respectively. Then, the processed sequences were submitted to the GenBank and published with the accession numbers MG063267 (PC), MG250598 (PP) and KY795028(CL).This

molecular identification of PP culture has confirmed it as *Aspergillus sydowii.* The CL culture *T. coalescens,* classified as filamentous fungi that also produced slimy yeast like colonies between 34 - 37°C.

Extraction of pigment from marine isolates

Extracellular pigments from PC, PP and CL were extracted, concentrated, lyophilized and quantified for the pigment production. The CL culture showed highest pigment production of 5.22 gram per 500mL followed by PP and PC produced 3.78 and 4.52 grams of lyophilized pigment per 500 mL of cultures, respectively. The lyophilized crude pigments were used for further characterization and evaluation of their biological activities.

Biochemical analysis of marine pigment

The total protein, phenol and flavonoid were estimated in the lyophilized pigments. The total protein content was very low in all PC, PP and CL extracts. The total phenol concentration was750µg/ml in PC, 630 µg/mL in PP and 815 µg/mL in CL pigment extracts. Later, the total flavonoid content was found about 213, 193 and 235 µg/mL in PC, PP and CL pigment extracts, correspondingly.

Thin layer chromatographic validation of marine pigments

The thin layer chromatographic (TLC) separation showed the pigments profile in two different solvent systems as depicted in table 2. Both solvent systems exhibited three prominent bands with different R_f values for the PC pigment extract. However, PP and CL showed only one spot with different R_f values for the same solvent systems. Therefore, the TLC result revealed that the PC pigment extract comprises a mixture of three different compounds, while PP and CL seem to have a single compound.

High performance liquid chromatography

The pigment extracts were also analyzed by HPLC to reveal their purity using a mixture of methanol, acetonitrile and water (75:5:20) as a mobile phase. The HPLC chromatogram showed only one major peak for PP extract at the retention time (RT) of 1.006 min for both, 254 and 370nm wavelength (Fig.1). CL pigment extract displayed a major peak at 1.175min and also displayed two minor peaks at 20.658 and 20.837min RT at 254nm.For the same CL pigment, the major sharp peak was observed at 1.176min and a minor peak at 0.865min of RT for 370nm(Fig. 2).

The HPLC result of PC pigment showed 3 major and 3 minor peaks at 254nm indifferent RT such as 0.998, 1.213, 1.516, 1.713, 15.915 and 18.823 min. Furthermore, the 370nm chromatogram exhibited one major and 3 minor peaks at the RT of 0.988, 1.225, 1.515 and 17.410min, respectively (Fig. 3). Therefore, the HPLC results revealed that the PC extract consists of a group of compounds but the other two extracts might be pure and contain a single compound.

Anti-bacterial activity

The pigment extracts were analyzed for their antibacterial effect against different human pathogenic bacteria. The PP and PC pigments were highly sensitive against all tested pathogens such as *Escherichia coli, Bacillus cereus*, *Shigella sp.*, *Salmonella typhi* and *Klebsiella pneumoniae*. But the pigment extracted from CL culture did not show any such sensitive activity against all five pathogenic bacteria. Moreover, the PP and PC pigment extracts showed better level of zone of inhibition against *E coli, Bacillus cereus* and *Shigella sp.* when compared to amoxicillin(Fig.4). Besides, *S. typhi* and *K. pneumoniae* species showed the moderate level of sensitivity for the same pigments in comparison with the positive control. This antibacterial property of pigments could be tapped for textiles, food and other beverages to protect from bacterial infections.

Free radical scavenging activities of marine pigments

The free radical scavenging property of extracted pigments were analyzed against DPPH and hydrogen peroxide (H_2O_2) radicals. The CL pigment showed the highest scavenging activity at 5mg/mL concentration for peroxide ions but the other two extracts PP and PC scavenged 65% of peroxide ions at 10mg/mL concentration in comparison with ascorbic acid positive control (Fig.5). PP and PC pigments did not show significant changes with respect to peroxide scavenging activity after increasing the dosage concentration to above 10mg/ml. This study reveals that the CL pigments can actively scavenge peroxide radicals at lower (5-10mg/ml) concentration. This property could be utilized to stabilize reactive species even in lower concentration and could control molecular misregulations.

The DPPH radical scavenging ability was also studied for CL, PP and PC pigment extracts. The PP and PC showed a significant DPPH scavenging activity in comparison to the ascorbic acid standard. The percentage inhibition of reactive DPPH radicals was estimated as 71, 60 and 52 for PC, PP and CL pigments, respectively (Fig. 6). The DPPH radical ion scavenging activity was increased with increasing concentration.In this study,the CL pigment was significantly scavenged the hydrogen peroxide ions and was not found effective againstDPPH ions. Likewise, PP and PC pigments showed effective reaction against DPPH, but not with H_2O_2. Therefore, based on the physicochemical nature, the free radical scavenging activity of the pigments varies depending on the source of isolates.

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Total reducing power potentials of marine pigments

The ferrous reducing potentials of the pigments were analyzed against standard BHT. The amount of Fe^{2+} complex in the test sample was estimated based on the reducing potential of the microbial pigments.15 mg of CL pigment reduced 50% of Fe^{3+} ions to Fe^{2+}complex in Perl's blue color when compared with standard BHT(Fig 7). PP and PC pigments showed only 30% of reducing activity as a maximum. The reducing power activity is directly proportional to the increasing pigment concentration. The CL pigment extract might have more effective iron reducing ability than PC and PP pigments.

Table 1. Biochemical Assimilation and hydrolytic enzyme producing properties of marine fugal isolates

Cultures	Assimilation assays			Hydrolytic enzyme production			
	Sugar	Urea	Nitrogen	Amylase	Cellulase	Lignin	Pectinase
PC	+	+	+	+	+	-	+
PP	+	-	+	-	+	+	-
CL	+	+	+	+	-	+	-

Table 2. Rf values of PC, PP and CL pigment extracts in thin layer chromatographic separation.

Pigments	R_f					
	Methanol : Water (1:1)			ACN: Methanol: Water (5:75:20)		
PC	0.273	0.527	0.600	0.391	0.609	0.870
PP	0.455	-	-	0.870	-	-
CL	0.782	-	-	0.739	-	-

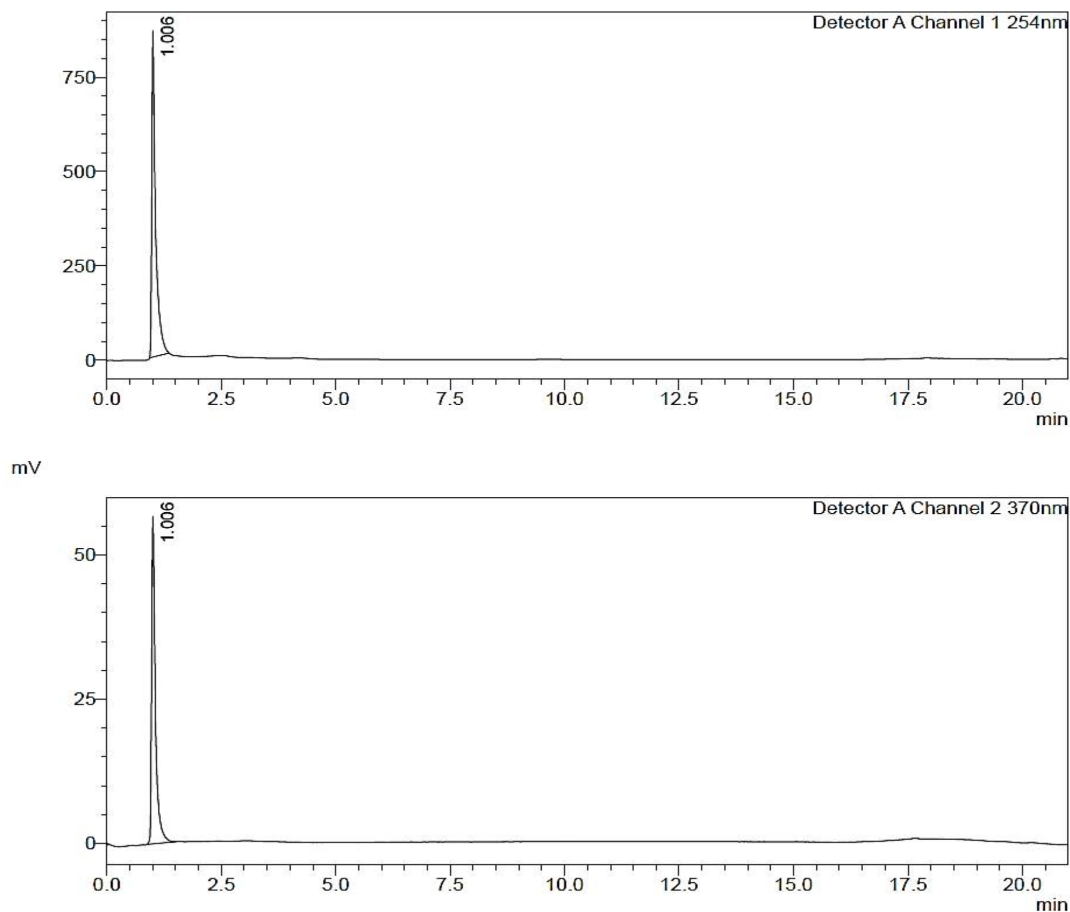

Figure1– HPLC chromatographic profile of PP pigment extract.

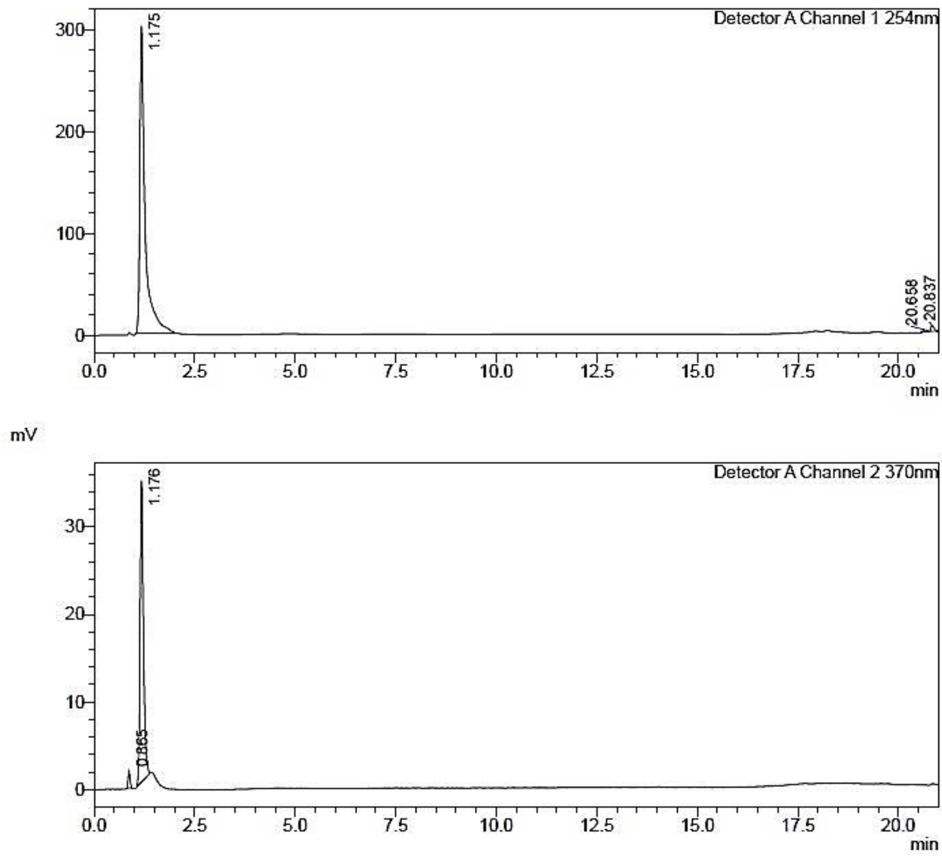

Figure 2 – HPLC chromatographic profile of CL pigment extract.

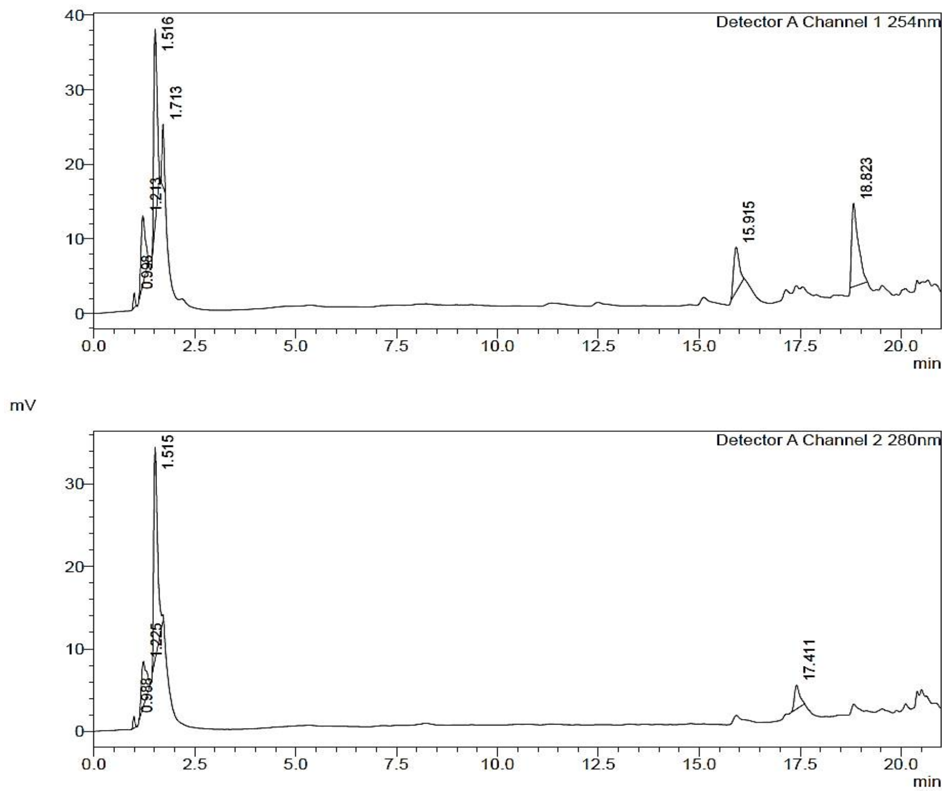

Figure3 – HPLC chromatographic profile of PC pigment extract.

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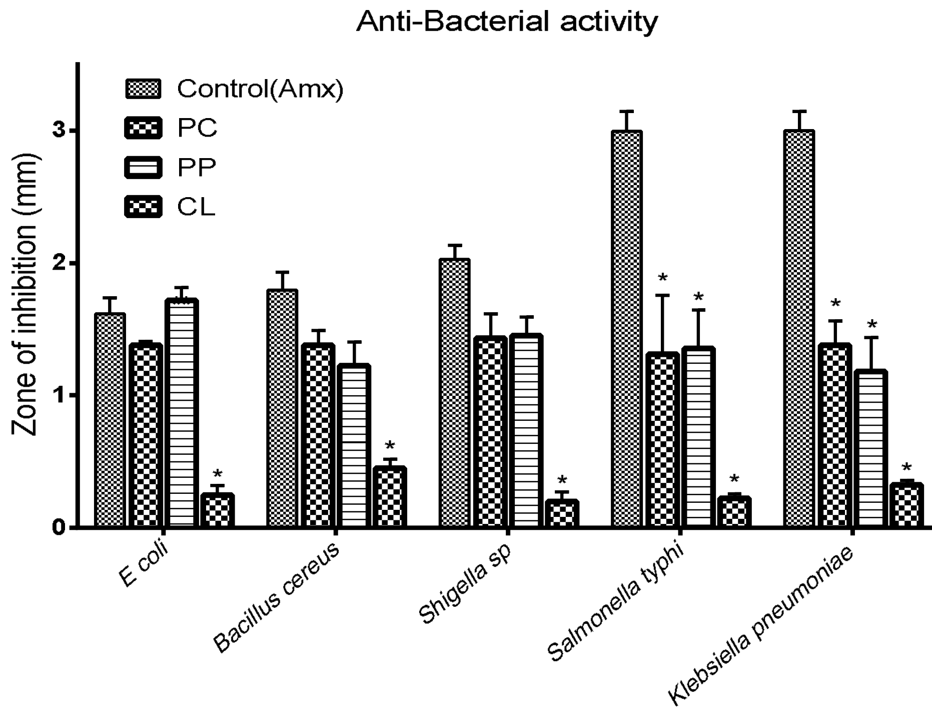

Figure4 . Anti-Bacterial activity of pigments extracted from marine fungal isolates. The bars represent the mean SD value of the replicates (n=3). Symbols *represent the statistical significance at $p< 0.01$ against standard amoxicillin (Amx).

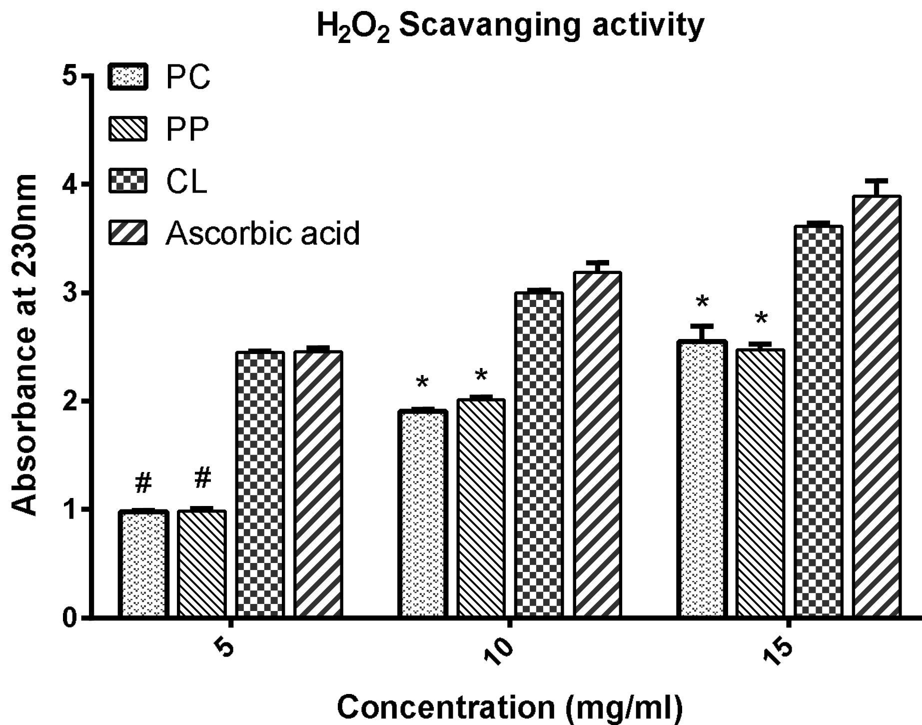

Figure5. Hydrogen peroxide radical scavenging activity of pigments extracted from marine isolates. The bars represent the mean SD value of the replicates (n=3). Symbols *represent the statistical significance at $p< 0.05$ and #represent the statistical significance at $p< 0.01$ against standard ascorbic acid.

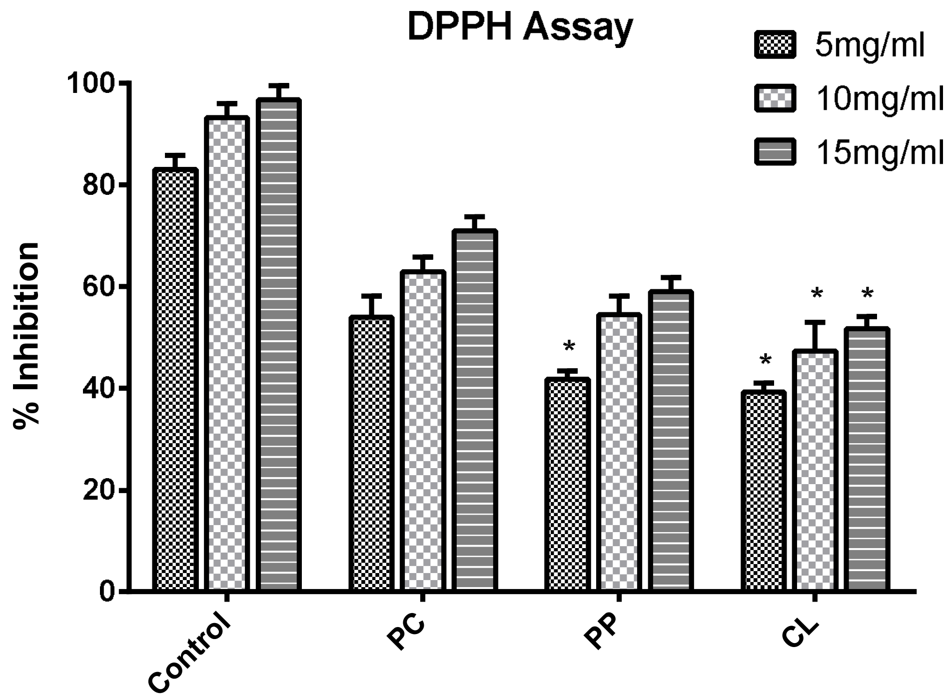

Figure6. DPPH radical scavenging activity of pigments extracted from marine isolates. The bars represent the mean SD value of the replicates (n=3). Symbols *represent the statistical significance at $p< 0.05$ against positive control (ascorbic acid) and PC pigment.

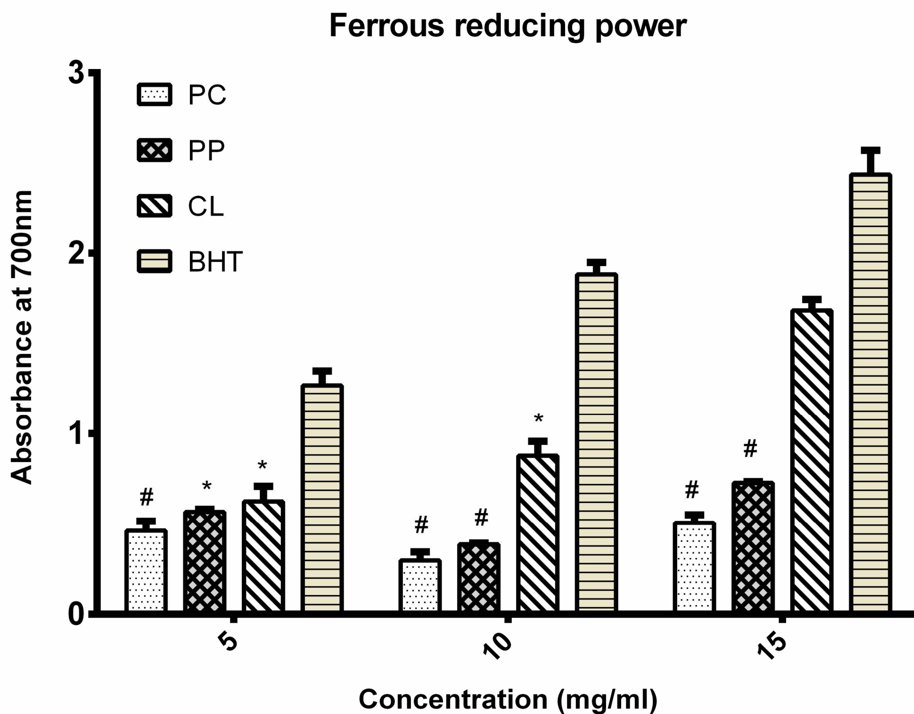

Figure7. Ferrous reducing power of pigments extracted from marine isolates. The bars represent the mean SD value of the replicates (n=3). Symbols *represent the statistical significance at $p< 0.05$ against standard BHT and #represent the statistical significance at $p< 0.01$ against standard BHT.

DISCUSSION

In recent years, natural pigments have been considered as an important source in various fields as colorants and substitutes for synthetic colors. Many synthetic colors are banned due to their allergenicity, carcinogenicity and other toxicological problems [14]. Microorganisms are also known resource for the pigment production due to their stability, availability, cost efficacy, easy downstream processing and yield. Recently, several studies have prominently projected the importance of marine fungi for their pigments as therapeutic lead molecules with potential biological activities[25].

In the present study, three different colored pigments such as green, red and greenish-black producing marine fungal species were isolated. The pigments have potent biological activities including antioxidant and antibacterial effects. The biological activities of the pigments revealed that they might have some active metabolites within them. These pigments have synthesized due to the stress induced metabolism of various physic-chemical factors of marine environment.

Several earlier studies have been reported the pigments producing microorganisms such as yeast, actinomycetes and fungal species in various marine samples from east coast of Tamilnadu [26-27].The present study also isolated the three different fungal species and evaluated for their biological activities. In addition, the marine isolates were analysed for their extracellular hydrolytic enzyme production and organic source assimilation properties. The marine organisms are often meant to adapt for an environmental crisis by producing intra or extracellular enzymes such as peptidases, phosphatases, cellulases, and oxidoreductase enzymes [1]. Extracellular enzymes play a crucial role to digest organic matters and to support growth and sustainability [28].The assimilation assay showed an elevated ability of PC and CL cultures to assimilate nitrogen and carbon sources. However, the PP culture did not grow in the urea supplemented medium. Nevertheless, PP was utilized the sugar and nitrogen source for its growth from the media which does not containing urea content. Moreover, carbon and nitrogen are the most important nutrients for the fungal growth[29].Therefore, the study cultures assimilated and metabolized the nitrogen and carbon sources certainly from the sole resource.

Molecular approaches are now developed to provide a more rapid tool in identification of microbes compared to traditional phenotypic methods. Molecular identification of fungi relied upon the amplification and sequencing of the internally transcribed spacer (ITS) region and D1/D2 region of the yeast genome, which was highly variable among species to species and within the same species [30-31]. In this study, the ITS sequence was amplified from isolated marine fungal genomic DNA by using specific ITS primers. The ITS amplicons were sequenced and the fungal species was confirmed as *Rhodotorula evergladensis, Aspergillus sydowii* and *Talaromyces coalescens*.

The isolated pigmented cultures wereidentified as belonging to the fungal phylum with spore forming in nature.Indeed,*Rhodotorula evergladensis* species was completely classified as unicellular microscopic pigmented yeast belonging to Basidiomycota phylum [32]. *Aspergillus sydowii* was reported as a human pathogenic fungus[33]and was found to occur in different marine habitats. Further, *Aspergillus sydowii* has also been studied for some novel secondary metabolites with anti-inflammatory and anti-diabetic activities[34]. Likewise, Talaromyces is the only known dimorphic genus in the fungus, producing filamentous growth at 25°C and exhibits yeast phenotype at 37°C[35]. Talaromyces possess medically important pigments and they displayed significant level of biological activities like radical-scavenging, inhibition of isocitrate dehydrogenase, antibiotic and/or cytotoxic activities[36].

The pigments derived from microbes have been attracting considerable importance in therapeutic uses due to their elevated biological activities. The pigment extracted from *T.ochracea* has exhibited an enhanced biological activity *viz.*, cytoxicity regulation, anti-inflammatory and antioxidant activities[37].The various research also reported that ascomycetous fungus can produce extracellular pigments with polar character and which can be extracted with polar solvents [38]. Therefore, 95% methanol was used to extract the pigments from the fungal isolates and the yield was 10g per 1 litre of culture medium.

The antimicrobial activity of the pigments was evaluated against various human pathogenic bacteria. Nascimento *et al*. demonstrated the synergistic effect from the association of different plant extract with known antibiotics against resistant bacteria as a novel therapeutic approach [39]. Treating MDR pathogenic infection is a big challenge. So, it is inevitable to evaluate different natural resources in identification of novel and highly sensitive drug molecules against microbial pathogens. In this context, the isolated pigments from marine microbes found to show antibacterial activity against five human pathogens *viz., E. coli, Bacillus cereus, Shigella sp., Salmonella typhi, Klebsiella pneumonia.* Karanjgaokar *et al*. have also reported the antimicrobial potentialof pigmentsfrom yeast against human pathogenic organisms such as*E. coli, S.aureus, K.pneumoniae, C.diphthriae* [40].In addition, microbial astaxanthin

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pigment from microbes showed effective antibacterial activity towards the pathogenic species *S. typhi*, *P.aeroginosa*, *B.subtilis* and *S.aureus* [41].
Furthermore, the marine microbial pigments extracted from different solvent systems were evaluated for anti-oxidant activity. DPPH is a free radical producing agent, which is well known for its' potential to evaluate the radical scavenging property of therapeutic molecules [42].The pigments extracted from marine yeasts were analysed for their DPPH and peroxide scavenging activity. Among the three fungal species *R. evergladensis* pigment showed highest activity(71%)for peroxide and DPPH ion scavenging assays and the other two pigments scavenged only 65% of peroxide ions as maximum. *T. coalescens* and *A. sydowii* pigments did not show such conspicuous effects for peroxide ion scavenging. But, *R. evergladensis* and *A. sydowii* showed better biological activity for ferrous reducing power. Poorniammal *et al*., (2018) also studied the scavenging activity of *Thermomyces* species pigments and found the similar effect against DPPH radical scavenging and reducing power property [43],whereas the standard BHA also exhibited 84.25% inhibition activity at the same concentration. Moreover, Michalowska and Stachowiak (2010) also evaluated the DPPH radical scavenging activity of *Sporobolomyces* species and observed the significant inhibitory effect on par with BHA standard [44]. The present study of *R. evergladensis* pigment also showed similar range of free radical scavenging activity. Thus, the study results conceived that the *Sporobolomyces sp. and R. evergladensis* pigments have the promising antioxidant activity and they can be used as an antioxidants in various applications.

CONCLUSION

The present study is concluded that the natural pigments of fungal species isolated from marine sediments have antibacterial, antioxidant and iron reducing properties. Among the three strains, the pigment from *R. evergladensis* species was found to possess effective antibacterial and antioxidant properties that could be harnessed for different industries like textiles, food processing and other beverages in near future. Therefore, it is highly deserved for further studies.

ACKNOWLEDGMENT

We thank the Faculty of Marine Sciences, Annamalai University, Parangipettai for providing infrastructure facilities for carrying out this research work.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

This research was supported by the University Grand Commission (UGC), India through Major research project Program (MRP–MAJOR–BIOT–2013–1747).

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