PRAJÑĀ - **Journal of Pure and Applied Sciences Vol. 27-28: 26 - 40 (2019-20)**
ISSN 0975 - 2595

Study of biosynthesis and statistical optimization of medium components for β-carotene production using marine *Paracoccus* sp. OC1 isolated from Gulf of Khambhat

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ABSTRACT

The present study aimed to isolate and identify a carotenoid producing microbial strain from the marine environment. The orange-colored microbial colony was isolated from marine water of the Gulf of Khambhat, Gujarat, India, and identified as a Paracoccus species using 16s rRNA gene amplification. Environmental factors viz. medium strength, pH, temperature, sugar, and aeration affecting the carotenoid production were optimized. Paracoccus spp. OC1 produced the highest pigment in media-Marine broth (MB) 1/10, pH-7, temperature-27ºC, sugar- sucrose, with incubation at static condition. Besides the five-fold increase in β-carotene production was achieved by optimization of meat extract, glycerol, ferric citrate, and potassium nitrate concentration using statistical designs like Plackett-Burman design and response surface methodology. The β-carotene was purified using thin layer chromatography and characterized using UV-Visible spectroscopy, FTIR spectroscopy, and mass spectroscopy. The presence of the lycopene β-cyclase gene responsible for β-carotene production was determined through PCR amplification.

***Keywords:** Carotenoids, Plackett-Burman Design, RSM, FT-IR, MS.*

INTRODUCTION

Natural products with diverse structures and applications gain importance in pharmaceutical, nutraceuticals, and food industries over synthetic products. One such product category is pigments of natural origin that include chlorophyll, melanins, carotenoids, etc. Carotenoids with a wide color range from yellow to red, present in plants, algae, fungi, and bacteria, are very attractive and widely used as a potential antioxidant in nutraceuticals. Carotenoids serve as a source of vitamin A (α-carotene, β-carotene, and β-cryptoxanthin), increasing the functioning of the immune system, preventing the cell from damage due to their antioxidant effect [1, 2]. This property is useful for preventing certain types of cancers in humans by serving as a hormone precursor, in photoprotection and increasing the growth rate of cells [3, 4]. Carotenoids play a major role in quenching singlet oxygen radicals and removing molecular oxygen from a biological system without producing any oxidizing products. Moreover, β-carotene traps peroxy free radicals in tissues at a low oxygen concentration [5, 6]. The growth of commercially used carotenoids increases at the rate of 2.3% annually with nearly $ 1.4 billion estimated market value in 2018. Specifically, the market value of β-carotene is expected to grow $334 million by 2018 with a 3.1% annual growth rate [6]. Large numbers of bioactive compounds bearing therapeutics applications were obtained from marine sources, as they have a highly beneficial effect on human health. Peng et al. [7] demonstrated the absorbance of carotenoids and their derivatives (e.g. fucoxanthin and fucoxanthinol) in the digestive tract of mammals which could enter in blood circulation system. Marine carotenoids like fucoxanthin and its metabolites fucoxanthinol, amarouciaxanthin A, and halocynthiaxanthin have antioxidant, anti-inflammatory, anticancer, anti-obese, antidiabetic effect and also have protective

effects on the liver, blood vessels of the brain, bones, skin, and eyes [8]. Therefore, the Gulf of Khambhat, a saline-alkaline site, was explored to obtain a pigment-producing microorganism. Depending upon the physicochemical factors viz., medium components, pH of media, temperature, aeration, presence of inorganic salts, and organic carbon source of the medium, the carotenoids producing ability of bacteria differ [9]. With excellent market value and applications of carotenoids, especially β-carotene, there is a need for scaling up the production using microbial source. Hence, the present study aimed to optimize parameters for carotenoids production, followed by developing medium formulation using statistical approach for increased carotenoid production by *Paracoccus* sp. OC1 isolated from Gulf of Khambhat. Carotenoid produced was separated and characterized using chromatographic and spectroscopic methods. The Lycopene β-cyclase, a key enzyme responsible for β-carotene production, was amplified from genome of *Paracoccus* sp. for the confirmation of β-carotene synthesis.

METHODOLOGY

Microorganism: Isolation and Identification

The water sample was collected from the Gulf of Khambhat (22.3°N 72.62°E) in a sterile bottle and stored at room temperature until used. The water sample was serially diluted and inoculated on various media like marine agar, Luria agar, and nutrient agar (Himedia, Mumbai, India), using the spread plate technique and incubated at 27°C for 4-5 days till colored colonies appeared for isolation of desired pigmented microorganism. The orange color colony was selected and maintained on marine agar by routine subculturing [10]. For the identification of bacteria, biochemical tests were performed and the 16S rRNA gene was amplified using universal forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer (5'-AAGGAGGTGATCCAGCCGCA-3') purchased from 1st BASE (Agile Life Science Technologies India Pvt. Ltd.). Amplified Gene product was sequenced at 1st BASE (Agile Life Science Technologies India Pvt. Ltd.). Using the Nucleotide BLAST search program [11] (http://www.ncbi.nlm.nih.gov/blastn) nucleotide sequence homology was searched and a neighbor-joining (NJ) tree with bootstrap value 500 was generated using the MEGA 4.0 software [12]. The sequence was submitted to GenBank [13].

Optimization of preliminary growth parameters and total carotenoids (TC) estimation

For studying primary growth parameters, 1% of actively growing culture was inoculated in different media and all flasks were incubated at 27°C for 7 days. Fermentation parameters like medium strengths (MB, MB 1/2, and MB 1/10), pH of media (pH 5, 7, 9, 10, 11, and 12), incubation temperatures (27°C, 37°C and 45°C) and carbon source (starch, sucrose, glucose, maltose, and glycerol; 1% of each sugar) were used for optimizing growth and pigment production. The effect of agitation was studied by incubating flasks on static (0 rpm) and shaking (150 rpm) conditions at 27°C. The intracellular pigments were extracted by the cell lysis method. For the determination of total carotenoids (TC) content of a cell, 1 mL of culture was centrifuged and the weight of the cell pellet was determined by the gravimetric method [14]. The pigment was extracted from the cell pellet using different solvents like methanol, acetone, chloroform, hexane, and ethyl acetate at 4°C until the cell pellet becomes colorless. The cell debris removed by centrifugation and supernatant with crude carotenoids was used for further analysis. All the tubes were kept in dark to prevent the oxidation of carotenoids [15]. Separation and characterization of carotenoids were done using chromatographic technique and spectroscopic analysis.

Identification of a gene involved in β-carotene biosynthesis

Under the present study, three enzymes lycopene β-cyclase, β-carotene hydrolase, and β-carotene ketolase playing role in carotenoid biosynthesis were amplified from the genomic DNA of *Paracoccus*. To identify these three enzyme, primer used were FP_LBC 5'-gcttgaggtgtcgctggt-3' and RP_LBC 5'-gtggaaaaaccggtgaaaga-3' for lycopene β-cyclase; FP_BCH 5'-gactgctgctgcgactcc-3' and RP_BCH

5'-gctccaaaactcccctctct-3' for β-carotene hydrolase and FP_BCK 5'-catcccctcatcttccttca-3' and RP_BCK 5'-caccaggccaggtaaggata-3'for β-carotene ketolase. The reaction was carried out in a 50 µl reaction mixture containing 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 25 pmol of Forward Primer (FP), and Reverse Primer (RP) of a particular enzyme, 50 ng DNA template, and 5 U Taq DNA polymerase. Amplification cycle parameters were set as 94 ˚C for 1 min for initial denaturation then 94 ˚C for 45 sec, 60 ˚C for 45 sec and 72 ˚C for 60 sec, for 35 cycles followed by a final extension of 10 min at 72 ˚C in a thermocycler. Amplified PCR products were analyzed using agarose gel electrophoresis (1.5 %) and compared with a 100 bp DNA ladder to determine the product size.

Screening of carbon and nitrogen source using a statistical approach

Plackett-Burman Design

The Plackett–Burman design, an effective technique for screening of 11 medium components [16–18], was used concerning their main effects on total carotenoids production. Based on our preliminary studies using One-Variable-At-a-Time (OVAT), the carbon and nitrogen sources were further used to show their main effect on TC production included in the design [19].

The effect of each variable was determined by equation (1):

$$E_{(xi)} = (\Sigma\ TCi^{+} - TCi^{-}) / N \text{----------(1)}$$

Where $E_{(xi)}$ is the concentration effect of the tested variable. TCi^{+} and TCi^{-} are the total carotenoids from the trials where the variable (*x*i) measured were present at high and low concentrations, respectively; and N is the number of trials divided by two.

Total eleven factors, glucose, starch, sucrose, glycerol, maltose, fructose, ferric citrate, meat extract, yeast extract, potassium nitrate, and ammonium nitrate were screened for their effect on the final product. The salinity was set up to 3.5% with artificial marine water in each run [20]. In the present study, a total of eleven components were screened using 18 experimental runs with six center point. TC production was carried out in triplicate and the average value was taken as the response. The factors showed a significant positive effect at 95% level ($P < 0.05$) were considered to have a significant effect on TC production and thus used for further optimization to formulate the final medium by response surface methodology (RSM).

Response Surface Methodology

Total four variables showing the highest positive effect in Plackett-Burman design were further used to formulate final media based on variable screened previously using central composite design (CCD) with a five-coded level. For four variables this design was made up to full 2^4 factorial design with the four cube points and total run number for CCD concerning the concentration of components could be decided by full factorial points $2^k$where *k* is the number of variables, center points η0 (η0>1) (six replicates) and two axial points for each variable (á=2^k/4, =2 for k=4) total design points (experiments) would be N=2^k+2^k+ η0=30 [21, 22].

The second-order polynomial coefficients were calculated and analyzed using the ''Design Expert" software (Version 9.0.3.1, Stat-Ease Inc., Minneapolis, USA) statistical package. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). For the informative analysis of variables, linear, mean, quadratic, quartic, and cubic terms of coded levels of glycerol, potassium nitrate, meat extract, and ferric citrate were tested. The level at which every term in the selected model should be significant was set at 5% [19]. The response surface plots were generated by the same software and response was calculated by using the following equation (2):

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_1\beta_2 AB + \beta_1\beta_3 AC + \beta_1\beta_4 AD + \beta_2\beta_3 BC + \beta_2\beta_4 BD + \beta_3\beta_4 CD + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 \text{----------(2)}$$

where Y is the response in terms of TC production, β_0 is the model intercept and β_1, β_2, β_3, β_4 are linear coefficient, β_{11}, β_{22}, β_{33}, β_{44} are squared coefficient and $\beta_1\beta_2$, $\beta_1\beta_3$, $\beta_1\beta_4$, $\beta_2\beta_3$, $\beta_2\beta_4$, $\beta_3\beta_4$ are interaction coefficient and A, B, C, D, AB, AC, AD, BC, BD, CD, A^2, B^2, C^2, and D^2 are level of the independent variable [23].

RESULTS

Isolation and Identification

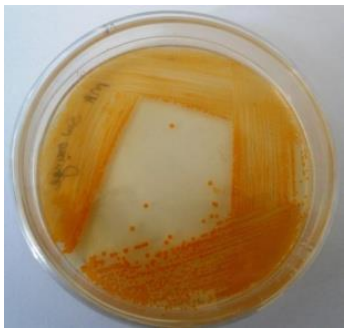

Fig. 1. **Growth of *Paracoccus* spp. on marine agar plate having orange pigmentation.**

The color of the colony was the basis of primary screening for the isolation of a carotenoid producing strain, therefore an orange-colored colony was isolated and maintained on a marine agar plate (Fig. 1) and labeled as OC1. Colonies were small, round with entire margin and convex elevation which showed a smooth surface with moist consistency.

Table : Result of Biochemical tests for *Paracoccus* spp., OC1

Biochemical Tests	Reaction
Gram Reaction	-
Spore Formation	-
Methyl Red Test	-
Voges- Proskauer (V-P) Test	-
Citrate Utilization Test	-
Indole Production Test	-
Hydrogen Sulphide Production Test	-
Phenylalanine Determination Test	-
Nitrate Reduction Test	-
Gelatine Hydrolysis Test	-
Ammonia Production Test	-
Urea Hydrolysis Test	-
Starch Hydrolysis Test	-
Casein Hydrolysis Test	-
Catalase Test	+
Dehydrogenase Test	-
Triple Sugar Iron Agar Test	-
Carbohydrate Utilization Test	
Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, Trehalose, Malibiose, Sucrose, L-Arabinose, Mannose, Inulin, Glycerol, Inositol, Sorbitol, Adonitol, Arabitol, Erythritol, Cellobiose,Xylitol, D-Arabinose	+
Raffinose, Sodium Gluconate, Salicin, Dulcitol, Mannitol, α-methyl D-glucoside, Rhamnose, Melezitose, α-methyl D-mannoside, ONPG, Esculin Hydrolysis, Malonate Utilization, Sorbose	-

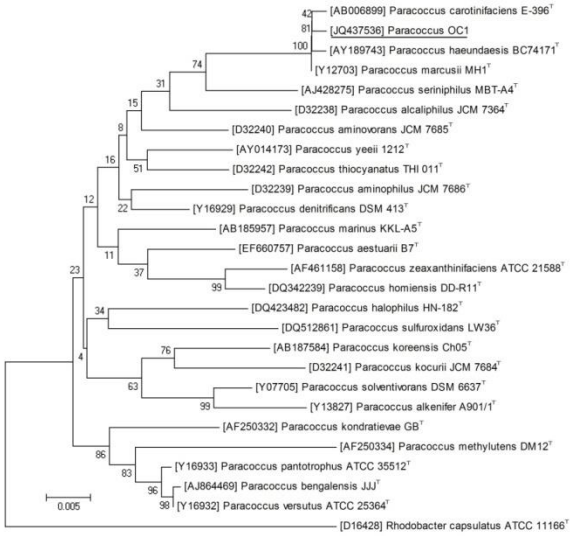

Fig. 2. **Phylogenetic neighbor-joining tree based on 16S rRNA gene sequences showing the isolates having closely relationship with *Paracoccus carotinifaciens* E-396 ^{T} and bootstrap values (expressed as percentages of 500 replications) greater than 50% are given at nodes. Bar 0.5% sequence variation.**

Microscopic observations suggested that strain OC1 was Gram's negative, nonmotile, rod-shaped, and nonspore forming. The biochemical characteristics of the OC1 strain are represented in Table 1. A comparison of the morphological and biochemical traits of OC1 with *Paracoccus* spp. [24] suggested that OC1 belonged to *Paracoccus* genus. For further identification of the strain, phylogenetic analysis was performed by aligning the 16S rRNA gene sequence of type strains reported till date belonging to *Paracoccus* genus with that of OC1 to construct a phylogenetic tree. Based on the phylogenetic tree, the type strain *P. carotinifaciens* E-396^{T} [AB006899] was the closest neighbor of *Paracoccus* OC1 with 99.35% similarity (Fig. 2). The 16s rRNA gene sequence was submitted GenBank nucleotide sequence database under the accession number JQ437536. Besides, *P. carotinifaciens* E-396^{T} was reported to produce astaxanthin [25] but our isolated strain can produce β-carotene, which is a different type of carotenoid, suggesting *Paracoccus* OC1 to be considered as a novel strain.

Preliminary Physiological Growth Parameter Optimization and Total Carotenoids Estimation

For biomass and carotenoid production, different physiological parameters were studied using OVAT method.

Table 2: Effect of preliminary growth parameter like medium strength, pH, incubation temperature, carbon source and aeration on wet cell weight and TC production.

	Wet Cell Weight (gm/L)	Total Carotenoids Weight (mg/L)
Media		
MB	28.33 ± 1.15	833.33 ± 288.67
MB1/2	20.33 ± 2.56	666.66 ± 188.67
MB1/10	14.5 ± 2.59	566 ± 148.58
pH of media		
pH 7	17.16 ± 0.28	565.33 ± 36.89
pH 8	21.16 ± 2.56	932.66 ± 73.54
pH 9	18 ± 1.32	867.33 ± 55.14
pH 10	17.66 ± 1.75	752 ± 102.05
Incubation Temperature		
27°C	28.33 ± 1.15	833.33 ± 288.67
37°C	17.66 ± 2.75	1166.66 ± 288.65
47°C	14.83 ± 6.71	545 ± 38.25
1% of each Sugar in medium MB		
Starch	22.5 ± 2.64	833.33 ± 288.67
Sucrose	18.33 ± 2.02	1166.66 ± 267.88
Glucose	19.16 ± 1.89	1090 ± 108.58
Maltose	20 ± 2	1000 ± 58.65
Glycerol	25.66 ± 3.25	1333.33 ± 577.35
Aeration		
Static Condition	28.33 ± 1.15	833.33 ± 187.36
Shaking Condition	16.5 ± 0.86	500 ± 87.36

Table 2 shows results of physico-chemical optimization for biomass and total carotenoids production. The biomass production was 26.16 g/L, 19.0 g/L, 12.83 g/L and corresponding carotenoid production was 833.33 mg/L, 666.66 mg/L, 500 mg/L in three different medium concentrations MB, MB (1/2), and MB (1/10) respectively. Microorganisms grew in the pH range of 7 to10, at pH-8, the highest biomass 21.16 g/L, as well as carotenoid production of 932.66 mg/L, was observed, whereas media having pH-5, 6, 11, and 12 were not conducive for the growth of *Paracoccus* sp. OC1. Isolate OC1 was growing at a temperature range of 27°C to 45°C and produced the highest biomass 28.33 g/L at 27°C and highest carotenoid 1166.67 mg/L when incubated at 37°C. Among five different sugars, glycerol supports the highest biomass (23.5 g/L) and total carotenoid (1333.33 mg/L) when incubated at static conditions.

Separation and characterization of β-carotene

Extracted carotenoid was analyzed by chromatographic and spectroscopic techniques. Concentrated methanolic extract of carotenoids was loaded on a TLC sheet using a glass capillary along with standard β-carotene (Hi-media) and developed using methanol: ethyl acetate (1:1 v/v) solvent system. The retention factor (R_f) of separated pigment was similar to standard β-carotene. The colored band separated corresponding to standard β-carotene was scraped and dissolved in methanol and used as a sample for spectroscopic analysis. In UV-Visible spectroscopy, three peaks at 425 nm, 450 nm, and 475 nm were obtained, which shows the signature spectral pattern of carotenoids [19, 26]. In Fourier Transformed Infra-Red (FT-IR) Spectroscopy, the spectrum exhibited peaks at 3436.54 cm^{-1} and 2923.50 cm^{-1} for asymmetric and symmetric stretching vibration of $-CH_2$ and $-CH_3$, 1633.77 cm^{-1} for a stretch of -C=C- (alkenes) repeats more than four to five times, 1028.78 cm^{-1} for trans conjugated alkenes -CH=CH- out of plane deformation mode which represents the carotenoid have similarity with the functional groups present in the structure of β-carotene [27]. Mass spectra of purified pigment showed a 100% mass to charge ratio at 538.25 (M^+H). Breemen reported molecular ion of β-carotene showed a signal of mass to charge ratio (m/z) at 536 (M) [28]. In another study, the mass spectrum of β-carotene predominance molecular ion (M^+H) showed m/z ratio at 537.33 [29]. From all the analytical techniques we proposed that the extracted molecule was β-carotene.

Amplification of gene involved in β-carotene biosynthesis

Out of all enzymes responsible for carotenoid biosynthesis, only three enzymes; lycopene β-cyclase, β-carotene hydrolase, and β-carotene ketolase were selected and their respected gene sequences were searched using KEGG (Kyoto Encyclopedia of Genes and Genomes) database [30]. Expected gene product size of lycopene β-cyclase, β-carotene hydrolase, and β-carotene

ketolase were 1050 bp, 918 bp, and 1599 bp respectively [31–33]. Figure 3 shows agarose gel containing PCR amplified products of selected genes with 100 bp DNA ladder and genomic DNA of isolate OC1. In PCR amplification, only lycopene β-cyclase gene was amplified with product size ~950 bp, and the expected gene size was 1050 bp, which showed the presence of the gene in genomic DNA of *Paracoccus spp*. OC1.

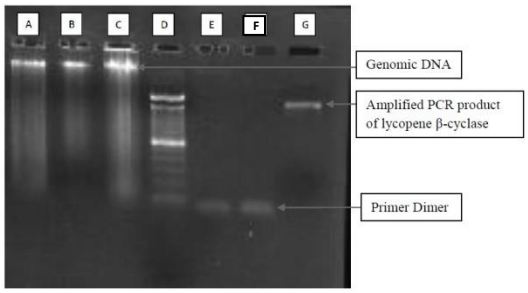

Fig. 3. Agarose gel electrophoresis of genomic DNA isolated from *Paracoccus* OC1 (A, B, C), 100bp DNA ladder (D), amplified PCR products for lycopene β-cyclase (G), β-carotene hydrolase (F) and β-carotene ketolase (E) in which only well G contain amplified gene where as well E and F showed only primer dimer, no amplification of gene.

Statistical Design

Plackett-Burman Design

Primary screening of physiological parameters and sugar utilization was performed by the OVAT method. Based on these results, eleven components were screened using the Plackett-Burman design.

Table 3: Effect of components used in Plackett-Burman design with their standardized effect and % contribution on TC production.

Variable	+ value (gm/L)	- value (gm/L)	Standardized effect	% Contribution
X1 (Glucose)	0.5	0.05	-83.33	3.05
X2 (Starch)	0.5	0.05	-59.33	1.55
X3 (Sucrose)	0.5	0.05	-208.67	19.12
X4 (Glycerol)	0.5	0.05	266.00	31.07
X5 (Maltose)	0.5	0.05	-22.00	0.21
X6 (Fructose)	0.5	0.05	-3.33	4.879E-003
X7 (Ferric citrate)	0.1	0.01	3.33	4.879E-003
X8 (Meat Extract)	2	0.2	90.00	3.56
X9 (Yeast Extract)	1	0.1	-106.00	4.93
X10 (Potassium Nitrate)	0.5	0.05	110.00	5.31
X11 (Ammonium Nitrate)	0.5	0.05	146.00	9.36

Table 3 shows the concentration of each parameter with its standardized effect on TC with a % contribution. Of the eleven tested components, glycerol had a maximum positive effect on TC production, followed by ferric citrate, meat extract, ammonium nitrate, and potassium nitrate. Table 4 shows the design matrix of each nutrient component tested at two concentrations, high and low concerning TC production ranging from 136 mg/L to 896 mg/L.

Table 4: Plackett-Burman design for eleven factors with two coded level (+ and -) with their respective TC production in each run.

Run	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	TC (mg/L)
1	+	+	-	+	+	+	-	-	-	+	-	688
2	-	+	+	-	+	+	+	-	-	-	+	366
3	+	-	+	+	-	+	+	+	-	-	-	544
4	-	+	-	+	+	-	+	+	+	-	-	652
5	-	-	+	-	+	+	-	+	+	+	-	340
6	-	-	-	+	-	+	+	-	+	+	+	896
7	+	-	-	-	+	-	+	+	-	+	+	724
8	+	+	-	-	-	+	-	+	+	-	+	464
9	+	+	+	-	-	-	+	-	+	+	-	136
10	-	+	+	+	-	-	-	+	-	+	+	824
11	+	-	+	+	+	-	-	-	+	-	+	472
12	-	-	-	-	-	-	-	-	-	-	-	480
13	0	0	0	0	0	0	0	0	0	0	0	532
14	0	0	0	0	0	0	0	0	0	0	0	652
15	0	0	0	0	0	0	0	0	0	0	0	792
16	0	0	0	0	0	0	0	0	0	0	0	852
17	0	0	0	0	0	0	0	0	0	0	0	652
18	0	0	0	0	0	0	0	0	0	0	0	668

Response Surface Methodology

The four components with the highest positive effect on TC production were used to further optimize and develop models using the central composite design. Table 5 shows the production of TC ranging from 1156 mg/L to 5884 mg/L when changing the different concentrations of the components.

Model development

Selection of sequential model by the sum of square

Appropriate selection of the model to calculate and estimate the response is of key importance. The quadratic model provided a significant value of P 0.0002 (<0.05) with fourteen degrees of freedom. Therefore, it was chosen to analyze the effect of the variables in terms of the main effect, the interactive and quadratic effects. The variance analysis (ANOVA) for the quadratic model was calculated from the response obtained for 30 runs.

Table 5: RSM design of four components at five concentration levels and their respective TC production (A=Glycerol, B=Potassium Nitrate, C=Meat Extract, and D=Ferric Nitrate with central value of 0.75, 0.75, 3.5 and 0.075 g/L concentration as a central value with six replicates).

Run	A (g/L)	B (g/L)	C (g/L)	D (g/L)	TC (mg/L)	
					Actual	Predicted
1	0.5	0.5	2.0	0.05	2548	2441.83
2	1.0	0.5	2.0	0.05	2800	2845.33
3	0.5	1.0	2.0	0.05	2952	2405.33
4	1.0	1.0	2.0	0.05	3008	2531.83
5	0.5	0.5	5.0	0.05	4484	4646
6	**1.0**	**0.5**	**5.0**	**0.05**	**5884**	**5404.5**
7	0.5	1.0	5.0	0.05	4676	4500.5
8	1.0	1.0	5.0	0.05	4660	4982.0
9	0.5	0.5	2.0	0.10	2672	2432.66
10	1.0	0.5	2.0	0.10	3100	2713.16
11	0.5	1.0	2.0	0.10	2668	2585.16
12	1.0	1.0	2.0	0.10	2668	2588.66
13	0.5	0.5	5.0	0.10	3060	2973.83
14	1.0	0.5	5.0	0.10	2980	3609.33
15	0.5	1.0	5.0	0.10	2980	3017.33
16	1.0	1.0	5.0	0.10	3832	3375.83
17	0.25	0.75	3.5	0.075	2296	2574
18	1.25	0.75	3.5	0.075	3136	3336.83
19	0.75	0.25	3.5	0.075	3472	3462.83
20	0.75	1.25	3.5	0.075	2704	3192.83
21	0.75	0.75	0.5	0.075	1156	1852
22	0.75	0.75	6.5	0.075	5060	4843.5
23	0.75	0.75	3.5	0.025	4052	4439.5
24	0.75	0.75	3.5	0.125	2732	2824
25	0.75	0.75	3.5	0.075	3536	3202.66
26	0.75	0.75	3.5	0.075	2900	3202.66
27	0.75	0.75	3.5	0.075	3248	3202.66
28	0.75	0.75	3.5	0.075	2884	3202.66
29	0.75	0.75	3.5	0.075	3152	3202.66
30	0.75	0.75	3.5	0.075	3496	3202.66

ANOVA showed that the model was very significant as the very high value of the F model (7.31) at a significant level of 1% eliminating the chance of noise. These considerations suggest good suitability of the second-order polynomial model designed to explain observed yields. The coefficients of the surface response model as outlined in equation (3) were evaluated. The student test showed that the two linear coefficients (C and D) and their interactions (CDs) were very significant, their values were P-values, P_C = <0.0001, P_D = 0.0007 and P_{CD} = 0.0026 respectively. However, to minimize errors, all coefficients were included in the model.

Total Carotenoids = (+235.18) + (2614.67 * Glycerol) – (502.67 * Potassium Nitrate) + (1094.15 * Meat Extract) – (5080.00 * Ferric Citrate) – (1108.00 * Glycerol * Potassium Nitrate) + (236.67 * Glycerol * Meat Extract) – (4920.00 * Glycerol * Ferric Citrate) –(72.67 * Potassium Nitrate * Meat Extract) + (7560.00 * Potassium Nitrate * Ferric Citrate) – (11086.67 * Meat Extract * Ferric Citrate) –(987.33 * Glycerol^2) + (500.67 * Potassium Nitrate^2) + (16.13 * Meat Extract^2) + (1.71667E+005 * Ferric Citrate^2)----------(3)

Table 6: ANOVA table for RSM (Coefficient of Determination (R2) = 0.8722, Adjusted R2 = 0.7529 and Predicted R2 = 0.3319* Significant at 1% level)

Source	**Sum of Square**	**df**	**Mean Square**	**F-value**	**p-value prob> F**
Model	0.00000021	14	0.00000156	7.31	**0.0002***
A-Glycerol	0.0000871	1	0.0000871	4.08	0.0617
B-Potassium Nitrate	0.00001094	1	0.00001094	0.51	0.4853
C-**Meat Extract**	0.00000013	1	0.00000013	62.82	**<0.0001**
D-**Ferric Citrate**	0.00000391	1	0.00000391	18.32	**0.0007**
AB	76729	1	76729	0.36	0.5579
AC	0.0000126	1	0.00001260	0.59	0.4544
AD	15129	1	15129	0.07	0.7938
BC	11881	1	11881	0.05	0.8168
BD	35721	1	35721	0.17	0.6884
CD	0.00000276	1	0.00000276	12.94	**0.0026**
A^2	0.00001044	1	0.00001044	0.49	0.4951
B^2	26857.19	1	26857.19	0.13	0.7279
C^3	36125.76	1	36125.76	0.17	0.6867
D^2	0.00003157	1	0.00003157	1.48	0.2429
Residual	0.00000320	15	0.00002137		
Lack of Fit	0.00000281	10	0.00002810	3.56	**0.0869**
Pure Error	0.00003949	5	78986.67		

The Lack of Fit F-value of 3.56 implies that it is not significant relative to the pure error. There is an 8.69% chance that this large Lack of Fit F-value could occur due to noise. A non-significant lack of fit is good, as we want the model to be fit.

Adequate Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. In our experiment, we found a ratio of 10.869, which indicated an adequate signal for the model. Therefore, this model was used to navigate the design space. ANOVA test showed that the coefficient of determination (R^2) for the production of total carotenoids were 0.8722 (Table 6), implying that 14.04% variance occurred could be explained by the model. The R^2 value should be between 0 and 1. The closer the R^2 to 1, the stronger the model, and the better it predicts the response. The predicted R^2 of 0.3319 was in reasonable agreement with the adjusted R^2 of 0.7529. This indicated a good agreement between the experimental and predicted values of carotenoids production.

3D Response Surface plots

The 3D surface response plots were generated for the response (TC) at any two independent variables while keeping the others at their 0 levels. Thus, six 3D plots were obtained by considering all the possible combinations (Fig. 4 a–e). Figure 4 reveals that with increase in glycerol (0.5 g/L), TC production also increases, whereas at higher potassium nitrate (0.5 g/L) and combinations of both, glycerol and potassium nitrate, it leads to decrease in TC production. Figure 4b represents meat extract enhanced increase in TC production. TC production was higher at a combination of glycerol (0.5 g/L) and meat extract (2 g/L) with a comparatively higher slope than only glycerol had. Figure 4c depicts the interaction of ferric citrate and glycerol. With an increase in the concentration of both the variables, TC production was increased and with a further increase in ferric citrate concentration and a mixture of both more than a central point, TC production decreased. Figure 4d represents the effect of meat extract, potassium nitrate, and their interactions. It is evident that meat extract increased the TC production and potassium nitrate led to a decrease in TC production. Interaction with low concentrations of potassium nitrate (0.05 g/L) and higher concentration (above the center point) of meat extract (2 g/L) resulted in increased productivity with higher slop. It could be depicted from Figure 4e that the variables, ferric citrate, and potassium nitrate, were showing the least variation with an increase in concentrations, both have a negative effect on TC. Figure 4f divulges the effect of meat extract, ferric citrate, and their interaction on the product. Meat extract illustrates the highest positive effect on TC production with increasing concentration, whereas at a lower concentration of ferric citrate, TC increases.

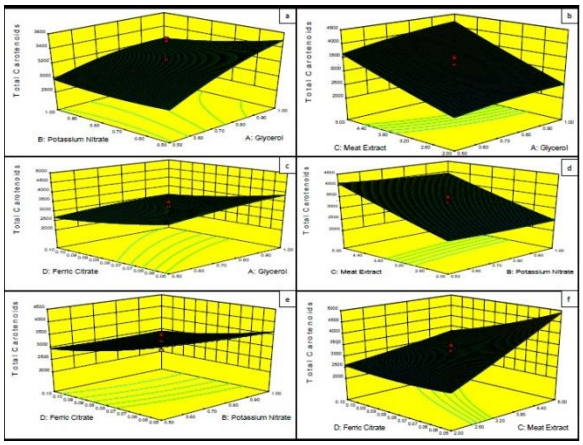

Fig. 4. 3-D response plot of *Paracoccus* OC1 on total carotenoids (TC) (a) effect of glycerol and potassium nitrate on TC, (b) effect of glycerol and meat extract on TC, (c) effect of glycerol and ferric citrate on TC, (d) effect of potassium nitrate and meat extract on TC, (e) effect of potassium nitrate and ferric citrate on TC and (f) effect of meat extract and ferric citrate on TC.

Validation of model

To confirm the predicted response from the medium composition obtained, the statistical model, as well as the regression equation, were validated under optimum conditions. It was found that total carotenoids production was increased and found maximum on 7th day, 5975 ± 346 mg/L, which was near to predicted value of 5404 mg/L with 101.54% yield of carotenoids and indicated that the model was validated for significant response near the predicted value.

DISCUSSION

The genus *Paracoccus* is metabolically versatile containing $C_{18:1}\omega7c$ as a major component of the cellular fatty acids, a gram-negative, catalase, and oxidase-positive and can grow at high-stress conditions [34]. Carotenoid containing *Paracoccus* strains were isolated from soil, as a contaminant from a nutrient agar plate, sediment, and seawater [24, 25, 34, 35]. In the present study, carotenoid producing *Paracoccus* spp. OC1 was isolated from a marine source. The

marine environment became a focus of natural products, because of its relatively unexplored biodiversity compared to terrestrial environments. The potential of marine natural products as pharmaceuticals was introduced by the pioneering work of Bergmann in the 1950s [36]. Marine microorganisms have not only been a tremendous source of biodiversity and chemical diversity but also have capacities to produce highly complex molecules with industrial-scale production of drugs [5, 37]. These novel chemicals from marine organisms that have demonstrated potential as new treatments for cancer, infectious diseases, and inflammation, suggest that there needs to be a greater focus on the development of pharmaceutical and nutraceutical products from marine sources [37].

The microbial system is easier to maintain and scale up than a plant system. The expanding market demand for carotenoids from natural sources has promoted the development of a large-scale production using on hand microorganisms as well as exploring newer sites to find outsources for such a valuable product with a decrease in production costs. Also, by changing physical parameters like medium strength, pH, temperature, and carbon source led to increase in carotenoid production. This is more beneficial for the industry than manipulation in the genetic makeup of an organism. A statistical design like the Plackett-Burman design is not only useful for screening of effect of components on the product, but also to minimize workload and the most precious time. After screening, to settle on the concentration of components for enhancing productivity as well as low cost medium formulation, response surface methodology is the most important [22]. The present study showed a sequential increase in TC production during stepwise optimization; initially, TC production was 1333.33 ± 577.35 mg/L after optimization of basic parameters like medium strength, pH, temperature, aeration, and basic sugar. The maximum nutrient supports the maximum growth of cells but does not support maximum secondary metabolites production. By comparing the yield factors (Ypx, mg of TC produced per gm of cell weight) of media, 10 times diluted media had the highest yield. Starvation of the nutrient supplement suppressed the cell division and enhanced carotenoids production. Results obtained are in corroboration with the Ibrahim, who reported that the MB1/10 media supports the highest carotenoid production [38]. The favorable pH for the growth of *Paracoccus* sp. OC1 was pH 8 which could be due adaptation of organism in the seawater with pH 8. However, Attri et al., [39] reported that medium of pH 6 supported the highest carotenoid production and acidic pH favors β-carotene production in *Monascus* sp. and in *Rhodotorula* sp. [40]. The present study showed the highest carotenoid production at neutral to slightly alkaline pH. Ibrahim [38] reported that the optimum temperature for carotenoid production for *Micrococcus* sp. was 30°C whereas 37°C was the optimum temperature for the highest biomass accumulation. These observations are in corroboration with our results for growth of OC1. However, the highest TC production was obtained at 37°C instead of 30°C. The temperature of the Gulf of Khambhat was also near to 25-30°C from which this strain was isolated and it may be required for the growth and activation of different enzymes involved in carotenoids biosynthetic pathway. At temperature of 45°C, the denaturation of enzyme structure might be the reason for decrease in carotenoids accumulation. Highest biomass and carotenoid production at 29-32°C were reported in *Rhodotorula glutini* [41], whereas higher β-carotene accumulation at 20°C was reported in *Phaffia rhodozyma* than 30°C [42]. This clearly indicates the strain specific effect of temperature on carotenoid production. The addition of glycerol supports higher β-carotene production in metabolically engineered *Escherichia coli* [9]. Glycerol can convert into pyruvate and acetyl CoA, both of which are precursors for the tetraterpenoid pathway. Thus glycerol enhances the accumulation of precursor that leads to higher carotenoid production. The addition of glucose, maltose, and sucrose leads to a decrease in carotenoid production as compared to glycerol. Supplementation of medium with glycerol and glycine resulted in higher total carotene accumulation in *Chlamydomonas acidophila*, an extremophile microalga [26]. With an increase in aeration, the color of pigment was changed from orange to dark brown. The higher carotenoid production with intensive aeration (1.3 L/L. min)

was observed in *Rhodotorula rubra* GED2 and *Lactobacillus casei* sub sp *casei* [15]. Aeration leads to pathway extension by incorporating oxygen molecules in the skeleton of the carotenoid molecule and can drive the pathway up to xanthophylls class.

The metabolic and genetic engineering approach is used for higher microbial carotenoid production but requires highly urbanized technology [43]. By changing nutritional parameters, the metabolite production can be increased and it becomes commercially feasible due to low-cost technology. Initial screening of the ingredients was done to understand the significance of their effect on the product formation and then a few better ingredients were selected for further optimization. Choudhary and Singhal [44] also used a statistical approach to enhance β- carotene production from *Blakeslea trispora*. Plackett-Burman design showed only glycerol, meat extract, ferric citrate, potassium nitrate, and ammonium nitrate had a positive effect on TC. Marine broth contains a peptic digest of animal tissue and the supplement of glycerol, which supports the highest carotenoid production. This supports our observations as both of these compounds had a positive effect on TC [9, 38]. Presence of inorganic salts like magnesium, manganese, calcium, zinc, potassium, and ferrous as a medium supplement showed a higher rate of carotenogenesis by serving as a cofactor of key enzymes like phytoene synthase [45]. In the present study also showed, isolate OC1 required a basal level of ferrous and potassium for carotenoid production. *Haematococcus pluvialis* showed the stimulation of phytoene synthase and carotenoid hydroxylase levels when grown in the presence of increased light intensity together with sodium acetate ferrous salt [46, 47]. Hyper-accumulation induced by ferrous ion was due to the generation of hydroxyl radical, which motivate cellular carotenoid synthesis, from the Fenton reaction ($H_2O_2+Fe^{2+}\rightarrow Fe^{3+}+HO^{-}+HO^{*}$) [48]. *Chlorococcum* and *Rhodotorula* strain also showed improvement in carotenoid yield in the presence of inorganic salts [49–51]. Basic cellular metabolic pathways like the tricarboxylic acid (TCA) cycle and its intermediates play an important role in carotenoid and lipid biosynthesis in microbes. Initially, citrate and malate were major components answerable for inspired carotenogenesis in *Rhodopseudomonas spheroids.* Later on, in *Blakeslea trispora* and *Xanthophyllomyces dendrorhous* also found carotenoid accumulation in the presence of citrate [52]. In *Sporobolomyces roseus*, cell growth and β-carotene production were observed when the medium was supplemented with succinate. Another report on carotenoid synthesis due to the presence of 0.2% (w/v) malate, citrate, α-ketoglutarate, fumarate, or oxalacetate in *Actinomyces crysomallus* was examined. Most of the *Paracoccus* species required nitrate as a final electron acceptor and can reduced nitrate into N_2 [53].

CCD with RSM is only applicable to a small number of variables (up to five) [22]. In the present study, four components were screened and achieved TC production range 1156 mg/L to 5884 mg/L using *Paracoccus* OC1 isolate, which was five-fold higher as compare to the previous optimization of fermentation parameters. A similar increase in reported 22.6% higher productivity of carotenoids in *Xanthophyllomyces dendrorhous* through statistical approach by Plackett-Burman and Uniform experiment design [54]. The medium optimization results suggest that carotenoid biosynthesis in *X. dendrorhous* is promoted by high C/N ratio+, low carbon and high nitrogen concentrations, and a slightly acidic condition when the cell growth is suppressed, whereas in *Paracoccus* sp., carotenoids production was only increased at low carbon and nitrogen concentration. A higher concentration of the nutritional medium components leads to cell growth but does not provide enough stress that is required for secondary metabolites (carotenoids) production. Wang et al., reported an increase of β-carotene (13.43 mg/L) yield, 34.17% compared to the control [55], and Malisorn and Suntornsuk also showed 2.7 g/L biomass and the maximum β-carotene of 201 μg/L, approximately 15% higher by response surface methodology (RSM) approach with a factorial design and CCD in *Rhodotorula glutinis* [56].

Commercially available carotenoid producing microorganisms can synthesize β-carotene, *Dunaliella salina* (10.35 mg/L), *Mucor rouxii*

(31.0 μg/g), *Dunaliella bardawil* (35.5 pg/cell), *Rhodotorula glutinis* mutant 32 (250 mg/L), *Blakeslea trispora* (420 μg/g) and *Phycomyces blakesleeanus* (1200 μg/g) whereas our isolate OC1 was able to produce 5884 mg/L. Previously *Paracoccus* strain is reported for astaxanthin production [24] whereas we report novel *Paracoccus* strain OC1 having the capacity to produce β-carotene and the first report on optimization using a statistical approach. This was evident in amplification of the genes involve in the production of caretenoids. β-carotene hydrolase and β-carotene ketolase genes were not amplified in the PCR reaction which suggested the absence of gene and due to this reason our isolate cannot produce astaxanthin. This result supported our previous results that extracted carotenoid was β-carotene and our isolate is a novel β-carotene producing *Paracoccus* strain.

The carotenoid biosynthetic pathway has been studied extensively. In the β-carotene biosynthetic pathway, phytoene synthase, phytoene desaturase, and lycopene β-cyclase genes are involved [57, 58]. Subsequently, β-carotene is used to synthesize xanthophyll types of carotenoids. Previously, *Paracoccus* species were reported for astaxanthin production [24] but our isolate produced only β-carotene. To prove this the presence of genes encoding key enzymes of the carotenoid biosynthetic pathway was amplified. Lycopene β-cyclase is an enzyme for the production of carotene molecule, β-carotene hydrolase, and β-carotene ketolase are two key enzymes responsible for the pathway driven towards xanthophylls [59]

CONCLUSION

Paracoccus strain is known for xanthophylls type of carotenoid production, whereas we have isolated and characterized a β-carotene producing strain which is confirmed by the amplification of the genes involved in the carotenoid production. Application of statistical methods for screening and optimization of the medium components successfully improve total carotenoid production.

Acknowledgments: Authors are thankful to Gujarat Council on Science and Technology (GUJCOST), Gujrat, India for the financial aid (File Number: GUJCOST/MRP/2014-15/1243). The authors are also thankful to Charotar University of Science and Technology (CHARUSAT) management for providing necessary facilities.

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