Research Article

Extraction, Characterization and Antimicrobial Properties of Pigments from Yeast, *Rhodotorula mucilaginosa* Isolated from the Mangrove Sediments of North Kerala, India

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ABSTRACT

Carotenoids are natural pigments having important role in food, feed, pharmaceutical and cosmetic industries. Their role as strong antioxidant and essential food coloring agent has received major attention during recent years. Though carotenoids are present in plants and animals, its production from microbes are more advantageous due to their faster growth rate, cheaper cultural requirements, ease of manipulation and extraction, and safety considerations. Among the microbes, pigmented yeasts are found to be the most reliable candidates for the large scale production of carotenoids due to their higher rate of growth and simple unicellular structure. In our study, pigmented yeasts, *Rhodotorula mucilaginosa* were screened, isolated and identified from mangrove sediments of various sites along North Kerala. The pigments were extracted using DMSO-acetone solvent in to petroleum ether and characterized using UV-Visible spectrophotometer and NMR spectrophotometer. The pigment present in *R. mucilaginosa* was found to be β-carotene. The antimicrobial effect of the extracted pigment and the whole yeast isolate was tested against potent pathogens *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Salmonella typhi, and Streptococcus pyogenes* using disk diffusion method. Antimicrobial activity in the form of inhibition zone was shown by the extracted carotenoid against *Staphylococcus aureus.* Also, the optimization of growth conditions for maximum biomass yield and hence maximum carotenoid production was also studied. *R. mucilaginosa* was found to have highest growth at pH 7, with 5 ppt salt concentration at 25 °C in malt extract broth and these conditions can be employed in their fermentation process for the large scale production of carotenoids.

Key words: Antimicrobial activity, β-carotene, Carotenoids, Mangrove sediments, Optimization, *Rhodotorula mucilaginosa*.

INTRODUCTION

Carotenoids are lipid soluble, naturally occurring pigments that belong to C40 class of terpenoids. They scavenge O_2 and peroxyl radicals, thus serving as membrane protective antioxidants. [1] The carotenoid pigments are ubiquitously present in the photosynthetic systems of phototrophic bacteria, algae and higher plants. In non-photosynthetic organisms like non-phototrophic bacteria and fungi, they help in protection against photo oxidative damages.[2] Carotenoids impart pleasing natural yellow to red colours which increases the acceptability of food.[3] The red carotenoid astaxanthin is often used as a feed pigment in aquaculture industry.[4] Some carotenoids are Vitamin A precursors, which reduce the risk of development of degenerative diseases such as cancer, cardiovascular diseases, cataract and macular degeneration.[5] The unique properties of

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carotenoids make them promising candidates in food, feed, pharmaceutical, and cosmetic industries and also in health sector.[6,7]

The different sources of carotenoids are still been investigated due to their various applications as natural pigments. In recent years, microbial carotenoids have received much attention than plant and animal carotenoids. This is due to their ease of increase in production by environmental and genetic manipulation, simplicity of extraction, stability, non-toxicity and quality of the final product.[8,9] A major drawback of using microorganisms for the commercial production of carotenoids is their high cost of production. But, this problem can be solved by using organisms with high pigment production, proper optimization of the process and usage of cheap nutrient sources for their culture.[10,11]

Coloured yeasts due to their unicellular nature and high growth rate are considered as the best source for large scale production of carotenoids compared to bacteria, algae and fungi.[12,13] The *Rhodotorula* species, belonging to strictly aerobic yeasts were studied to have peculiar biochemical characteristics like production of large amount of glycogen, lipids, acetic acid, acetaldehyde and carotenoids during different phases of their growth. Nowadays, they are widely used in food and pharmaceutical industries and also in waste water treatment.[14,15] Among the *Rhodotorula* species, *Rhodotorula mucilaginosa* is one of the most potential pigment producing yeasts which can be fermented using cheap industrial by-products and wastes as nutrient sources.[16-18] This makes them a promising candidate in the biotechnological and commercial production of carotenoids.

Against this backdrop, the present study aimed at isolation and identification of yeast *Rhodotorula mucilaginosa* from the mangrove sediments of Northern Kerala. The pigments from the isolate were extracted, characterized and checked for its antimicrobial activity. The optimization of growth conditions of *R. mucilaginosa* for maximum carotenoid production was also performed.

MATERIALS AND METHODS

Isolation of yeasts

Sediment samples were collected from the mangroves of the 5 districts along North Kerala coast during the period 2018-2019. Approximately 10-20g of sub surface sediment was collected using hand core method and was transferred aseptically into sterile polythene bags. The collected samples were transported in ice boxes and processed within 4 hr of collection. For the isolation of yeasts, spread plate method was performed in Wickerham's agar supplemented with 200 mg/l chloramphenicol in duplicates.[19] The plates were incubated at 18 ± 2°C for 7 days and the colonies developed were purified by quadrant streaking and transferred to malt extract agar slants for further studies.

Screening and identification of pigmented yeasts

After isolation, the colonies which showed yellow to red colouration were selected, purified, and coded for future identification. Out of 486 yeast isolates obtained during the study period, 24 isolates showed pigmentation at high intensity.

The isolates were later subjected to morphological, biochemical and molecular characterization. In morphological characterization, colony characteristics on malt extract agar and microscopic appearance of methyl blue stained smear, under 40x and oil immersion (100x) in compound microscope were observed. In biochemical characterization, urea hydrolysis, sugar fermentation (MOF - Microbial Oxidation Fermentation test), fatty acid hydrolysis, nitrate assimilation, starch like substance production, citric acid production, Diazonium Blue B reaction (DBB) and growth at 37°C were performed.[20] The identification of these isolates were later confirmed by sequencing of ITS region with ITS primers (Forward ITS 1: 5' -TCC GTA GGT GAA CCT GCG G- 3' and Reverse ITS 4 - 5' -TCC TCC GCT TAT TGA TAT GC- 3').[21,22] The amplified fragment of approximately 580 bp containing the ITS 1, 5.8 S and ITS 2 regions were used for the sequence similarity search using NCBI BLAST. The yeast strains identified as *Rhodotorula mucilaginosa* were then selected for further studies.

Extraction and characterization of pigment

R. mucilaginosa broth culture was swab inoculated in to Yeast Extract Sucrose (YES) agar plates and incubated at 28 ± 2°C for 7 days. The cells were harvested with physiological saline and the cell suspensions were centrifuged for 10 min at 12,000 rpm. The pellet formed was frozen at -20°C for carotenoid extraction.[23] Pellet was then suspended in 5 ml of preheated DMSO by vortexing and incubated at room temperature overnight. The suspension was centrifuged, DMSO phase was collected and the pellet was washed 3 times with 10 ml acetone. All coloured DMSO and acetone phases were pooled together and transferred to extraction funnel containing an equal volume of light petroleum (Boiling point range 30-75°C), 10 ml distilled water, and 5-10ml saturated NaCl (in case of poor phase separation). Carotenoids were then extracted into the light petroleum

phase by gentle rotation and washed 3 times with an equal volume of distilled water, adding saturated NaCl and stored in light petroleum at 4°C. The extracted pigments were subjected to UV-Vis spectrophotometry. After evaporating at 40°C the pigment extract was subjected to NMR spectrometry with acetone as solvent, for identification of the pigment.

Determination of antimicrobial activity

Whole yeast isolate of *R. mucilaginosa* and their extracted pigment were used to investigate the antimicrobial activity against potent pathogens. Type cultures of *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Salmonella typhi, and Streptococcus pyogenes* procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India were used as the test pathogens. The antimicrobial activity of the whole yeast was assessed by checking the inhibitory effect of *R. mucilaginosa* on the growth of pathogens using dual culture method. Kirby Bauer disk diffusion method using Mueller Hinton agar was used for checking the antimicrobial activity of pigment.[24]

Optimization of growth parameters for the production of carotenoids

Various growth parameters like culture media, pH, salinity and temperature were optimized for the carotenoid producing *R. mucilaginosa* isolates. The yeast colonies were inoculated into malt extract broth, grown at 28 ± 2°C for 48 hr and the optical density of the culture suspension was taken at 500 nm with the help of a UV-VIS spectrophotometer. Later the OD was adjusted to 1 by dilution with sterile water and 10µl of this cell suspension was used as inoculum. All the cultures for this study were performed in triplicates and incubated for growth at 28 ± 2°C for 5 days except for temperature optimization. The optical density/absorbance was measured using UV-VIS spectrophotometer and the wavelength at 500 nm was used for further calculations. The mean absorbance value of the triplicate samples and their standard deviation was calculated.

Media optimization: The inoculum was cultured in two separate liquid media viz Malt Extract media and Glucose Yeast Peptone broth (Wickerham media) in triplicates.

***pH optimization*:** Malt extract broth was prepared at pH 5, 6, 7, 8 and 9 in triplicates, inoculated and incubated as mentioned.

***Salinity optimization*:** Malt extract broth at pH 7 of different salinities 5 ppt, 10 ppt, 15 ppt, and 20 ppt were prepared in triplicates, inoculated and incubated as mentioned.

***Temperature optimization*:** Malt extract broth at pH 7, 5 ppt salinity were prepared in triplicates, inoculated and incubated at temperatures 20°C, 25°C, 30°C, 35°C and 40°C.

RESULTS

Screening and identification of pigmented yeasts

A total of 24 isolates showed orange/red/pink pigmentation. The red coloured isolates identified as *R. mucilaginosa* were pure cultured and studied further. Colony characteristics on malt extract agar and microscopic appearance of methyl blue stained smear under 40x and oil immersion (100x) in compound microscope were analyzed in morphological examination of the isolates. The colonies of *R. mucilaginosa* were smooth and mucoid in nature and showed orange to red colour in malt extract medium (Figure 1, Table 1). Microscopic examination showed oval shaped, ahyphated cells which are asexually reproducing with the help of multi-lateral budding (Figure 2a, 2b). The results of biochemical characterization are summarized in Table 2. The amplification and sequencing of the ITS region of the yeast DNA confirmed the species as *R. mucilaginosa* when compared with the GenBank database with 100% sequence homology. The sequence obtained was deposited in the GenBank with accession no MT 131387.

Characterization of the pigment

The pigments from *R. mucilaginosa* were extracted completely with DMSO: acetone solvent from the

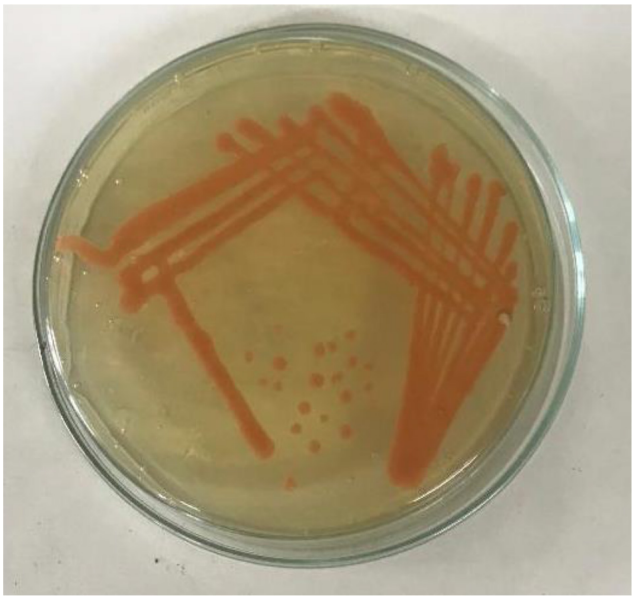

Figure 1: Pure culture of red yeast *R. mucilaginosa* on Malt Extract agar medium.

Table 1: Colony characteristics of *R. mucilaginosa*.

Sl. No.	Characteristics	*R. mucilaginosa*
1	Colour	Orange/Red
2	Size	Medium (2-3mm)
3	Single colony shape	Round
4	Appearance	Smooth and glossy
5	Texture	Mucoid
6	Margin	Entire
7	Elevation	Raised/convex
8	Sedimentation	Positive
9	Cell shape	Ovoid
10	Spore formation	Negative
11	Pseudo-true-mycelium formation	Negative/ahyphated cells
12	Budding	Multilateral

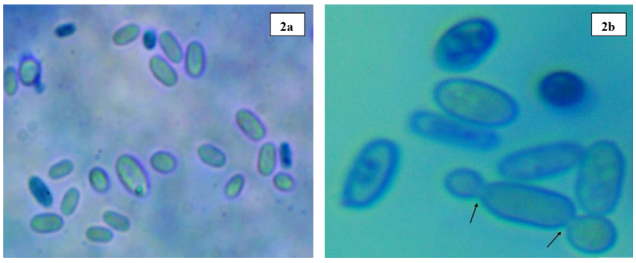

Figure 2: Microscopic appearance of *R. mucilaginosa* under 100x (oil immersion) magnification. 2a) Cell morphology – oval shaped cells 2b) Arrows indicate multilateral budding.

Table 2: Biochemical characterization of *R. mucilaginosa*.

Sl. No.	Tests	Results
1	Urea hydrolysis	Present
2	Glucose fermentation (MOF test)	Absent
3	Fatty acid hydrolysis	Absent
4	Nitrate assimilation	Absent
5	Starch like substance production	Absent
6	Citric acid production	Absent
7	Diazonium Blue B reaction (DBB)	Present
8	Growth at 37°C	Present

frozen pelleted cultures. The absorbance of the pigments was measured primarily in the 400nm–550nm wavelength range to confirm the presence of carotenoids. The appearance of a three peak profile which is the typical characteristic of carotenoids was observed in the visible absorption spectrum. Later, the oven dried carotenoids were subjected to NMR analysis

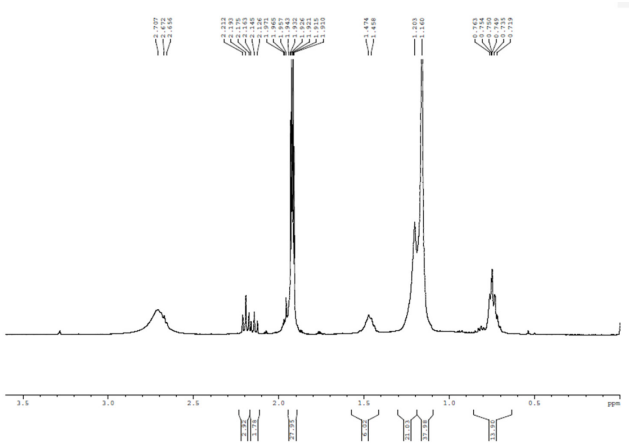

Figure 3: NMR spectrum of extracted carotenoids in solvent acetone showing peaks corresponding to that of β-carotenoid.

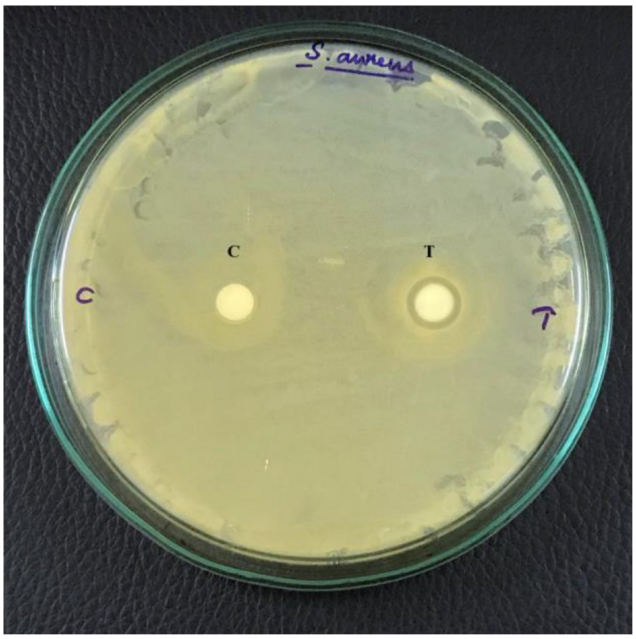

Figure 4: Antimicrobial activity of *R. mucilaginosa* by disk diffusion method. C - Control disc with petroleum ether, T - Test disc with extracted carotenoid.

which confirmed the carotenoid present in our sample as β-carotene (Figure 3).

Antimicrobial effect

Both *R. mucilaginosa* whole yeast isolate and their extracted pigment were tested for antimicrobial activity against bacterial cultures of *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Salmonella typhi, and Streptococcus pyogenes* using disk diffusion method. The whole yeast isolate did not show any antimicrobial effect against the tested potent pathogens. But, the extracted

carotenoid pigment of *R mucilaginosa* showed zone of inhibition against *Staphylococcus aureus* measuring 10 mm in diameter (Figure 4). The result clearly indicates that the antimicrobial effect of this particular species is attributed to the carotenoids present in them rather than any other factors.

Growth optimization for pigment production

The optimum growth conditions for maximum biomass yield that subsequently cause for maximum carotenoid production of *R. mucilaginosa* was determined. The growth/ biomass in the culture media were evaluated on the basis of turbidity obtained by measuring the absorbance at 500 nm wavelength. *R. mucilaginosa* culture showed highest growth in Malt extract broth compared to Glucose Yeast Peptone broth (Wickerham media). Therefore, malt extract broth was chosen as the growth media for further optimization procedures. When the pH of the media was pre adjusted from 5-9, maximum growth was observed at pH 7. Low growth was seen at pH 5 and increased when the pH was increased until 7 and then it dropped again when the media became more basic (Figure 5a). The optimized pH 7 was maintained for the culture media for further procedures. In the case of salinity, the cultures showed maximum growth in media which has 5 ppt NaCl concentration. The growth significantly decreased as the salinity was increased (Figure 5b). The salinity of the culture medium was maintained as 5 ppt for rest of the process. The optimum temperature needed for the maximum growth of *R. mucilaginosa* was found to be 25°C. At all other incubation temperatures including 30°C which is normally employed, they showed reduced growth (Figure 5c). Hence from this study, the optimum growth conditions needed for the maximum growth and biomass yield of *R. mucilaginosa* was found to be pH 7, with 5 ppt salt concentration at 25°C in malt extract broth.

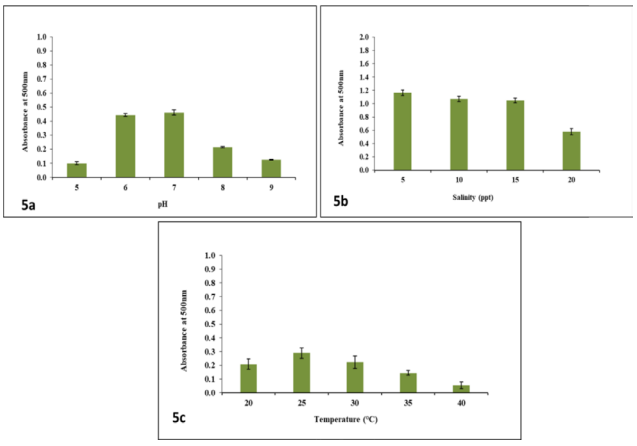

Figure 5: Optimization of growth conditions for of *R. mucilaginosa*. 5a: pH; 5b: Salinity; 5c: Temperature. Values represented as Mean ± SD.

DISCUSSION

The production of carotenoids by yeasts depends mainly on two factors, namely the strain of yeast used and the culture conditions that influence their growth and metabolic process.[25] *R. mucilaginosa* has been identified as a potential source of carotenoids among coloured yeasts in many studies.[26] Due to its high growth rate and wide substrate specificity, it is comparatively easy to achieve large biomass which could be directly added as feed or additive in other industries. They can be also cultured easily at laboratory and in pilot plants as they adapt well to different environmental and cultural conditions.[27]

The carotenoid present in the yeast *R. mucilaginosa* isolated in the present study was found to be β-carotene. The absorption maxima of carotenoids are at visible region ranging between 400 – 550nm which produces a typical three peak spectrum.[28] This characteristic spectrum is due to the presence of highly conjugated double bond system present in the pigment.[29] The carotenoid extracted from *R. mucilaginosa* in our study also showed the three peak pattern and later the analysis of NMR spectra obtained confirmed it as β-carotene. β -carotene is an important high value compound in global market due to its application in aquaculture, chemical, pharmaceutical, and alimentary industries. β-carotene is used as a natural food colouring agent as well as provitamin A food supplement due to its antioxidant properties. Besides, it is also used as additives in beverages, juices, and lipophilic substances like butter, cheese and margarines.[1]

The bacterial strains due to the virtue of differences in their cell wall composition like charge, composition and structure of lipids and lipopolysaccharides show variable susceptibility to carotenoid pigments.[30] Manimala and Murugesan showed that the chemistry and composition of the carotenoid pigments of yeast has a significant influence on its antimicrobial activity and suggested its use as a potential antibiotic drug.[31] The pigments of yeast belonging to *Rhodotorula* are studied to have strong antimicrobial properties.[32] In our study, the carotenoid extracted from *R. mucilaginosa* showed inhibitory action against pathogen *Staphylococcus aureus*. This suggests that the inhibitory effect/ zone against the pathogen was produced purely due to the β-carotene extracted from *R. mucilaginosa*. Further studies on β-carotene of *R. mucilaginosa* and the factors affecting its antimicrobial

properties would be necessary to substantiate its role as an antibiotic.

The total biomass of yeast in a culture is found to be directly proportional to their carotenoid yield. So, for maximum pigment production, there should be high growth rates which are achieved only if cultural conditions are optimal.[18] In this study, the conditions for the culture/growth of *R. mucilaginosa* was optimized for maximal pigment production and they showed highest growth rate at pH 7, with 5 ppt salt concentration at 25°C in malt extract broth. The concentration of pigments can be varied by changing culture conditions like pH, temperature, salt concentration, light and carbon and nitrogen sources.[33] The optimal conditions for growth and carotenoid production of each pigmented yeast strain should be well studied for large scale fermentation and there by cost effective biotechnological production of carotenoids.

CONCLUSION

The present study focused on the isolation of carotenoid producing red yeast *R. mucilaginosa* from the sediments of mangrove ecosystem, their pigment extraction, characterization, antimicrobial activity and growth optimization. The results showed that the carotenoid present in the species is β-carotene which possessed strong antimicrobial activity against pathogenic bacteria *Staphylococcus aureus*. The optimum cultural conditions for maximum growth rate and thereby maximum carotenoid production was studied to be pH 7, with 5 ppt salt concentration at 25°C in malt extract broth, which could be easily achieved even in normal laboratory set ups. Hence, our study emphasize on the use of yeast *R. mucilaginosa* as the safest and most cost effective source for large scale carotenoid production that can be utilized in food, feed, pharmaceutical and other related industries.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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