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Optimization of Carotenoid Production from *Microbacterium oleivorans* SAA1517and its Application as Fish Feed

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ABSTRACT: Carotenoids are structurally and functionally diverse groups of pigments produced by plants, animals and microorganisms. These compounds have received considerable attention due to their sustainable nature and potential health benefits. The present study was carried out to screen carotenoid producing bacteria from diverse soil samples and optimize their yield. The most challenging aspect of the study was distinguishing between carotenoid-producing bacteria and non-carotenoid pigment-producing bacteria. In addition, the extraction of carotenoids posed some difficulty as it necessitated the use of organic solvents. The samples were collected from farm fields, mangrove and garden rhizosphere regions. Among the 18 isolates, *Microbacterium oleivorans* SAA1517, was identified as the most potential carotenoid producer based on spectrophotometric (showing λmax at 465nm), qualitative (ring test) and TLC (Rf value of 0.65) analysis. The gene sequence of this isolate was submitted to the gene bank (accession no LC224329). The novel strain produced a dark orange pigment and showed maximum yield in Luria Bertani medium containing galactose and sodium nitrate in 3:1 ratio. The pigment production was optimum at pH 7, temperature 30°C, shaker conditions (120rpm) and presence of 2% inoculum size (0.9 $O.D_{545nm}$). Methanol was the most effective solvent for extraction of pigment. The antioxidant, antibacterial activity of the pigment, and its efficacy as feed supplement using goldfish were also evaluated. It showed good anti-oxidation potential with IC_{50} 4.2µg/mL and significant antibacterial activity against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Proteus mirabilis* and *Pseudomonas aeruginosa*. Determination of survival rate, weight gain, specific growth rate and feed conversion rate further confirmed that carotenoid supplements significantly improved the growth rate of goldfish. In the future, enhancing the fermentation conditions can significantly enhance the production of carotenoid pigments, which hold great promise as a natural and sustainable ingredient for fish feed in aquaculture. Additionally, incorporating bacterial carotenoids into fish feed has the potential to reduce the reliance on synthetic pigments and mitigate the environmental impact of aquaculture.

Keywords: Carotenoids, *Microbacterium oleivorans*, optimum, pigment, screening.

INTRODUCTION

Carotenoids are a group of natural pigments produced by micro-organisms, animals and plants (Maoka, 2020). They are structurally and functionally diverse secondary metabolites and occur in different shades of yellow, orange, pink or red (Britton *et al*., 2004). Carotenoids find extensive applications in food and cosmetic industries as coloring agents (Lourenco-Lopez *et al*., 2021). They also have significant medical importance due to their antioxidant and immunity boosting potential (Hughes, 1999). Studies have also indicated anticancer and anti-obesityproperties of carotenoids in animal models (Ávila-Román *et al*., 2021; Gammoneand D'Orazio, 2015).

Carotenoids can be produced very easily using chemical methods providing stable, vibrant and diverse shades of pigments. However, synthetic carotenoids are complex structures, which make them difficult to decompose in natural environment. Consequently, they persist in soil and water contributing to extensive pollution caused due to xenobiotics (Sliwka and Partali 2012). Natural sources of carotenoids like plants and insects with pigmented epidermal layer suffer from drawbacks of yield optimization due to the impact of climate and growth conditions which are difficult to control and monitor (Mezzomo and Ferreira 2016). Contrary to the synthetic, animal and plant sources of carotenoids, micro-organisms are an economicalchoice, and their growth can be easily controlled and optimized in a small laboratory space. For these reasons, there is a great emphasis on studies on production and optimization of carotenoids produced by novel microbial strains. Given the increasing demand for natural pigments worldwide, utilizing fermentation processes involving bacteria, fungi, and yeast can efficiently and effectively enhance the production of carotenoids at a large scale. This approach offers the potential to replace synthetic carotenoids with a sustainable and economically viable alternative, suitable for industrial and commercial applications.

Structurally, carotenoids are poly-isoprenoid compounds occurring mainly as carotenes or xanthophylls. Carotenes have simple structure consisting of carbon and hydrogen bonds. Xanthophylls, on the other hand, are more complex structures (oxygenated hydrocarbon derivatives) containing at least one hydroxyl, keto, epoxy, methoxy, or carboxylic acid groups. Carotenoids also occur in the form of flavonoids (anthocyanins) and tetrapyrrole (chlorophylls and phycobiliproteins) rings (Milani *et al*., 2017; Kushwaha *et al*., 2014). All carotenoids are soluble in lipids and show sensitivity to oxygen, heat, acids, alkali and light intensity (fluorescent, ultraviolet as well as white light). Most commonly acknowledged function of carotenoids is their ability to convert retinal into retinol (provitamin A). As humans cannot synthesize carotenoids, we are dependent on plant and dietary sources for fulfilling our vitamin A requirements. Thus, carotenoids are vital sources for eye sight and immunity functions in humans (Meléndez-Martínez *et al*., 2022; Kamal *et al*., 2021).

The production of β-carotene by an algae *Dunaliella* sp. is a well-developed technology used for preparation of nutrient supplements and pharmaceutical products (Wolf *et al*., 2020; Tafreshi and Shariati 2009). Similarly, β-carotene obtained from fungi *Blakeslea trisporais* is used in cosmetics, beverages and as poultry feed (Papadaki and Mantzouridou 2021). Among bacteria, carotenoid production is reported in diverse species of *Flavobacterium Corynebacterium*, *Agrobacterium*, *Bacillus*, *Micrococcus*, *Bradyrhizobium*, *Brevibacterium*, *Gordonia*, *Altermonas*, *Flexibacter* and *Dietzia* genus (Sastry *et al*., 2016). Yet many bacterial species remain unexplored for their potential to produce carotenoids. The objectives of the present study were to screen and isolate a novel carotenoid producing organism from a natural source, optimize the metabolite production and study the applications of crude carotenoids as antibacterial and antioxidant agent, and as a feed supplement for gold fish.

The antioxidant properties and potential benefits for fish health make bacterial carotenoids an attractive option for various applications, including as a natural antioxidant supplement and as a component of fish feed. The sustainable and cost-effective nature of microbial fermentation further adds to the potential of bacterial carotenoids as a valuable resource.

MATERIALS AND METHODS

Media and Chemicals. All chemicals and nutrient media used in the present study were of highest purity and analytical grade. They were purchased from Hi-media, India Ltd. and Difco laboratories.

Isolation and screening of carotenoid producing bacteria. The soil samples were collected from farm fields, mangroves and rhizosphere regions of local gardens for screening of carotenoid producing bacteria. The sample suspensions were prepared and suitably diluted to obtain well isolated colonies on Nutrient Agar (NA) plates. The spread plate technique was used for isolation and plates were incubated at Room Temperature (RT; ~30°C) for 24-72 h. After incubation, the colonies were observed for production of pigments (red, orange and yellow). Their characteristics were studied and pure cultures were maintained on NA slants at 40°C.

Extraction of pigments. The culture density was adjusted to 0.9O.D. at 540nm and an inoculum size of 2% was adjusted in 50 ml growth medium (nutrient brothadjusted topH7). The flasks were incubated under shaker (120 rpm) and light conditions at RT for up to 3 days. The pigments produced by bacteria were harvested by centrifuging the growth medium at 8,000 rpm for 15 min. The cell pellet was suspended and washed with sterile saline and centrifuged again for 15 min at 4,000rpm. The cell pellet was then suspended in 5 ml methanol and vortexed vigorously (1 min) for extraction of pigment. To ensure complete extraction of pigment, the above solution was incubated in a water bath at 60°C for 15 min until all visible pigments were extracted from cell pellet. After incubation, the dissolved pigment was cooled and centrifuged at 4,000 rpm for 15 min. The cell pellets were discarded carefully after autoclaving. The supernatant containing the extracted pigments was analyzed for presence of carotenoid using preliminary tests like ring test (qualitative test) and Thin Layer Chromatography (TLC) analysis. The carotenoid production was confirmed based on UV- Vis spectrophotometric analysis (Maheshwari, 2013).

Confirmation of carotenoid production.

Ring test. The appearance of blue colorat the interface on treatment of 5 ml of the methanolic pigment extract with 85 % sulfuric acidindicated the presence of carotenoids (Shatila *et al*., 2013).

TLC analysis. The extracted pigment was concentrated using a rotary evaporator. To 2 ml of concentrated pigment, 5 ml of petroleum ether was added and the solution was partitioned with equal volume of 90% methanol in a separating funnel with intermittent rigorous shaking. The epiphase and hypophase solutions (50µL) were used as samples for TLC. Silica gel coated on glass sheets were used as stationary phase and a solution of methanol: benzene: ethyl acetate (5: 70: 25) was used as mobile phase. Relative Rf value of 0.65 indicated presence of carotenoids (Bhat and Marar, 2015; Laxmi *et al*., 2012).

Identification of potential carotenoid producing bacterial strain. The morphological features of all pigmented colonies on NA plates were studied. Preliminary identification of all strains was done based on cultural, morphological and biochemical tests. The potential carotenoid producer strain was confirmed by 16s rRNA gene sequence analysis. PCR based 16S rRNA gene amplification and sequencing of the isolated bacterium was carried out using universal primers at Sai Biosystems Private Limited, India.

Optimization of growth conditions for production of carotenoids. The standard parameters used in our study were nutrient broth medium (pH 7), 30℃, shaker conditions (120rpm) and 1% inoculum adjusted to 0.1 $O.D_{540nm}$. The optimization of each of these growth parameters was done by applying one factor at a time

(OFAT) approach. In this method, one variable of the system is changed at a time while keeping the others constant. Dry cell mass was also measured and controls were maintained without inoculum.

Since growth medium is the basic requirements for microbial processes, we initiated the optimization by screening different media that supported maximum cell density and pigment production. Besides nutrient broth, Luria Bertani broth, M9 broth, Basal medium and polypeptone yeast extract glucose broth (PPYG) were screened for this purpose (Laxmi *et al*., 2012; Godinho and Bhosle 2008). Another important characteristic of carotenoid production is the exposure to light. Hence, the effect of light and dark conditions on carotenoid production was also studied by incubating the growth medium near the window (12 h light and 12 dark period) and in dark incubator respectively. The varying physicochemical parameters optimized in our study included the incubation time (24h, 48h, 72h, 96h, 120h, 144h, 168h and 192h), optical density of test isolate ($O.D_{540nm}$0.2, 0.4, 0.6, 0.8 and 0.9), inoculum size (0.25%, 1.0%, 2.0%, 3.0%, 4.0%, 5.0%, 6.0% and 7.0%), temperature (5°C, 20°C, 25°C, 30°C, 37°C, 40°C, 45°C and 55°C), pH (4-12) and aeration (static and 120rpm) (Bhat and Marar 2015; Laxmi *et al*., 2012; Godinho and Bhosle 2008; Voaides and Dima 2012).

The influence of 1% carbon sources like glucose, mannitol, maltose, glycerol and sucrose were studied on carotenoid production under previously optimized conditions. Similarly, the effect of 1% nitrogen sources like ammonium nitrate, ammonium choride, ammonium sulphate, sodium nitrate and potassium nitrate was also studied. In addition, the effect of C/N ratios (1:1, 2:1, 3:1, 4:1 and 5:1) was observed (Bhat and Marar 2015; Voaides and Dima 2012).

Optimization of extraction method. Different solvents (acetone, methanol, ethanol, petroleum ether and ethyl ether) were investigated for their ability to extract the intracellular water insoluble carotenoids. For this purpose, the carotenoid producer was grown under optimized conditions and the cells were harvested by centrifuging at 8,000 rpm for 15min (Bhat and Marar 2015). The pigments were extracted by following the steps described in section 2.3.

Application of carotenoids

Anti- bacterial activity. The anti-bacterial potential of the carotenoid was tested against six test organisms viz., *Staphylococcus aureus*, *Psuedomonas aeruginosa*, *Streptococcus pyogens*, *Salmonella typhi*, *Proteus mirabilis* and *Escherichia coli* using well diffusion method. To carry out the procedure, 20 ml of sterile molten Mueller and Hinton agar was bulk seeded with 0.1ml test culture ($0.1O.D_{545nm}$). Wells of 6mm were made using a sterile borer and 50 µl of the carotenoid extract was added. The petri-plates seeded with test cultures, and containing the extracts was incubated at 37°C for 24 h. The diameter of zone of inhibition was measured in mm (Mohana *et al*., 2013).

Antioxidant activity. The antioxidant activity of the isolated carotenoid pigment was determined using DPPH radical scavenging assay. The desired dilutions of the carotenoid extract were made using methanol (1–5 mg/ml) and reacted with DPPH (40 µg/mL) in dark conditions for 30 min. The carotenoid pigments in 3 mL of methanol without DPPH served as blank and methanol solution of DPPH (devoid of pigment) served as control. Ascorbic acid was used as standard for comparison of antioxidant activity. The absorbance of the solutions was recorded using UV-VIS spectrophotometer at 517 nm (Mohana *et al*., 2013). Percent inhibition of DPPH radicals was calculated by following formula:

$$\text{Antioxidant activity} = \frac{A(\text{control}) - A(\text{sample})}{A(\text{control})}$$

Where A is the absorbance of the sample

Feed supplement using gold fish as experimental model. Five gold fish were kept in experiment tank and another five were kept in control tank. Initial weight and length of all the fishes were measured. The fish feed available in market was fed to fishes (thrice a day) maintained in control tank. Similarly, the fish feed was mixed with the carotenoid extract and fed to fishes maintained in experiment tank. The standard length and weight of the fish were measured every week for 3 weeks. On the last day of the experiment, final weight and length of all the fishes was noted and calculations were done to determine the weight gain, specific growth rate, average daily growth, feed conversion rate and survival rate using the formulas represented below (Promya and Chitmanat 2011; Chiu and Liu 2014).

$$\text{Weight gain (\%)} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

$$\text{Specific growth rate (\%/day)} = \frac{\text{Fish weight at end of experiment} - \text{Fish weight at the beginning of experiment}}{\text{Number of days of experiment}} \times 100$$

$$\text{Average daily growth (g/fish/day)} = \frac{\text{Fish weight on day 2} - \text{Fish weight on day 1}}{\text{Fish weight on day 1}}$$

$$\text{Feed conversion rate} = \frac{\text{Weight of feed}}{\text{Increase of fisht weight}}$$

$$\text{Survival rate (\%)} = \frac{\text{No. of surviving fish}}{\text{Initial number of fish}} \times 100$$

Statistics. All of the experiments were carried out in triplicates, and the mean values with standard deviation were reported.

RESULTS

Screening and Identification of potential carotenoid producer. The spread plate technique of soil samples collected in our study showed presence of diverse bacterial species with a variety of pigments (Fig. 1a). Among the isolated cultures, eighteen isolates showed production of yellow, orange, pink or red pigments. Hence, pure colonies of these isolates (Fig. 1b) were obtained and characterized for presence of carotenoids. The λ_{max} of pigments extracted from these isolates is represented in Table 1. The methanolic extract of isolate F2 showed three peaks at 440, 465 and 495 nm on subjecting it to UV- Vis spectrophotometric analysis, which is a characteristic absorption spectrum of a carotenoid. Hence, isolate F2 was selected for further studies. The preliminary biochemical tests and 16s rRNA gene sequence analysis confirmed the strain as *Microbacterium oleivorans* SAA1517 (NCBI accession no. LC224329). It produced dark orange colored pigment.

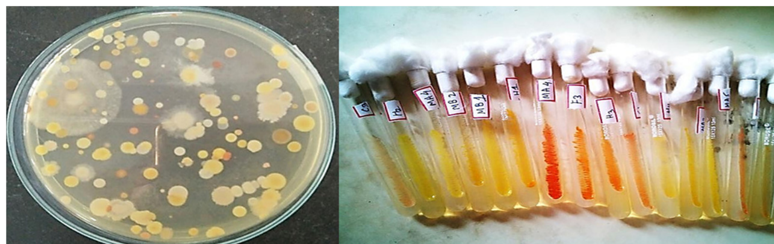

Fig. 1. Pigment producing bacteria obtained on nutrient agar plate using (a) spread plate technique and (b) pure cultures of isolated strains obtained in our study.

Table 1: λ_{max} and absorbance of pigments obtained from isolated bacteria.

Sr. No.	Isolate	λmax	Absorbance
1.	MA1	465 435 415	0.664 0.728 0.523
2.	MA2	450	0.502
3.	MA5	465 415 435	0.321 0.342 0.251
4.	H1	465 435 415	0.616 0.638 0.446
5.	H2	465 435 415	1.001 1.083 0.840
6.	H3	495 465	0.165 0.204
7.	C1	495 465	0.215 0.236
8.	C2	495 465	0.295 0.323
9.	F2	465 495 440	1.498 0.765 1.493
10.	F3	460	0.294
11.	MB1	465 435	0.261 0.290
12.	MB4	465 440 415	0.341 0.372 0.295
13.	C4	440 360 345	0.688 0.745 0.620
14.	C12	445	0.311
15.	C10	465 440 405	0.325 0.375 0.378
16.	C8	565	0.062
17.	C3	465 435	0.359 0.378
18.	C6	465 435	0.310 0.368

Optimization of growth conditions for production of carotenoids. During optimization of bacterial growth and yield of carotenoids, we first analyzed the primary growth requirements of *M. oleivorans* SAA1517 i.e., the nutrient medium and effect of light and dark conditions (Fig. 2). Maximum yield of dry cell mass as well as carotenoids was obtained in LB medium and presence of light. The optimization of carbon and nitrogen sources further showed maximum yield of carotenoid in presence of galactose and sodium nitrate, respectively, in 3:1 ratio (Fig. 3). The optimum physicochemical parameters (Fig. 4) for cell growth and carotenoid production were identified as 120h, pH 7, 30°C and shaker conditions (120rpm). The absorbance (carotenoid production) and cell mass showed similar pattern of increase/decrease when growth medium was inoculated with lower inoculum size (1% at 0.8 $O.D_{545nm}$). Initial inoculum of 1% at 0.8 $O.D_{545nm}$ produced 1.5g% cell mass in 120h.Optimum inoculum size (2% at 0.9 $O.D_{545nm}$) produced 3.2g% cell mass in same time (Fig. 5). At higher than optimum inoculum size and culture density, a considerable increase in cell mass was observed, with simultaneous decrease in absorbance.

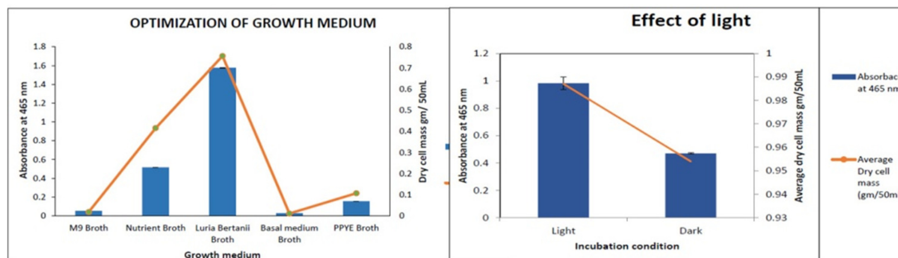

Fig. 2. Optimization of the basic growth parameters of *M. oleivorans* SAA1517.

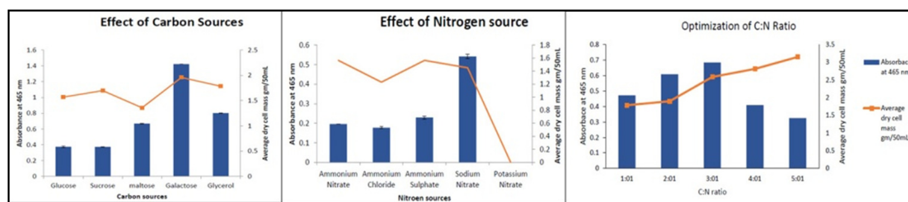

Fig. 3. Optimization of carbon and nitrogen sources for pigment production by *M. oleivorans* SAA1517.

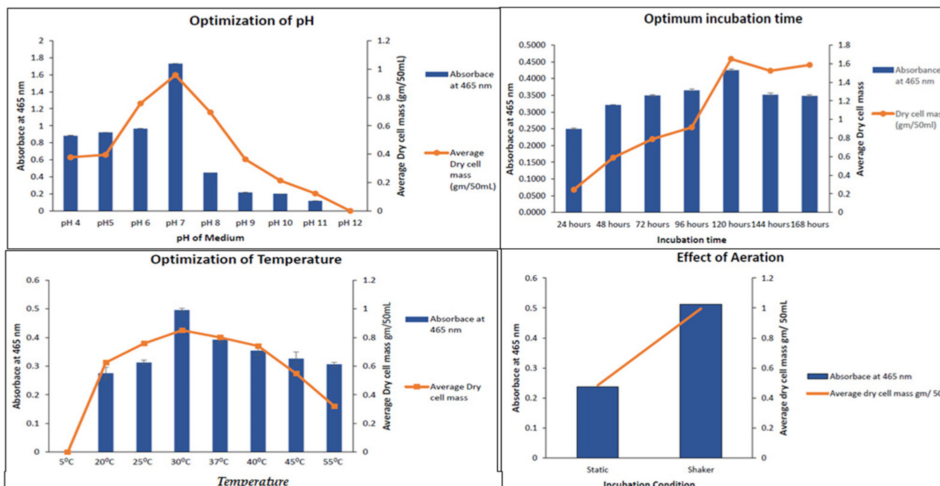

Fig. 4. Optimization of physicochemical parameters for pigment production by *M. oleivorans* SAA1517.

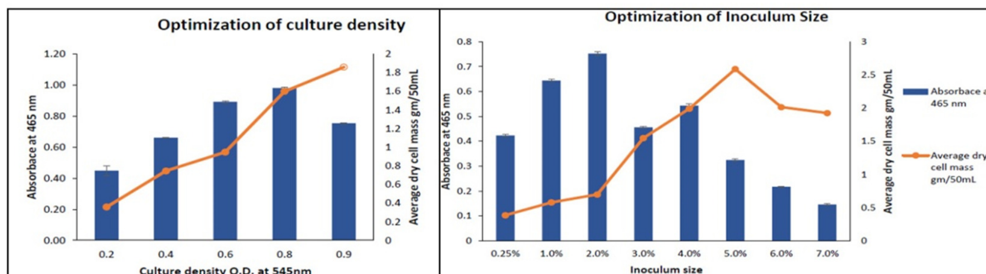

Fig. 5. Optimization of culture density and inoculum size for pigment production by *M. oleivorans* SAA1517.

Optimization of extraction method and characterization of carotenoid. The pigment from *M. oleivorans* SAA1517 was extracted optimally in methanol (Fig. 6). It was further confirmed to be a carotenoid based on U.V Vis spectrophotometric analysis, ring test and TLC (Fig. 7). As observed in the figure, it showed characteristic peaks at 440, 465 and 495 nm (Fig. 7a). The methanolic pigment extract reacted with concentrated sulfuric acid to produce a dark blue ring (Fig. 7b). The pigment was extracted in the epiphase of TLC solvents which showed an orange spot at Rf value 0.79 (indicating that the carotenoid has a carotene structure) whereas no coloured spot was observed in hypophase sample (Fig. 7c).

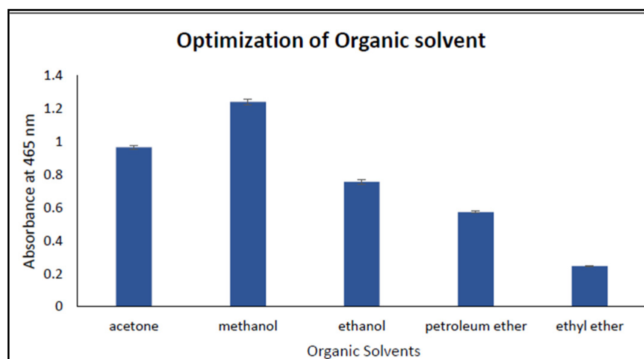

Fig. 6. Optimization of organic solvent for extraction of pigment produced by *M. oleivorans* SAA1517.

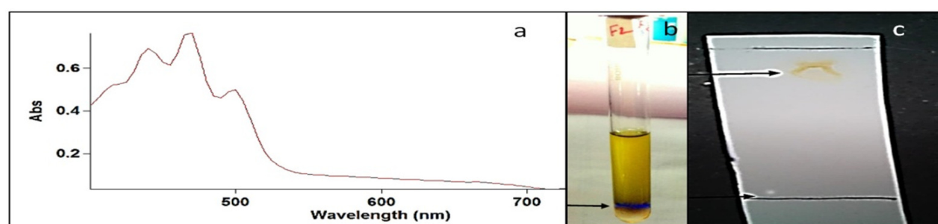

Fig. 7. Confirmation of carotenoid production based on (a) U.V Vis spectrophotometric analysis showing characteristic peaks (b) ring test showing dark blue color and (c) TLC showing orange spot at Rf value 0.79.

Application of carotenoids. The three most important applications of carotenoids were evaluated in the present study. The extracted carotenoid showed significant antibacterial activity against *S. aureus* (12 mm), *S. pyogenes* (10 mm), *P. mirabilis* (9 mm) and *P. aeruginosa* (11mm) whereas it showed no activity against *S. typhi* and *E. coli*. The DPPH activity of carotenoids is represented in Fig. 8. The IC_{50} values of ascorbic acid (standard) and carotenoid samples were 1.26 and 4.2 ug/mL respectively. The effect of carotenoid as a nutrient supplement in fish feed was tested using gold fish as experimental model. The different parameters of growth rate are indicated in Table 2. The experimental doses of feed containing carotenoid extract had no effect on the standard body length of the gold fish, but it significantly increased body weight after 3 weeks of supplementation.

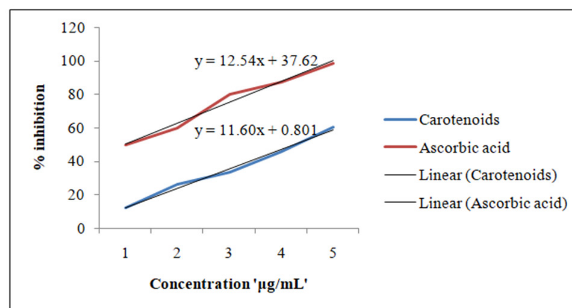

Fig. 8. Antioxidant activity of carotenoids in comparison with ascorbic acid during DPPH assay

Table 2: Effect of carotenoid as dietary supplement in growth enhancement of gold fish.

	Weight gain (%)	Specific growth rate (%/day)	Average daily growth; (g/fish/day)	Feed conversion rate (units)	Survival rate (%)
Test	12.59	0.3784	0.042	0.444	100
Control	2.03	0.3736	0.007	0.078	100

DISCUSSION

In recent years, consumers increasingly seek sustainable products and as a result, the industries are forced to accept and improvise sustainable practices. In this attempt, the interest in natural pigments from microbial sources has increased worldwide (Galasso *et al*., 2017; Torregrosa-Crespo *et al*., 2018). The present study was carried out to screen for a potential carotenoid producing bacterial strain from natural sites and optimize the yield of secondary metabolite.

The characteristics of carotenoids produced by microorganisms vary between different species (Kushwaha *et al*., 2014). Since the microorganisms are adapted to distinct conditions occurring in a habitat, the micro-ecosystem uniquely influences their growth and metabolic activities (Gupta *et al*., 2016). Hence, in the present study, soil samples were screened from different environmental conditions of farm field, mangrove and rhizosphere region of plant from local garden to isolate the most efficient carotenoid producing bacteria. It was identified as *Microbacterium oleivorans* SAA1517. Similar to the present study, the spread plate technique was employed to isolate carotenoid producing strains of *Rhodopseudomonas faecalis* PSB-B and *Salinicoccus* sp. M KJ997975 from garden and mangrove soil samples respectively (Zhang and Qing-Ping 2015; Bhat and Marar 2015).

M. oleivorans is a rod shaped, gram-positive and aerobic actinobacteria commonly isolated from soil samples across the world (BacDive strain identifier tool, 2022). They are rarely associated with infections in humans (Kim and Lee 2012). Instead, *Microbacterium* spp. are more commonly reported for their potential to bioremediate soil contaminated with oil and xenobiotic compounds due to continuous exposure to environmental pollutants over time (Avramov *et al*., 2016; Schippers *et al*., 2005; Kaushik *et al*., 2012). *M. oleivorans* characteristically produce yellow pigments but are rarely studied for carotenoid production. A recent study reported pigment production from *M. oleivorans* strains MV-19, MV-5 and UAC-76 isolated from Ratargul swamp forest soil (Anzum *et al*., 2022). However, the pigment was not characterized in their study. To the best of our knowledge, this is the first study demonstrating potential of *M. oleivorans* SAA1517 for carotenoid production.

Carotenoids absorb light in the visible region between 400 and 500 nm. It has been demonstrated by Fieser, in 1950, that carotenoids containing polyene system of 10 or 11 double bonds form three distinct bands at 440, 465 and 495 nm. Hence, carotenoids can be easily characterized using a U.V-Vis spectrophotometer and measurement of their absorption spectrum (Fieser, 1950; Bridoux, 2008). Another confirmatory test for detection of carotenoids was suggested by Mrak *et al*. (1949); Karrer and Jucker (1950). They reported that C40 carotenoids typically react with concentrated sulfuric acid to form blue or violet ring at the interface. For characterization of carotenoids into carotenes and xanthophylls, TLC has been suggested as the most suitable technique. Xanthophylls do not contain hydroxyl group in their structure and hence, during partitioning of pigment in petroleum ether and methanol mixture, it remains in hypophase. On the other hand, the carotenes contain two hydroxyl groups and hence are dissolved in the epiphase. When carotenes in epiphase migrate with the solvent front, the mono-hydroxylated compounds migrate to an intermediate distance and di-hydroxylated carotenes remain close to the base of the chromatography sheet (Mishra and Singh 2010). Hence, based on U.V Vis spectrophotometric assay and ring test, the pigment produced by *M. oleivorans* SAA1517 was confirmed to be a carotenoid. It was further characterized as a mono-hydroxylated carotene based on TLC observations. Using the above techniques, dark yellow carotenoids (Rf value of 0.77) obtained from *Microbacterium arborescens* AGSB was identified as a mono-hydroxylated carotene and light yellow carotenoids (oRf value of 0.38) obtained from*Micrococcus luteus* strain BAA2 was identified as di-hydroxylated carotene (Godinho and Bhosle 2008; Surekha *et al*., 2016).

Carotenoids are vital components of the bacterial cellular membrane (Seel *et al*., 2020). However, under natural conditions, the amount of carotenoid produced by bacteria is insufficient for industrial and commercial purposes. Hence, optimization of growth conditions for bacteria by providing necessary nutrients and growth conditions, and maximizing the yield of carotenoids is a primary step in screening potential carotenoid producers. *M. oleivorans* SAA1517 showed optimum yield of carotenoid in LB broth (pH 7) containing galactose and sodium nitrate in 3:1 ratio inoculated with 2% inoculum size (0.9 $O.D_{545nm}$) on incubation at 30°C and shaker conditions (120rpm) for 120h. The synthetic mineral medium like M9, Basal and PPYG supported cell growth but carotenoid production was lower as compared to NB and LB medium. This indicates that carotenoid production is an energy demanding process which is met by ingredients of complex media (Mezzomo and Ferreira 2016; Martínez-Cámara *et al*., 2021). Also, a decrease in carotenoid production was observed with increase in culture density (beyond optimum concentrations). This observation indicates that cell growth is preferred over production of secondary metabolites by *M. oleivorans* SAA1517under conditions of nutritional stress (Veerwal *et al*., 2007; Saini and Keum 2017). Similar observations were reported in *Rhodotorula* spp. & *Sarcina* spp. (EL-Banna *et al*., 2012). Interestingly, in spite of the energy demanding process, *M. oleivorans* SAA1517 produced carotenoids proportional to its growth in the pH range of 4 to 11; though the growth was significantly low in the alkaline range (pH 8 -11).Generally, bacteria isolated from natural soil show little tolerance to changes in pH for carotenoid production (Dyaa *et al*., 2022; Montero-Lobato *et al*., 2018). Optimum pH between 6.5 and 7, and sensitivity to changes in pH is reported in *Rhodotorula* sp. strain ATL72, *Deinococcus xibeiensis* R13 and *Rhodotorula mucilaginosa* (Tian *et al*., 2019; Sharma and Ghoshal 2019; Dyaa *et al*., 2022).

On optimization of nutritional and physicochemical conditions, the cell mass of *M. oleivorans* SAA1517 increased by 53.125%. In another study, *Rhodotorula*

sp. strain ATL72 showed optimum production of carotenoids in medium containing malt extract (3.7 g/L), fructose (7.7 g/L), urea (9 g/L), NaCl (35 g/L), and yeast extract (1 g/L). The optimum incubation conditions were 27.5 °C, pH 6.7, and 180 rpm. This strain showed 90.64% increase in carotenoid production under optimum conditions (Dyaa *et al*., 2022). Similarly, carotenoid yield from *Deinococcus xibeiensis* was 84% higher under optimum conditions of 30°C, pH 7.0 and fructose/ tryptone (C/N) ratio of 1:5 in 48h (Tian *et al*., 2019).

Besides the physicochemical and nutritional parameters, light and dark conditions have a significant impact on carotenoid production. The photosynthetic microorganisms are commonly reported to produce large concentration of carotenoids (since it is a photosynthetic pigment). However, light alters the metabolic activities by inducing carotenogenesis in non-photosynthetic bacteria too (Goodwin, 1980; Igreja *et al*., 2021). During light conditions, the activated cytochromes enable alternate electron transfer pathways in bacteria triggering formation of photo-oxidized metabolites. These metabolites induce carotenogenic enzymes in non-photosynthetic bacteria (Sieiro *et al*., 2003; Batra, 1967). This explains the significantly higher yield of carotenoids in light conditions as compared to dark conditions in a non-photosynthetic actinobacteria like *M. oleivorans* SAA1517.

The antibacterial and antioxidation potential of carotenoids are well reported in literature (Fiedor and Burda 2014; Bhatt and Patel 2020; Nabi *et al*., 2020). The carotenoids obtained in our study showed moderate antibacterial ability. Its antioxidant ability was low but comparable to ascorbic acid. Carotenoids extracted from *Rhodotorula glutinis* showed antimicrobial activity against food pathogens (Konuray and Erginkaya 2015). The IC_{50} value (4 µg/ml) of carotenoids produced by *Thermus filiformis* was comparable to ascorbic acid (Mukherjee *et al*., 2017). Different fractions of carotenoid obtained from *Cerrena unicolor* were prepared in another study which showed 20-60 µg/ml IC_{50} value. The low molecular weight sub-fraction (with unidentified components) was reported to be most effective with its IC_{50} value comparable to ascorbic acid and greater than trolox (antioxidant standards) in the above study (Matuszewska *et al*., 2018).

Although moderate antibacterial and antioxidant potential was identified for carotenoids produced by *M. oleivorans* SAA1517 as compared to published data, it showed great potential as a nutrient supplement for gold-fish in the present study. As indicated in Table 2, 83.87% increase in body weight of gold fish was observed within 3 weeks of supplementation with carotenoids which was equivalent to 1.26% specific growth rate per day. Also, over 82.43% higher feed conversion rate was observed in gold fish fed with carotenoid supplement as opposed to those fed with fish meal. To the best of our knowledge, no other study has reported such distinct characteristic of carotenoids to improve body weight within a short span (3 weeks) in animal models.

Similar to our study, Red Tilapia, African Sharptooth Catfish and Fancy Carp fish fed with carotenoid supplements, of Spirulina and Cladophora algae respectively, for 2-3 months showed higher average daily growth rate and specific growth rate compared to controls. The carotenoid content of fish meat in above studies was also higher than controls (Promya and Chitmanat 2011; Chiu and Liu 2014). Carotenoid supplements have also been reported to improve phagocytic activity and overall immunity in common carps (Anbazahan *et al*., 2014).

In conclusion, considering the great demand of pigments in various industries, the use of microbial sources for its production is a practical as well as sustainable strategy to meet the market demands. Though the microbial production studies are in preliminary stages at present, it is a potential biotechnological tool. In this aspect, genetic engineering to strain improvement, genome engineering and fermentation strategies to scale-up production to industry level etc. play vital roles in maximizing the yield of microbial secondary metabolites. The carotenoid production ability of *M. oleivorans* SAA1517 was improved by 53.125% under optimized conditions in the present study. Comprehending the fact that the optimization studies carried out in 250ml shaker flasks under laboratory conditions enhanced the carotenoid production significantly in a non-photosynthetic bacterium, it is a potential candidate for scale up studies using larger fermenters.

CONCLUSIONS

Considering the great demand of pigments in various industries, the use of microbial sources for its production is a practical as well as sustainable strategy to meet the market demands. Though the microbial production studies are in preliminary stages at present, it is a potential biotechnological tool. In this aspect, genetic engineering to strain improvement, genome engineering and fermentation strategies to scale-up production to industry level etc. play vital roles in maximizing the yield of microbial secondary metabolites. The carotenoid production ability of *M. oleivorans* SAA1517 was improved by 53.125% under optimized conditions in the present study. Comprehending the fact that the optimization studies carried out in 250ml shaker flasks under laboratory conditions enhanced the carotenoid production significantly in a non-photosynthetic bacterium, it is a potential candidate for scale up studies using larger fermenters.

FUTURE SCOPE

The scope of this study can be extended by testing its antimicrobial activity on various pathogenic strains. A thorough understanding of the regulation and pathway of carotenoid production will help us develop defined bioprocess for the enhance production of the desired carotenoid pigment. This study will also help us to explore the potential application of a naturally occurring source of carotenoid as a feed additive in aquaculture.

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