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Fermentative production of carotenoids from marine actinomycetes

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ABSTRACT

Background and Objectives: In marine actinomycetes, carotenoid production occurs in constitutive, light-dependent or cryptic manner. The present work deals with the fermentative production of carotenoids from marine actinomycetes.

Materials and Methods: Marine actinomycetes namely *Streptomyces* strain AQBMM35 was isolated from the marine sponges *Mycale mytilorum* collected from South West coast of India using ISP media. The *Streptomyces* isolates were characterized for their colony characteristics, morphological properties, physiological and biochemical properties and were tentatively identified. Fermentation of the strain under fluorescent white light was carried out for the production of carotenoids. UV spectrum, TLC and HPLC analysis were done for the confirmation of carotenoids.

Results: The characteristics studied strongly suggest that the strain AQBMM35 belongs to the genus *Streptomyces* sp. It has been found that *Streptomyces* strain (AQBMM35) fermenting under fluorescent white light produced carotenoids. Spectrophotometric analysis of the carotenoid fraction revealed a peak at 280 nm. TLC analysis of the carotenoid extract showed the presence of phytoene (R_f of 0.81). HPLC confirmed the production of phytoene when compared with standards.

Conclusion: The fermenting sponge-associated *Streptomyces* isolate (AQBMM35) produced carotenoids namely phytoene. If this symbiotic *Streptomyces* strain, from which secondary metabolite like carotenoids are derived, can be cultured under light, then it can be used for mass production of precursor pigment and it can be used as an antioxidant and also as a food additive.

Keywords: Marine actinomycetes, *Streptomyces*, Fermentation, Carotenoids, Phytoene.

INTRODUCTION

Marine microorganisms produce unique and novel secondary metabolites unlike those found in the terrestrial organisms and display interesting biological activities. Microbial biosynthesis of natural products is an emerging area of metabolic engineering and industrial biotechnology that offers significant advantage over conventional chemical methods or extraction from biomass. Carotenoids have received considerable attention because of their interesting properties like their potential beneficial effects on human health (1). Due to their useful biological activities, carotenoids have several applications especially in the food and pharmaceutical industries. Currently, commercial carotenoid production is mostly carried out by extraction from plant tissues or chemical synthesis; however, microbial production also has great potential in terms of both the efficiency of production and the diversity of molecular species. In recent years, the interest in production of natural carotenoids by microbial fermentation has increased. Carotenogenic microbes such as *Xanthophyllomyces dendrorhous*, *Bradyrhizobium* sp. *Flavobacterium* sp. *Brevibacterium aurartiacum* and *Blakeslea trispora* have been investigated for large-scale production (2).

Carotenogenesis in actinomycetes occurs in a constitutive, light-induced or cryptic manner. In many species of terrestrial *Streptomyces*, carotenogenesis occurs only by photo-induction and in a cryptic manner. To date, study on carotenoid production in *Streptomyces* has been performed with *S. griseus*, *S. setonii* and *S. coelicolor* (3). The production of carotenoids in marine sponge associated *Streptomyces* was recently reported (4). In the present study, an attempt was made to fermentatively produce carotenoids from *Streptomyces* strain (AQBMM35) isolated from marine sponges.

MATERIALS AND METHODS

Collection of sponges. The marine sponge *Mycale mytilorum* was collected at a depth of 5 -10 m by

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SCUBA diving from the Vizhinjam port, situated about 16 km to the south of Trivandrum at 8° 22' 30" N latitude and 76° 59' 16"E longitude in the South-West coast of India. The marine sponge was transported by storage on ice to the laboratory in minimum possible time to avoid external microbial contamination and excessive proliferation.

Fixation and preservation of sponge. The collected sponge was frozen immediately which to a certain extent fixes the colour of the live material. The sponges were again fixed in 10 % formalin for 24 hours after which the specimens were transferred immediately to 70 % ethanol and stored in airtight glass containers (5).

Spicule preparation. Spicules are tiny spike-like structures composed of either silica or calcite. The spicules were prepared by boiling a fragment of sponge in a test tube with ten times the volume of fuming nitric acid until the cellular material was dissolved. The sample tube was filled with distilled water and the spicule was allowed to settle to the bottom of the tube for at least 2 hours. The water was removed by pipetting and this step was repeated three times. Then the tube was rinsed with 95 % alcohol in the same way (three times). Subsequently, the spicules were suspended in 1-2 ml alcohol and poured onto a slide. After the alcohol evaporated, the slide was dried at 60 °C. Finally, the slides were mounted under Camera Lucida microscope and photographed (5).

Isolation of *Streptomyces* from marine sponges. The sponge extract was obtained by squeezing the sponges gently with a glass stick. Aliquots (1 ml) of each sponge extract were diluted with sterilized seawater. A quantity of one millilitre of the dilutions were mixed with 20 ml of sterile glycerol asparagine agar media (ISP-5) and incubated at room temperature (28 ± 2 °C) for seven days. Rifampicin (2.5 µg/ml) and amphotericin B (75 µg/ml) were added to ISP-5 media to inhibit bacterial and fungal contamination, respectively. The total *Streptomyces* population in the plates were counted (6).

Enumeration and maintenance of strain. The isolated strain (AQBMM35) was maintained as ISP-5 media slant culture at 28±2 °C (20). The inocula used in all the experiments were seven day old cultures unless otherwise stated.

Characterisation of the strain (AQBMM35). The strain was characterised by nutritional, morphological, physiological and biochemical properties which are detailed below (7-9). The microorganism was characterised by acid-fast staining and by Gram's staining techniques. The isolate was also studied by employing various parameters which are detailed below. In the nutritional uptake experiments given below, the culture was inoculated into the basal medium + nutrient and incubated at 28 °C for 7 days. The biomass thus obtained was separated from the broth, dried and weighed. The weight of the biomass was expressed in grams.

Utilization of carbon source. Various carbon sources (glucose, xylose, arabinose, rhamnose, fructose, galactose, raffinose, mannitol, inositol, sucrose) were mixed at a concentration of 1 % each to 10 ml of basal mineral salt medium.

Influence of amino acid. Various amino acids (glycine, cystine, alanine, tryptophan, valine) were mixed at a concentration of 0.1 % each to 5 ml of basal mineral salt medium.

Sodium chloride tolerance. Sodium chloride at varying concentrations (1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9, and 10 %) was added to 5 ml of the basal medium (10).

Melanoid production. The culture was streaked onto peptone-yeast extract-iron agar (ISP-6) slants and incubated at 28 °C for 48 h.

Pigmentation of mycelia and spore morphology. The culture was grown on a Petri dish containing casein-starch-peptone-yeast extract (CSPY) agar medium with a cover slip inserted at an angle of 45º. The cover slip was removed after 7 days of incubation, air dried and observed under the scanning electron microscope (11).

Fermentative production of carotenoid pigments. The isolate AQBMM35 was inoculated in 500 ml Erlenmeyer flasks containing 100 ml of Bennett's sugar medium (yeast extract, 0.1 g; meat extract, 0.1 g; NZ amine, 0.2 g; dextrose, 1 g; sterilized sea water, 100 ml; pH 7.2) and incubated at 180 rpm at 28º C for 120 h in a rotary shaker with fluorescent white light (compact fluorescent light (CFL) lamp emitting 15 W of power) exposure which was fitted inside the incubator. The fermented broth was lyophilized and then extracted with a mixture of 50% (v/v) ethyl ether and methanol. The extract was cleared by centrifugation, dried under a stream of nitrogen, and resuspended in petroleum ether (12). The resulting extract was concentrated under pressure and subjected to photometric analysis with a UV spectrophotometer. Qualitative analysis was performed by silica gel thin-layer chro-

Table 1: Physiological, biochemical properties and carbon utilization of the strain AQBMM35.

Physiological and biochemical parameters	Results
Growth temperature	22 - 45°C
pH	4 –10
Starch hydrolysis	+
Production of H_2S	-
Degradation of cellulose	+
Liquefaction of gelatin	+
Coagulation of milk	+
Peptonization of milk	+
Degradation of urea	+
Citrate utilization	+
Indole production	-
Catalase	-
Carbon source	**Utilization**
No carbon source (negative control)	-
Xylose	+
Arabinose	+
Rhamnose	-
Fructose	-
Galactose	+
Raffinose	+
Mannitol	-
Inositol	-
Sucrose	+
Glucose (positive control)	+

matography with a solvent mixture of petroleum ether: acetone (19:1, v/v) as mobile phase and the development was observed under ultraviolet lamp. Separation of carotenoids was also carried out by HPLC on a C_{18}, 3µm column with acetonitrile: methanol: 2-propanol (40:50:10). The flow rate was 0.8 ml/min. The eluted fractions were monitored using a photodiode array detector in the wavelength range of 200 to 600 nm. An authentic phytoene was purchased from Sigma and used as the standard.

RESULTS

In the present investigation, fermentative production of carotenoids from the *Streptomyces* associated with marine sponge was carried out. For the identification of marine sponge *Mycale mytilorum*, the sponge tissues were preserved and photographed. The spicules were separated from sponge tissue, fixed, drawn and photographed through the Camera Lucida microscope. They have sigma and tylostyle type of silicious spicules.

The isolated strain (AQBMM35) was characterized by morphological, physiological and biochemical properties. The isolated strain was slow growing, chalky, folded and aerobic. Mycelial colour pattern of strain was of white series. The aerial mycelium was

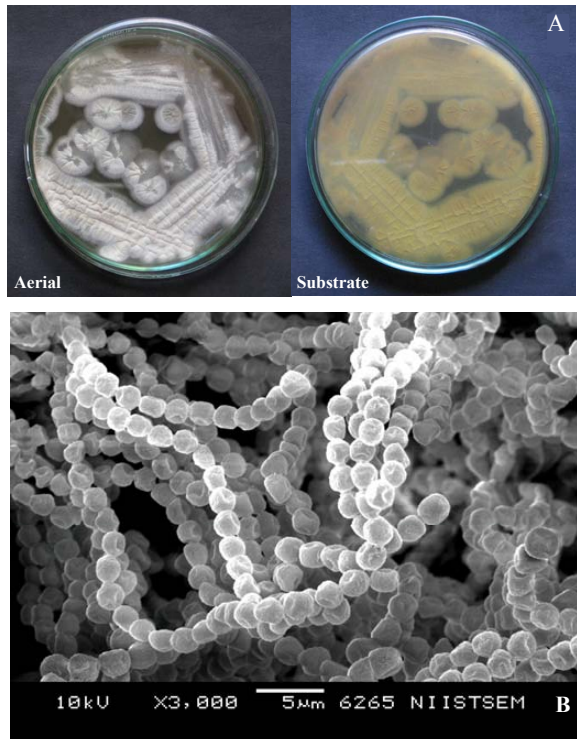

Fig. 1. The mycelial colouration (A) and scanning electron micrograph (B) of strain AQBMM35

white and vegetative mycelia bearing light yellow coloration in ISP-5 media (Fig. 1A). The strain was acid-fast negative and found to be Gram-positive. The strain does not produce melanoid pigments. The scanning electron microscope results showed the spore morphology as having a smooth surface and rectiflexibiles (RF) hyphae (Fig. 1B). The morphological characteristics indicated that the strain AQBMM35 may be actinomycete.

The nutritional characteristics of the strain AQBMM35 were studied. The carbon utilization of the strain showed positive growth in media containing glucose, xylose, arabinose, galactose, raffinose, sucrose and others resulted negative. The amino acids glycine and tryptophan positively influence the growth of the strain (Fig. 2). The strain AQBMM35 exhibited profuse growth and maximal biomass at the sodium chloride concentration of 6 % (Fig. 2). The AQBMM35 strain was able to grow in 22-45°C, pH 4-10. It was able to hydrolyze starch and cellulose, liquefy gelatin, utilize citrate, solidify milk and peptone, but could not produce hydrogen sulphide. The strain exhibited positive urease and negative catalase activity. The results of physiological and biochemical characteristics of the strain AQBMM35 are shown in Table 1. According to the morphological, nutritional, physiological, and biochemical characteristics of the strain we obtained,

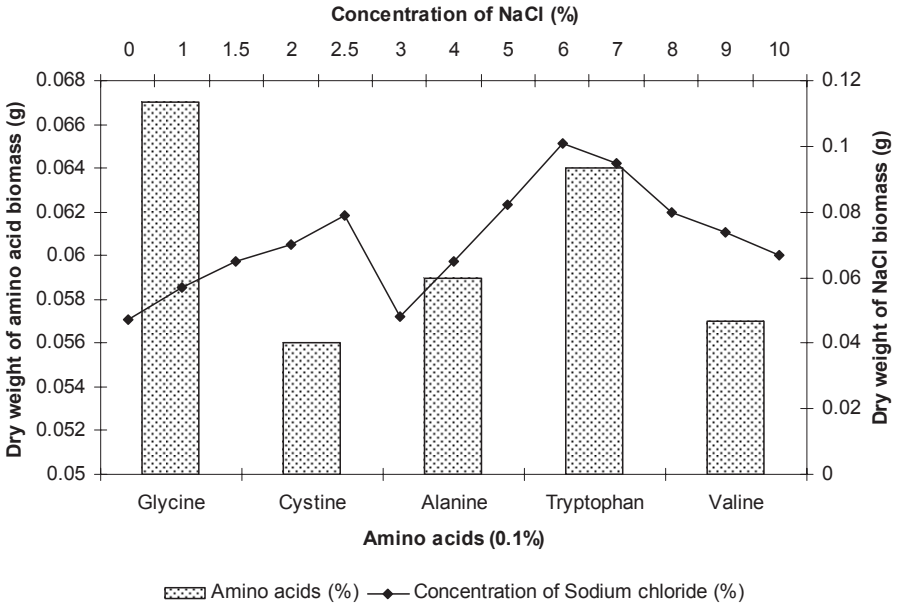

Fig. 2. Influence of amino acids and sodium chloride concentrations on the growth of the strain AQBMM35.

AQBMM35 was classified to be a species belonging to the *Streptomyces* genus.

Fermentation of *Streptomyces* strain AQBMM35 under fluorescent white light resulted in the production of carotenoids and was found to be initiated after 4 days of growth under illuminated conditions. Upon UV spectral analysis of the carotenoid extract, we found maximal absorbance peak at 280 nm (Fig. 3A). Thin layer chromatographic analysis of the carotenoid extract revealed the presence of a single

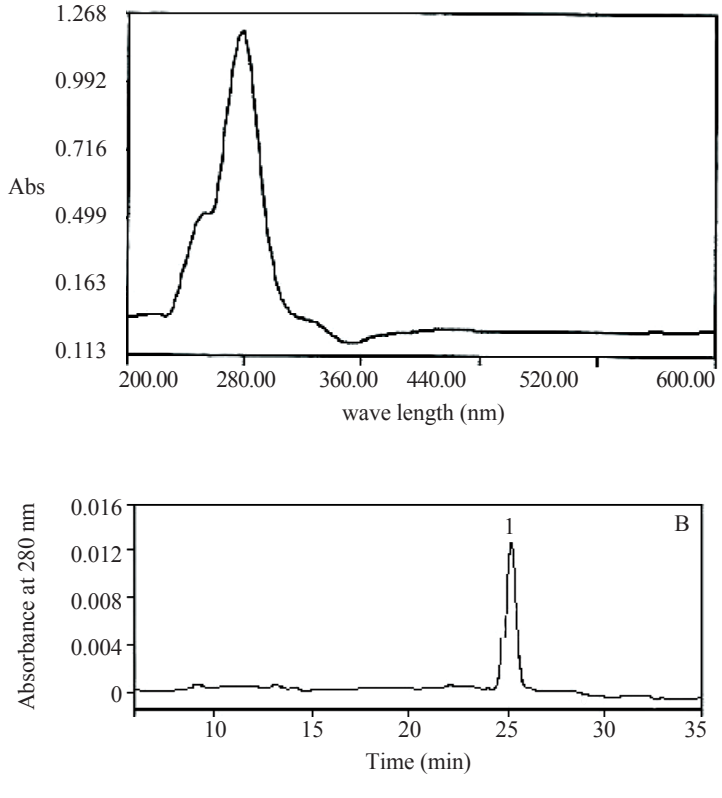

Fig. 3. UV spectrum [A] and HPLC analyses [B] of carotenoid extract showing the production of phytoene.

spot with R_f value of 0.81. This was further confirmed by HPLC analysis in which a single peak was produced which corresponds to phytoene when compared with the standards (Fig. 3B).

DISCUSSION

Marine sponges have been in recent focus because they form close associations with a wide variety of microorganisms and are a rich source of biologically active secondary metabolites (13). In the present investigation, the marine sponge collected was identified as *Mycale mytilorum* belonging to the class *Demospongiae Sollas*. Each species has unique spicules and according to that classification of species done (5). Microbial associates can comprise as much as 40% of sponge tissue volume, with densities in excess of 10^9 microbial cells per ml of sponge tissue, several orders of magnitude higher than those typical for seawater. In a recent report, nearly 94 isolates of *Streptomyces* were found to be associated with four species of marine sponges (14).

The strain AQBMM35 was characterized by morphological properties, nutritional uptake, physiological, biochemical properties and all these results suggest the strain to be classified under *Streptomyces* genus as reported earlier (7-9). The strain showed typical morphology of Streptomycetes when analyzing the shape and spore chains under scanning electron microscope as described earlier (11). The *Streptomyces* classification system was mainly dependent on characteristics such as the form of spores, the spores on the selection of accessories, melanoma and classified them into different clusters and could be divided into smaller groups according to their use of carbon (7-8).

The industrial production of carotenoids from the natural extracts was less when compared with synthetic manufacturing (15). In *Streptomyces*, carotenoid production is a widespread metabolic activity which occurs in a constitutive, light-dependent, or cryptic manner. Some *Streptomyces* sp. are naturally carotenogenic while some are not. The ability of some strains to produce these metabolites would provide newer and novel carotenoids which can ultimately be used for industrially useful products. The *Streptomyces* strain (AQBMM35) light-induced fermentation exhibited carotenoid production. Spectroscopic analysis, TLC and HPLC results confirmed the production of colorless carotene and phytoene. The results of the present finding correlate with previous findings of light-induced carotenogenesis in *Streptomyces griseus*, *Streptomyces coelicolor*, *Myxococcus xanthus*, *Mycobacterium* sp., and *Brevi-bacterium* sp. (3).

In conclusion, biotechnology plays a crucial role for large scale fermentative production of carotenoids. The large-scale production of carotenoids using microorganism is a viable option to chemical methods. Although good progress has been made in the development of a production host strain for enhanced carotenoids production through metabolic engineering, only a few studies have attempted the optimization of culture conditions for large-scale production of carotenoids. In the present context, the sponge-associated *Streptomyces* isolate (AQBMM35) fermentation in the presence of fluorescent white light enhanced carotenoid production. Further studies on mass production of this carotenoid and its use as an antioxidant and feed additive have to be carried out.

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