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Characterization of Marine *streptomyces sp. T1027* Producing β-Carotene Under Light Induction

V. Subhash Chandra Bose[1], B. Vishnupriya[2], K. Selvam[2]

1. Bharathiar University Coinbatore-46, Tamilnadu, India

2 Dr.N.G.P. Arts and Science College, Coimbatore-48, Tamilnadu, India.

ABSTRACT

Marine *Streptomyces sp.T1027* obtained from South coast of India was discovered to produce and accumulate β-carotene under the influence of light in liquid shake flask culture. The accumulation of β-carotene was growth associated and controlled by light, aeration, carbon and substrate solubility. Starch was the ideal substrate for maximum growth (4.5-6.1 g l^{-1}) and β-carotene accumulation (92-130 μg g^{-1}). Rapid extraction was done in Dimethylsulphoxide, Methanol (1:1) Solvent mixture. The organism was sensitive to a variety of antibiotics; in turn it was able to inhibit the human pathogen *Corynebacterium diphtheria*. Commercial substrates with high soluble starch content were found to produce maximum biomass and β-carotene production.

Keywords: Light induced carotenogenesis, Starch, β-carotene, Marine *Streptomyces sp. T1027*, DMSO: Methanol,

*Corresponding Author Email: Subash.cbose8@gmail.com

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INTRODUCTION

Microbial biosynthesis of natural pigments is an emerging area in metabolic engineering and industrial biotechnology that offers significant advantage over conventional chemical methods or extraction from biomass. The inherent biosynthetic capacity of variety of different organisms enables the effort for developing systems to produce bio colorants and pigments commercially from biological hosts. The production of carotenes from microbial sources has been an area of intensive investigation due to problem of seasonal and geographical variability in plant origin and limitations in chemically synthesized carotenoids especially due to complex pathways involved in chemical synthesis and mixtures of stereoisomers, which are not active as natural isomers resulting in undesired side effects their potential benefit to human health as a pro vitamin and antioxidant [2, 4]. The isolation of keto-carotenoids from human blood plasma and from the egg yolk and egg products of animals indicates the significance of carotenoids in diet and health. The antioxidant and free radical scavenging activity of carotenoids is well studied. Carotenoids act as an aesthetic and appealing food grade coloring agents and animal feed supplement and more recently as cosmetic and pharmaceutical compound[9].

Carotenogenisis and their pathways has been extensively studied eukaryotes and prokaryotes and the nucleotide sequences of carotenoid biosynthesis genes were first reported in the phototrophic bacteria *Rhodobacter capsulatus* and also in *Erwinia uredovora, Erwinia herbicola, Synechococcus sp.*, *Anabena sp., Myxococcus xanthus* and also *Brevibacterium linins* which is known to produce aromatic ring containing carotenoids[11].

The production of Carotenoids have been studied in terrestrial actinomyces especially in organisms like *Streptomyces coelicolor, S. griseus, S. setonii,* which were designated to be cryptic in nature since the exact mechanism is unknown. The light induced carotenogenesis in *Streptomyces coelicolor A3 (2)* has been extensively studied and the biosynthetic gene cluster and its sigma factor that directs light induced photodependent carotegenesis has also been discovered. Novel carotenoids have also been reported from *Streptomyces mediolani.* Many marine isolates of Streptomyces sp. have obtained from marine sediments, fishes and sponges which have been recently been explored for the production of carotenoids[15, 18]. Carotenoid biosynthesis in several carotenogenic Streptomyces species like *S. mediolani S. odorifer, S. lividans and S. coelicolor* typically yields the rare carotene isorenieratene The *Streptomyces griseus* strain JA3933 is non-carotenogenic under natural conditions, the presence of several carotenogenic functionally intact but not expressed genes has also recently been demonstrated[12].

Actinomycetes to date remain the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of about half of the discovered bioactive secondary metabolites notably antibiotics, antitumor agents, immunosuppressive agents and enzymes. Around 23,000 bioactive secondary metabolites produced by microorganisms have been reported and over 10,000 of these compounds are produced by actinomycetes, representing 45% of all bioactive microbial metabolites discovered. Among actinomycetes, around 7,600 compounds are produced by Streptomyces species. Many of these secondary metabolites are potent antibiotics, which has made Streptomycetes the primary antibiotic-producing organisms exploited by the pharmaceutical industry[4].

The oceans are highly complex environments and harbor a divers assemblage of microbes that occur in environments with extreme variations in pressure, salinity, temperature and low nutrition, besides this it is estimated that only 1% of marine microorganisms have been cultured or identified[7]. Among the genera of marine actinobacteria, the genus Streptomyces is represented in nature by the largest number of species and varieties about 80% of the total actinomycetes species, which differ greatly in their morphology, physiology and biochemical activities. Marine Streptomyces occur in different biological sources such as fishes, molluscs, sponges, seaweeds and mangroves, besides seawater and sediments[16].

In the present study marine *Streptomyces sp. T1027* producing β-carotene under light induction was characterized along with various physiological factors determining the amount of β-carotene production under light.

MATERIALS AND METHODS

Culture

The strain used in this study was isolated from marine sediments from South coast of India and identified as *Streptomyces sp.T1027* and it was found to produce β-carotene under Light induction.

Media and growth conditions

Streptomyces sp.T1027 was grown in Starch Casein Nitrate agar containing per liter of distilled water, 10 g soluble starch, 2.0 g $KHPO_4$ (anhydrous), 2.0 g $NaNO_3$, 2.0 g NaCl, 0.3 g Casein, 0.05 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4$, 0.02 g $CaCO_3$, 1.0 ml trace salts solutions, and 20 g agar at pH 7.2 ± 0.2, autoclave sterilized at 121^oC for 20 min at 100 kPa pressure. Unless otherwise stated, experiments were carried out in 250 ml Erlenmeyer flasks with a working volume of 100 ml in basal mineral salts media containing per liter of distilled water, 2.0 g $KHPO_4$ (anhydrous), 2.0 g $NaNO_3$, 2.0 g NaCl, 0.05 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4$, 0.02 g $CaCO_3$, 1.0 ml trace salts solutions agar at pH 7.2 ± 0.2. The aerial spores obtained from 7 days old culture were used as spore suspension for all the experiments[10].

Light induced carotenogenesis

Light induced carotenogenesis was initiated by inoculating the test organism in 100 ml of basal mineral salts media containing 10 g starch $l^{-1}$and incubated at 150 rpm at 28^{o}C for 5days in a incubated rotary shaker under continuous illumination with a under 500 lux incandescent light at 28^{o}C fitted inside incubator. Control flasks were maintained wrapped in foil. The pigmented mycelial pellets were filtered in Whatmann no.1 filter paper and the mycelia were blotted and the water content was reduce by drying in an oven at 40^{o}C for 30 min to avoid pigment break down and stored at 4^o C for future process. The mycelial pellets were solvent extracted and scanned for the presence of carotenoids in a Spectrophotometer from 300 nm-500 nm. The presence of distinct primary and secondary peak at 450 nm and 470 nm indicates the presence of β-carotene. Dry weight was estimated from equal volume of pellets dried at 90^{o}C in an oven[20].

Extraction and analysis of β-carotene

The semi dried mycelia was directly extracted with solvents DMSO, Methanol, Ethanol, Butanol, Chloroform and Petroleum ether[8]. The β-carotene were extracted in a mixture of 50 % (v/v) Methanol and Dimethylsulphoxide (DMSO) then the residues were cleared by centrifugation at 6000 rpm and the pigment was further extracted in petroleum ether in a separating funnel. The dry weight was determined by weighing an equal volume of the oven dried mycelia balls. The extracted β-carotene from each experimental sample was diluted to appropriate volume as to obtain the optimum optical density value (≤ 0.9) for the solvent used in the β-carotene extraction. After proper dilution, the optical density was measured at 400-500 nm. Total β-carotene in the sample was then estimated by using the formula given below

Total carotenoid content (µg/g) = $A \bullet V \bullet d / E^{1\%}_{1cm} \bullet W$

A = Absorbance

V = Volume of Extract (ml)

d = dilution

$E^{1\%}_{1cm}$ = coefficient of absorbance (2592 for petroleum ether)

W = weight of sample (g)

Cultural studies

The morphological characteristics of mycelial growth at different cultural conditions were observed in liquid - static; submerged - shake flask and solid state culture media's[6]. The microscopic morphology of the above cultural conditions was also observed.

Optimization of culture conditions

The influence of incubation time (1- 7 days), initial pH (4 - 10), incubation temperature (10, 28, 37 and 45°C) and concentration of NaCl (0.5 % to 5.0 %) were studied in the Mineral salts basal media. The total biomass yield and pigmentation of *Streptomyces sp.T1027* were studied in Mineral basal salts media under illumination with a Carbon to Nitrogen ratio (C: N ratio) of 5:1. The ability of the isolate to utilize common carbon substrates including Starch, Crystalline Cellulose, Carboxymethyl Cellulose (CMC), Pectin, Chitin and Lignin was analyzed. Dextrose, D-Fructose, D-Galactose, D-Mannitol, D-Mannose, D-Ribose, D-Sorbitol, Inositol, Lactose, Maltose, Starch, Sucrose and Xylose at 10 g/l were evaluated for carbon source utilization and β-carotene production[13]. Yeast extract (2.8 g l^{-1}), peptone (2.4 g l^{-1}), casein (4.4 g l^{-1}), urea (0.64 g l^{-1}), Sodium Nitrate (2.0 g l^{-1}), Potassium Nitrate (2.0 g l^{-1}), Ammonium Nitrate (0.8 g l^{-1}) and Ammonium tartrate (1.9 g l^{-1}) were the different nitrogen sources evaluated[17]. The growth conditions for *Streptomyces sp.T1027* were optimized with different C: N ratio by having various concentrations of glucose (0.25, 0.5, 1.0 and 2.0 g l^{-1}) and a single concentration $NaNO_3$ (2.0 g l^{-1}) as nitrogen source.

Antibiotic sensitivity test

Antibiotic sensitivity test of the selected isolate was performed according to the Kirby-Bauer method. The test organism was streaked on Muller Hinton Agar and the susceptibility resistance of the test organism to various types of antibiotics was tested by observing and measuring the zones of inhibition[1].

Antimicrobial activity

Antimicrobial activity of the actinomycete was screened by conventional cross-streak method[19]. Single streak of the actinomycetes was made on Nutrient agar medium and incubated at 28 °C for 4 days. After observing a good ribbon like growth of the actinomycetes, the human pathogens namely *Escherichia coli*, *Klebsiella*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphyloccous aureus*, *S. epidermis*, *Corynebacterium diphtheriae* and *Candida albicans*, which were obtained from the Clinical Laboratory, in Kovai Medical Center and Hospital, Coimbatore, were streaked at right angles to the original streak of actinomycetes, and incubated at 37°C. The inhibition zone was measured after 24–48 h. The antimicrobial activity was determined based on the zones of inhibition.

Production in commercial substrates

The ability of the test organism to produce and accumulate β-carotene s in various commercial agro substrates was tested by growing the test organism in basal mineral media containing 1% of the commercial substrates. The various agro products are Wheat flour, Rice flour, Tapioca Flour, Corn Flour, Wheat husk, Paddy husk, Milled maize, Groundnut cake, Cotton cake and Gram husk. The substrates are tested for their potential suitability to act as commercially viable substrates for β-carotene production [14].

RESULTS AND DISCUSSION

Light induced carotenogenesis

The microscopic morphology of the Marine Actinomycete *Streptomyces sp.T1027* was also observed (Plate 1). The culture grown in the presence of light was found to produce intense yellow mycelial pellets but the same culture grown in dark conditions exhibited the formation of colorless mycelial pellets (Plate 2). Spectrophotometric analysis of petroleum ether extract revealed the λ_{max} value at 450 nm for cultures grown in light and no peaks were observed for the test culture grown in dark.

Plate 1. a. 100X magnification of aerial spores *Streptomyces sp.T1027* Plate 1. b. 400X magnification of submerged mycelia of *Streptomyces sp.T1027.*

Plate 2. Growth and presence of β-carotene production in light in submerged culture

As shown in Figure 1, Photo induces caroteogenesis was initiated within 48 hours of incubation in the shake flask culture. The formation of β-carotene pigment was found to be growth associated and the accumulation of β-carotene pigment was found to be directly proportional to the growth rate and exposure to light. The increase in total carotenoid content was found to linearly increase with the increase in biomass yield. The accumulation of β-carotene was maintained at a constant rate with the increase in biomass and the cessation in the increase of biomass was associated with the cessation in β-carotene accumulation. This indicates that the carotenoid production in the test organism was growth associated. The various growth phases of the mycelia were not found to increase the β-carotene accumulation and intensity.

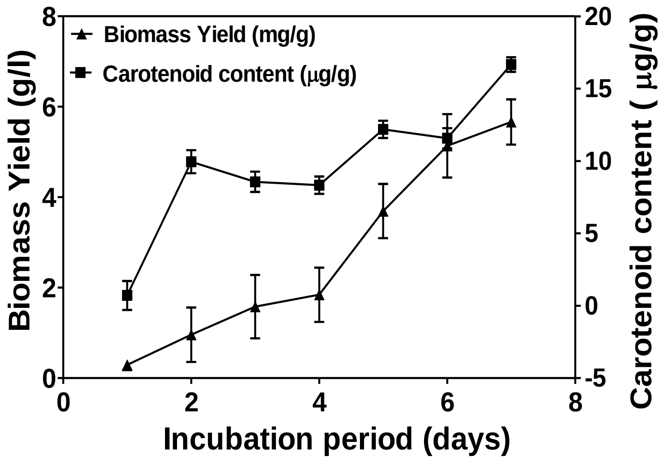


Figure.1 Accumulation of β-carotene was proportional to increase in biomass but was independent of the growth phase of mycelia

The test organism was observed to initiate and maintain carotenoid accumulation in proportion to resist the light stress on reaching a critical juncture were the light stress has been adequately absorbed by the accumulated β-carotene, the β-carotene accumulation ceases and a uniform coloration results. The formation of spherical mycelial pellets also was able to confirm this mode of β-carotene accumulation. The newly formed mycelial pellets which were found to be white and on growing and maturing they were able to accumulate and retain a uniform yellow color and the final pellet size was found to be uniform throughout the culture.

In *S. coelicolor*, the transcription of the carotenoid biosynthesis gene cluster is specified by LitS, a photo-inducible sigma factor, conformational alteration of LitR upon receiving the illumination signal determines it's binding to DNA and the MerR family transcription regulators In *Myxococcus xanthus*, a Gram-negative gliding bacterium it involves a complicated mechanism that causes activation of an extracytoplasmic function sigma factor (CarQ), which leads to the sequestration of a MerR family transcriptional regulator (CarA) that represses the expression of the carotenoid biosynthesis genes in the dark[17].

Cultural studies

Accumulation of carotene was observed in submerged shake flask culture. The static liquid and culture media produces white aerial mycelia which mature into brown filaments. The differentiation of growth patterns and characteristic in different types of culture media indicates that the β-carotene accumulation was found to be maximum in the submerged shake flask culture. The aeration provided in the shake flask culture was also found to influence the formation of yellow mycelial pellets, aeration in the submerged culture was also found to an important factor in the growth and accumulation of β-carotene. The formation of aerial mycelia was found to inhibit the formation of β-carotene; solid culture media promoting copious aerial mycelia were unable to accumulate β-carotene. Photo induction, aeration, growth morphology, media composition, nature of substrates and light penetration were found to plays a significant role in the production of β-carotene.

Extraction and analysis of β-carotene

Complete extraction was obtained within 30 min at 60^{o}C for raw samples and instant extraction within 5 min was obtained for samples stored at 4^{o}C. The β-carotene extracted in DMSO/Methanol was re extracted in Equal volume of petroleum ether in a separating flask and scanned at 300 nm-500 nm and the λmax values of primary peak at 449 nm and secondary peak at 476 nm was obtained for confirmation.

On selection with various solvent combinations, DMSO/Methanol (50:50) was found to be the quickest and ideal method to completely extract all the β-carotene from the test sample of *Streptomyces sp.T1027*. This was also observed in β- carotene extraction from carrots *Daucus carota*, were it was found that solvent type, time, temperature and treatment plays a significant role in carotene extraction and content It was found that extraction at 60^{o}C, fresh carrots yielded 39.8 μg $g^{-1}$of β-carotene and on freezing yielded 64.5 μg g^{-1}on four hour incubation[8]. The λmax values obtained corresponding to β-carotene in standard extractions. The stability of the β-carotene extract in Petroleum ether was less than 30 min at room temperature, on the contrary good stability was observed in DMSO/ Methanol and it was stable up to 48 hrs.

Optimization of Culture conditions

The cell dry weight increased gradually with increase in initial pH of the media. The organism exhibited maximum growth at pH 8.0 (5.959 g l^{-1}) and maximum β-carotene yield (23.71 μg g^{-1}) at pH 7.0 (Figure 2). The isolate was able to grow at temperatures 28^{o}C and 37^{o}C the yield of carotene was similar 23.56 μg $g^{-1}$and 22.14 μg g^{-1} respectively. The biomass yield at 28^{o}C was

5.83 g l^{-1} and at 37°C was 5.21 g l^{-1} and produce β-carotene, no growth was observed at 10°C and 45°C. The organism was able to with stand salt concentration up to 5% where it exhibited mild growth but good growth & β-carotene production was observed till 3% concentration (Figure 3).

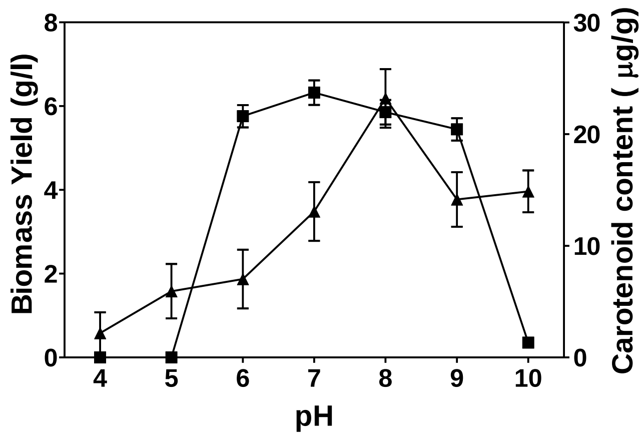


Figure.2 Effect of pH on accumulation of β-carotene in the optimum pH range of *Streptomyces sp.* from pH 6-9.

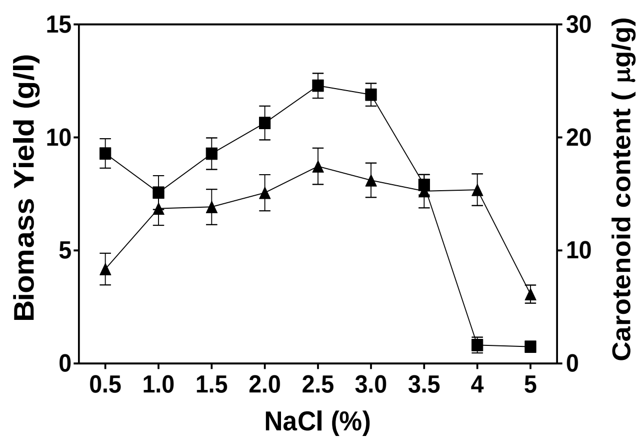


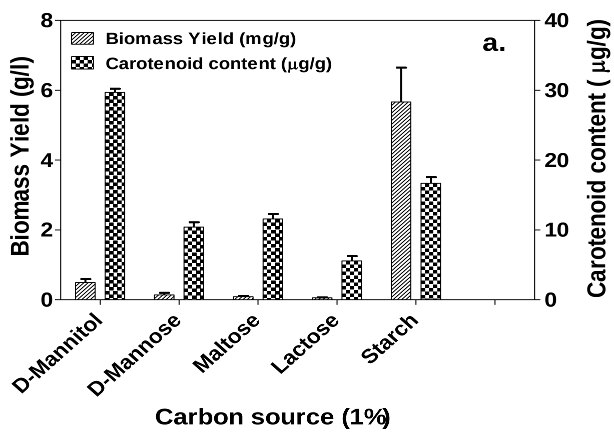
Figure.3 Influence of NaCl stress. Growth associated β-carotene accumulation under salt stress. No significant variation in β-carotene accumulation due to salt stress

The accumulation of carotenes through the utilization of major carbon substrates were analyzed under illumination in a shake flask culture. Starch was found to be the ideal substrate for the maximum yield of biomass and β-carotene accumulation, the biomass yield was found to be 4.5-6.1 g l^{-1} which forms about 50% of the total carbon substrate added. The total carotenoid content was found to be 92-130 µg g^{-1}. Chitin was also found to accumulate carotenes but could not be quantified. CMC and Crystalline Cellulose exhibited growth but no β-carotene accumulation was observed. No growth was observed in pectin and lignin. Assimiliation of different sugars as carbon source by the isolate for growth and pigment production shows that Mannitol (29.07 µg g^{-}

^{1}) has the highest β-carotene yield and the biomass yield was maximum in starch (5.664 g l^{-1}). There was variation in the accumulation of β-carotene based on the type of the carbon source, intense accumulation of carotene was observed in mannitol and only mild accumulation was observed in lactose. Growth was observed in D-Glucose, D-Galactose, Inositol, and mild growth was observed in Sucrose, D-Mannose and no growth was observed in Fructose, D-Ribose, Pectin and Xylose. It is inferred that the nature of the carbon source influences the production and accumulation carotenes. Soluble starch was found to be highly suitable for inducing maximum growth and β-carotene production and relatively high concentrations of starch can be utilized for growth and β-carotene production, the soluble nature of starch effectively aids in light induced carotenogenesis (Figure 4a).

Among the various nitrogen sources Peptone gave the maximum biomass yield (4.97 g l^{-1}) and maximum β-carotene production was obtained in Casein acids hydrolysate (31.244 μg g^{-1}) the nature of the nitrogen source was not a significant factor influencing the production and accumulation of β-carotene. The entire nitrogen source used here exhibited β-carotene accumulation at various degrees. Compounds with nitrate group were more conducive to β-carotene production than compounds with ammonia functional groups, ammonia compounds seem to give less biomass yield than nitrate groups (Figure 4b)

Maximum growth (0.45g l^{-1}) and β-carotene production (16.12 μg g^{-1}) was observed in a concentration of 0.5% the concentration of Glucose at 1% inhibited carotene production and the growth was also retarded.



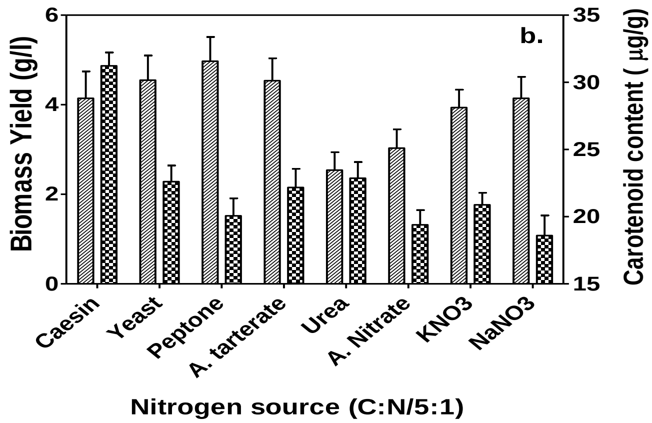


Figure.4 Growth and β-carotene production in marine *Streptomyces sp.T1027*. a) Influence of Carbon source. b) Influence of Nitrogen source

Antimicrobial activity

The isolate was able to inhibit the pathogen *Corynebacterium diphtheria.* No zones of inhibition were observed against. *Escherichia coli*, *Klebsiella*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphyloccous aureus*, *S. epidermis,* and *Candida albicans* in the Nutrient agar plates. It is interesting to note that *Corynebacterium diphtheria* also belong to the Phylum Actinobacteria. (Plate 3)

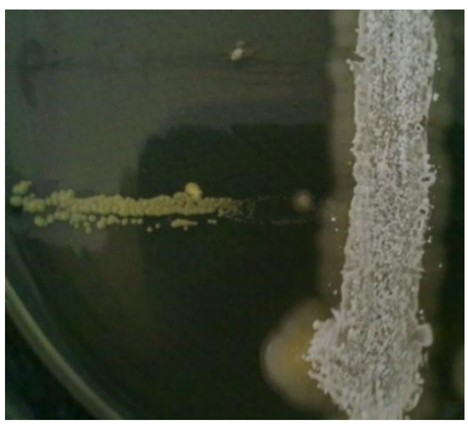


Plate 3. Antimicrobial activity of *Streptomyces sp., T1027 against Corynebacterium diptheriae*

Production of β-carotene in Commercial Agro Substrates

The growth and β-carotene production on various commercial agro substrates were tested. The biomass yield and β-carotene production were 2.839 g/l and 11.03 µg/ml in Potato starch, 3.306 g/l and 7.561 µg/ml in Tapioca starch and 1.174 and 7.71µg/g in Corn starch respectively.(Figure 5) Other substrates exhibited growth but there was no carotenogenesis, this may be primarily due to the lack of diffusion of light and also the insoluble nature of the other substrates.

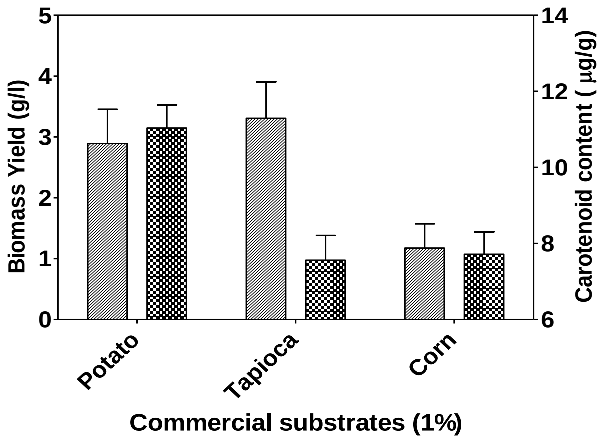


Figure.5 Growth and β-carotene production in marine *Streptomyces sp.T1027* from commercial substrates

CONCLUSION

We have studied the ability of β-carotene accumulation in marine *Streptomyces sp.T1027* under light induction. The pattern of growth and β-carotene accumulation in standardized mineral basal salts media in which starch was found to yield the maximum growth (4.5-6.1 g l^{-1}) and β-carotene accumulation under light induction at 23-30 µg g^{-1} yielding about 50% of the total carbon source added. Nitrates were found to be the best nitrogen sources for growth. The Extraction with DMSO: Methanol (50:50 v/v) was the ideal solvent mixture for extraction from Streptomycetes semi dried mycelial pellets. In turn it was able to inhibit the human pathogen *Corynebacterium diphtheria.* The ability to accumulate β-carotene and the aesthetic nature of the organism can be exploited in probiotics, dietary supplements and aquaculture. Further studies are required for maximizing β-carotene production in *Streptomyces* sp., and their application.

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