https://doi.org/10.33472/AFJBS.6.13.2024.2462-2478

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Elevating carotenoid production from marine *Bacillus infantis*

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Article Info

Volume 6, Issue 13, July 2024

Received: 04 June 2024

Accepted: 01 July 2024

Published: 26 July 2024

doi: 10.33472/AFJBS.6.13.2024.2462-2478

ABSTRACT:

The ocean, a mother of all life, supports a vast microbial diversity, with marine bacteria being key producers of valuable molecules like carotenoids with various valuable biological properties and applications. The present research aimed to study the effect of one-factor-at-a-time (OFAT) such as pH, temperature, media (marine broth and nutrient broth), carbon sources (maltose, lactose, glucose, and sucrose), nitrogen sources (peptone, yeast extract, ammonium sulfate, and urea), and salts ($MgCl_2$, $MgSO_4$, $CaCl_2$, and NaCl) on growth and pigment production. Response Surface Methodology (RSM) was used to further improve lactose, yeast extract, and magnesium sulfate significant components selected from the OFAT technique. The optimized medium consisted of 5 g/l lactose, yeast extract, and $MgSO_4$. The maximal biomass in RSM optimized medium was 12.58 g/l and the carotenoid yield was 147.58 $\pm$ 2.48 mg/l, in the validated model. When compared to the unoptimized medium, we discovered that the optimized fermentation medium allowed a 3.45-fold increase in carotenoids and a 1.97-fold increase in biomass production of *B. infantis*. This is the first research on carotenoid production by marine *Bacillus infantis*, offering a novel source of natural carotenoids. The result suggests that the Response Surface Methodology improved yield and helped control the fermentation process.

Keywords: One factor at a time (OFAT), Fermentation media, Response Surface Methodology (RSM)

1. Introduction

It is thought that the most basic forms of life descended from this "primordial soup," that is the ocean, which is considered the mother of all life. Because marine life prospers in a distinct climate, these organisms develop adaptation mechanisms. The products of these defense mechanisms, such as the carotenoids that bacteria produce to protect themselves from UV radiation, may be beneficial to humans in a variety of ways [1]. Carotenoids are pigments with a yellow-to-red tint that are produced via the terpenoid biosynthesis pathway [2]. About 750 naturally occurring carotenoids have been reported among these, more than 250 are of marine origin and show therapeutic and industrial significance due to their biological activity and interesting structural diversity [3, 4]. Marine *B. infantis* isolated from the Gulf of Khambhat is the potential source of carotenoids [5]. Strong antioxidant, antiproliferative, and anti-inflammatory properties are exhibited by marine carotenoids, which can be employed as ingredients in nutraceuticals and cosmetics to treat disorders linked to oxidative stress or as a skin photoprotectant to minimize the harmful effects of solar UV radiation [6].

The most commonly sold carotenoids are generated industrially through chemical synthesis [7], which is simple to accomplish and results in stable, vibrant, and diverse pigment hues. However, this increases production costs and waste material production, which may have detrimental effects on the environment [8, 9]. Natural carotenoid-based goods have seen a surge in demand over the past 10 years due to growing customer concern over the health of both humans and the environment. It is typically not possible to standardize the seasons and geographic regions that affect the industrial production of carotenoids that are derived from terrestrial plants [10]. When compared to plants or manufactured goods, natural carotenoids can provide various advantages in terms of expense, timeframes, and yields [11]. For these reasons, biotechnological research on the production of carotenoids from marine organisms has significantly risen [12].

Over the past few decades, a variety of studies on the carotenoids generated by microbes have become accessible. These include microalgae *Haematococcus* and *Dunaliella* the bacterial strain *Bacillus, Paracoccus, Exiguobacterium, Corynebacterium,* and *Rhodococcus*, fungus *Blakeslea trispora*, *Monascus purpureus*, and yeasts, among others of the genera *Rhodotorula*, *Sporobolomyces*, and *Phaffia* [13–16]. Several *Bacillus* species have been recorded for carotenoid synthesis thus far, for example. *B. clausii* produces β-carotene [17], *B. megaterium* produces C30 carotenoids 4,4'-diaponeurosporenic acid and 4,4'-diapophytofluene [18], and *B. firmus* synthesizes C30 carotenoid derivative 4,4'-diapolycopene-dioate esters [19].

The commercial success of the fermentation process has been attributed to the optimal selection of media, which has been achieved through remarkable developments in medium optimization. Numerous investigations demonstrated that temperature, pH, and medium composition are important factors that influence the synthesis of carotenoids [16][16, 17]. Moreover, the production of targeted metabolites typically requires species-specific tailoring of many factors, and the microbial production of carotenoids frequently does not demand all of the ingredients in complex media [20] To produce carotenoids profitably, fermentation technology should be improved, specifically concerning the media and physical conditions. By using the traditional "one-factor-at-a-time" method, medium optimization entails modifying one element at a time while making precise fixes to the others. For subsequent optimization, only the most productive components with positive significance may be chosen. Others that are not as important or have a negative impact on the response value could be excluded from further optimizations [21]. The central composite design (RSM-CCD) combined with response surface methodology has been widely utilized to improve the production of potential compounds, investigate the interactions among variables, and determine the ideal circumstances for a desired response [22]. Additionally, RSM analyses the connections between the independent variables and the

experimental response, fitting the observational response(s) to a polynomial function, and describes the impact of the independent factors on the processes, either separately or in combination. Nonetheless, research into improving the medium composition and fermentation conditions for the synthesis of carotenoids seems to be a viable strategy for improving the commercialization of the target metabolites. However, the optimization of carotenoid synthesis by *Bacillus infantis* using statistical design is still not fully researched and needs further investigation. We have already documented the extraction, separation, and characterization of carotenoids from *B. infantis* [5]. Following the sequential statistical design of the experiment strategy, the current study aims to determine an optimum medium for enhancing the carotenoid production from *B. infantis* and to examine the impact of media components and physical factors on carotenoid and biomass productivity.

2. Materials and Methods

2.1 Microorganism

Bacillus infantis (Accession number: OP601610) was isolated from seawater in the Gulf of Khambhat (22.2397° N, 72.7659° E) and maintained on a nutrient agar plate containing (per liter): 1.5 g beef extract, 1.5 g yeast extract, 5 g peptic digest of animal tissue, 5 g sodium chloride, 15 g agar. Every month, a single colony was transferred to a new sterile nutrient agar plate, cultured for four days, and then stored in refrigeration at 4 °C.

2.2 Preparation of inoculum

A pure culture of *Bacillus infantis* was inoculated into 100 ml of nutrient broth and kept at 30 ± 2 °C in a static environment for 24 hours at the logarithmic growth phase. An inoculum (1 % v/v) with an optical density of 0.6 at 600 nm was employed in each experiment.

2.3 Measurement of cell biomass

Biomass (g Dry cell weight/l) was calculated as previously described by Kanzy et al. 2015. Using the REMI C-24 cooling centrifuge, 1 ml of the culture was centrifuged for 10 minutes at 10,000 rpm in a pre-weighed centrifuge tube. The cells were washed with distilled water after the supernatant was removed. Dry cell weight was determined by drying the wet pellet to a consistent weight in an oven at 80°C and then subtracting the pre-weight of the centrifuge tube [23].

2.4 Extraction and quantification of pigment

The pigment was extracted using a modified method of Soni et al. 2023. In summary, the cells were incubated in nutrient broth under static conditions at 30 °C for 7 days. The cell pellet was subsequently obtained by centrifugation. Following a distilled water wash, the pellet was again centrifuged. The resultant cell pellet was suspended in 5 ml of 0.5 M Tris HCl (pH 7) 5 ml of 5% SDS, and 20 ml of distilled water were added. A Qsonica sonicator was used to ultrasonicate the mixture at 50 AMP for 20 minutes on ice. Followed by sonication the solution was centrifuged for 20 minutes at 10,000 rpm, to produce an orange-colored supernatant and a colorless pellet [5]. The colored supernatant was treated with an equal amount of cold acetone to eliminate SDS, proteins, and lipids and centrifuged at 6000 rpm for 20 minutes [24]. After being resuspended in methanol, the colored pellet was vortexed for 30 seconds and centrifuged at 6000 rpm for 20 minutes. The colored supernatant was dried and the white pellet was discarded. The total carotenoid concentration (µg/g) and mg/l were calculated using the following formula Eq. 1 and 2 respectively [25, 26].

The total carotenoid content (µg/g) = 1000ADV / 0.16W Eq. 1

A = absorbance at λmax, D is the dilution ratio, V = total volume of extract, 0.16 is the extinction coefficient of carotenoids, and W = dried weight of *Bacillus infantis* (g).
Carotenoid yield (mg/l) = total carotenoid content (µg/g)
Eq. 2

2.5 Screening of media components and physical factors by OFAT experiments

To assess the effects of media components (C-source, N-source, and salt) and physical factors (temperature and pH) on carotenoids and biomass production by *B. infantis*, the usual one-factor-at-a-time (OFAT) optimization strategy was used. Every experiment was conducted in triplicate, with the results shown as mean ± SD.

2.6 Effect of media on growth and pigment production

For the growth of the organism and the production of pigment, two different media nutrient broth and marine broth were utilized. Therefore, a specific size inoculum as described above was inoculated in a 250 ml Erlenmeyer flask each containing 100 ml of media, and incubated at 30 °C for 7 days under shaking conditions to determine the effect of media on growth and pigment production. The optical density at 600 nm and dry cell weight was used to quantify the growth of the organism in two distinct media. The pigment production was measured using OD at 492 nm, and the total carotenoid content was calculated using the formula given in Eq. 1.

2.7 Effect of aeration on growth and pigment production

All experiments were conducted in nutrient broth. The flasks were inoculated with inoculum (1% v/v) and incubated for 7 days at 120 RPM in shaking and static conditions at 30 °C. Biomass and pigment production were calculated for up to 7 days, as previously stated.

2.8 Effect of temperature and pH on growth and pigment production

For determination of optimum temperature and pH for growth and pigment production, 100 ml of nutrient broth containing inoculum was incubated at four different temperatures (27 °C, 30 °C, 37 °C, 47 °C) and four different pH (5, 7, 8, 9) and incubated in static condition for 7 days. The production of pigment was calculated based on Equation 1.

2.9 Effect of carbon and nitrogen source on growth and pigment production

The effects of various carbon supplements (1%), such as glucose, maltose, lactose, and sucrose, as well as nitrogen supplements (1%), such as peptone, yeast extract, ammonium sulfate, and urea, on the production of pigment, and biomass were studied. The fresh inoculum inoculated in the nutrient broth supplemented with different carbon and nitrogen sources was incubated at 30 °C for 7 days in static condition. The calculations for pigment production and biomass were performed as previously mentioned.

2.10 Effect of mineral salts on growth and pigment production

Nutrient broth (100 ml) was supplemented with 1 % of different salts including magnesium sulfate ($MgSO_4$), sodium chloride (NaCl), magnesium chloride ($MgCl_2$), and calcium chloride ($CaCl_2$). All of the flasks were inoculated with the fresh inoculum, which was then incubated for seven days in a static environment at 27 ± 2 °C. The quantification of growth and pigment production was carried out as previously described.

2.11 Statistical optimization of media components by RSM

The Response Surface Methodology was employed to optimize the medium composition (lactose, yeast extract, $MgSO_4$) and to enhance biomass and carotenoid production using a central composite design. Every experiment was carried out in triplicate, and the response value was determined by averaging the yield of carotenoid and biomass. A complete factorial central

composite design consisting of 2^4 = 16 plus 4 center points and a star point was used for a total of 20 experimental runs. As indicated in **Table 1**, the factors were examined at five different experimental levels. The dependent response in the study was evaluated as total carotenoids (mg/g) and biomass (g/l). Regression analysis was utilized to ascertain the coefficients of a polynomial of second order. To evaluate the significance of the variation in the obtained biomass and carotenoid yield, analysis of variance (ANOVA) was used.

Table 1 Code values for independent variables

Independent variable	Variable level Code				
	-α	-1	0	+1	+α
Lactose (g/l)	1.59	5	10	15	18.04
Yeast extract (g/l)	1.59	5	10	15	18.04
$MgSO_4$ (g/l)	3.29	5	7.5	10	11.7

The second-order polynomial model was utilized to calculate the responses, and the interaction between the independent variables was represented by a quadratic equation. (Eq. 2).

$$Y=x_0+\sum_{i=1}^{3}a_i x_i+\sum_{i=1}^{3}a_{ii}x^2{}_i+\sum_{i=1}^{3}\sum_{i<j}a_{ij}x_i x_j \quad \text{Eq.2}$$

Where Y is the predicted response, X_0 is the intercept term, X_i is a linear coefficient, X_{ii} is the quadratic coefficient X_{ij} is the interactive coefficient and X_i and X_j depict the independent variables as coded values. The 3D surface plots were developed by altering the concentrations of two components while holding the concentrations of the remaining components constant. All statistical investigations were carried out using the Design Expert Software (Version 10, State-Ease).

2.12 Statistical analysis

All experiments were carried out in triplicate. Data are expressed as mean ± standard deviation (SD). The graph pad prism software (Windows-based) was used for the preparation of the graph for the OFAT experiment.

3. Results

3.1 Effect of media on biomass and pigment production

When the two media nutrient broth and marine broth examined for biomass and pigment production were compared. The biomass production was 7.96 ± 0.25 g/l and 6.36 ± 0.20 g/l in nutrient broth and marine broth respectively. Nutrient broth produced 25.4 ± 2.39 mg/l, and marine broth produced 24.23 ± 2.62 mg/l of total carotenoid on 3rd day of incubation in shaking conditions as shown in **Fig 1a**. Therefore, compared to marine broth, there was a 4.60% increase in carotenoid output in nutrient broth. As the highest pigment production was observed in nutrient broth, it was used in subsequent optimization experiments.

3.2 Effect of aeration on growth and pigment production

In contrast to pigment production, aeration increases biomass production as compared to a static condition. Maximum biomass production was 5.96 ± 0.85 g/l on the second day of shaking conditions, compared to 3.26 ± 0.20 in static conditions. The maximum carotenoid production measured in a static condition was 42.59 ± 4.01 mg/l on the sixth day of incubation; in a shaking environment, it was 21.64 ± 4.57 mg/l on the third day, and it rapidly decreased to 9.44 ± 0.32 mg/l on the sixth day as represented in **Fig 1b**. The static condition enhanced

the total carotenoid yield by 96.34%. Aeration may therefore impact the formation of pigment, but growth was promoted by shaking conditions.

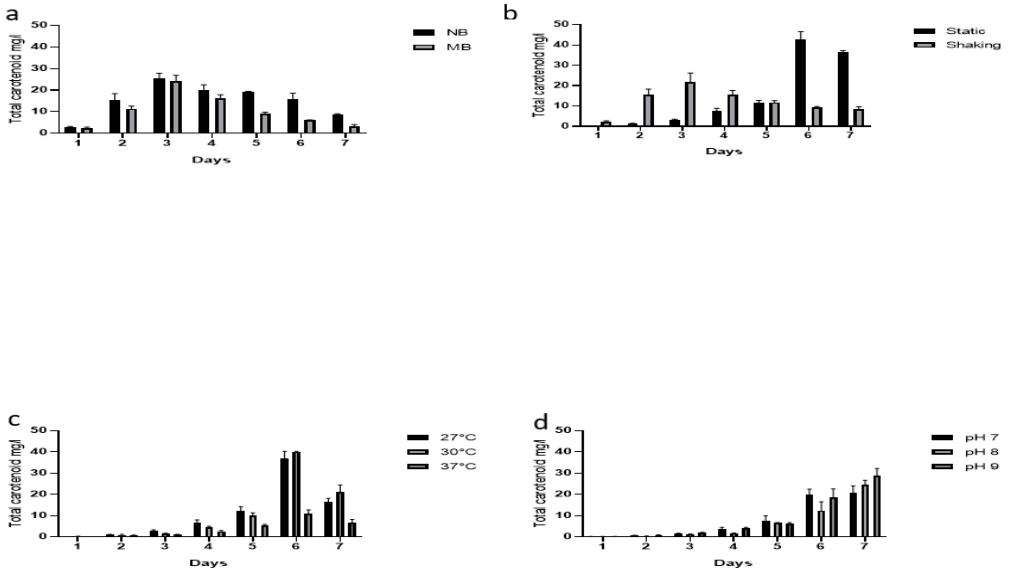

Fig 1. Effect of different parameters (a) media (Nutrient and marine broth), (b) aeration, (c) temperature, and (d) pH on the growth of the organism.

3.3 Effect of temperature and pH on growth and pigment production

Biomass production was 5.53 ± 0.39 g/l and 5.40 ± 0.36 g/l at 27 °C and 30 °C respectively. Except 47 °C, pigment synthesis was observed at all temperatures tested. Maximum carotenoid production was observed at 40.07 ± 0.21 mg/l at 30 °C as depicted in **Fig 1C**. The growth of the organism was slower and pigment synthesis was not observed at 47 °C.

The formation of pigment was also impacted by pH. Alkaline pH was shown to produce the maximum amount of pigment and biomass while acidic pH (pH 5) prevented growth and production of pigment. From pH 7 to pH 9, the biomass production was nearly the same. The maximum carotenoid synthesis determined on the seventh day of incubation was 24.48 ± 2.14 mg/l and 28.82 ± 3.30 mg/l, at pH 8 and 9 respectively as shown in **Fig 1d**.

3.4 Effect of carbon and nitrogen source on growth and pigment production

In general, the formation of pigment was hindered by the carbon source supplied in the nutrient broth. When lactose was added to the nutrient broth provided the maximum amount of total carotenoid when compared to glucose, sucrose, and maltose; however, the yield of carotenoid and biomass was reduced when compared to the nutrient broth alone. Lactose served as an additional carbon source in the nutritional broth that produced the maximum biomass of 3.36 ± 0.15 g/l. The production of carotenoids in the presence of lactose was only 10.36 ± 0.68 mg/l as illustrated in **Fig 2a**. Thus, based on the results obtained, it was hypothesized that additional sugar as a carbon source prevents the production of carotenoids in *B. infantis*.

In a similar vein, when compared to nutrient broth alone, nitrogen supplementation in the broth decreased the amount of pigment produced. The yeast extract produced 4.23 ± 0.35 g/l biomass on the seventh day of incubation. Yeast extract supported the highest pigment production of 14.54 ± 1.29 mg/l on the seventh day of incubation among the studied nitrogen sources, as shown in **Fig 2b**.

3.5 Effect of salts on growth and pigment production

All of the mineral salts that were investigated were able to support growth and pigment formation. $MgCl_2$ produced the highest biomass production on the fifth day of incubation, about 3.46 ± 0.40 g/l. On the other hand, on the seventh day of incubation, the maximum pigment production recorded was 12.75 ± 1.07 mg/l in the presence of $MgSO_4$ as illustrated in **Fig. 2c**. In comparison with the nutrient broth alone, the yield of pigment synthesis and biomass was decreased in presence of 1 % salt concentration. Additionally, the production of carotenoid and biomass was not significantly enhanced by other mineral salts as calcium and sodium.

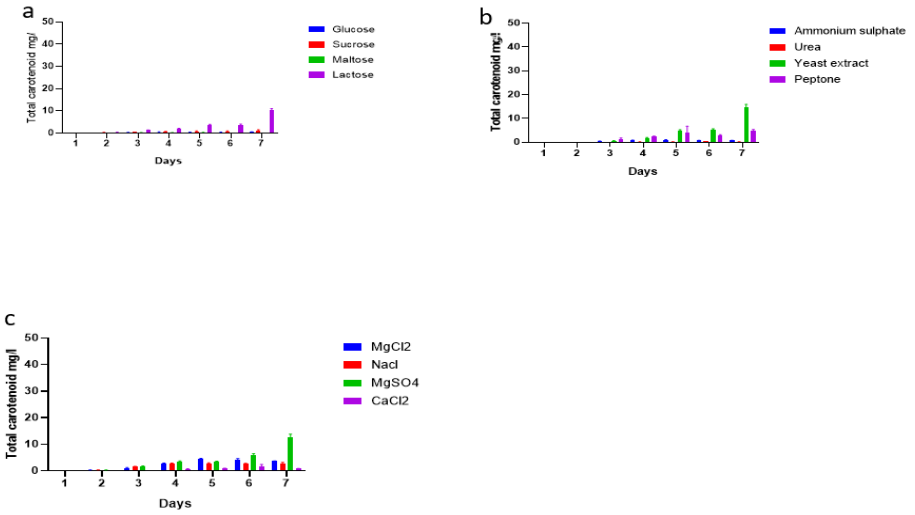

Fig 2. Effect of different parameters (a) carbon source, (b) nitrogen source, and (c) salts on the pigment production.

3.6 Optimization of media components by RSM

Table 2 Central composite design of distinct variables containing estimated and experimental carotenoid and biomass yields.

Run	Lactose (g/l)	Yeast Extract (g/l)	$MgSO_4$ (g/l)	Experimental cell wt. (g/l)	Predicted cell wt. (g/l)	Experimental pig wt. (mg/l)	Predicted pig wt. (mg/l)
1	10	10	11.7045	8.49	8.25	124.29	119.50
2	10	10	7.5	11.58	12.20	66.93	72.49
3	10	1.59104	7.5	7.32	6.21	112.43	96.81
4	10	10	3.29552	7.7	7.28	105.56	112.03
5	10	10	7.5	13.2	12.20	51.74	72.49
6	5	15	5	6.7	7.0	70.75	63.84
7	15	15	10	9.36	9.27	100.24	98.35
8	5	5	10	4.56	5.5	90.51	102.19

9	5	15	10	7.96	7.77	69.88	61.42
10	15	5	10	6.6	6.68	60.06	65.75
11	15	5	5	5.59	6.20	46.95	54.19
12	10	18.409	7.5	7.89	8.34	36.29	53.65
13	10	10	7.5	11.89	12.20	76.09	72.49
14	1.59104	10	7.5	7.41	6.63	69.35	70.54
15	5	5	5	6.58	7.12	147.12	147.79
16	10	10	7.5	12.58	12.20	80.51	72.49
17	10	10	7.5	11.87	12.20	89.02	72.49
18	10	10	7.5	11.98	12.20	70.98	72.49
19	18.409	10	7.5	7.02	7.14	22.46	22.97
20	15	15	5	6.98	6.49	56.53	43.62

Table 3 ANOVA of the experimental findings for the response biomass (g/l) in the central composite design quadratic model.

Source	**Sum of Squares**	**Degree of freedom**	**Mean Square**	**F-value**	**p-value**
Model	120.24	9	13.36	22.03	< 0.0001
A-Lactose	0.3150	1	0.3150	0.5193	0.4876
B-Yeast Extract	5.45	1	5.45	8.99	0.0134
C-MgSO4	1.15	1	1.15	1.89	0.1990
AB	0.0496	1	0.0496	0.0818	0.7807
AC	2.15	1	2.15	3.55	0.0889
BC	2.70	1	2.70	4.46	0.0609
A^2	50.78	1	50.78	83.72	< 0.0001
B^2	43.59	1	43.59	71.87	< 0.0001
C^2	35.34	1	35.34	58.27	< 0.0001
Residual	6.07	10	0.6065		
Cor Total	126.30	19			

R^2 = 0.9520, Adequate precision = 12.0787

Table 4 ANOVA for the experimental findings for the response pigment (mg/l) in the central composite design quadratic model.

Source	Sum of Squares	Degree of freedom	Mean Square	F-value	p-value
Model	15329.01	9	1703.22	8.79	0.0011
A-Lactose	1164.71	1	1164.71	6.01	0.0341
B-Yeast Extract	3036.34	1	3036.34	15.67	0.0027
C-MgSO4	775.06	1	775.06	4.00	0.0734
AB	2692.31	1	2692.31	13.90	0.0039
AC	1633.06	1	1633.06	8.43	0.0157
BC	931.82	1	931.82	4.81	0.0531
A^2	1194.41	1	1194.41	6.17	0.0324
B^2	13.45	1	13.45	0.0694	0.7975
C^2	3374.35	1	3374.35	17.42	0.0019
Residual	1937.29	10	193.73		
Cor Total	17266.30	19			

R^2 = 0.8878, Adequate precision = 12.68

The RSM was employed to optimize the medium compositions (lactose, yeast extract, and $MgSO_4$) to increase biomass and carotenoid production. Table 2 displays the experimental design along with the corresponding yields for each response. Both biomass and carotenoid productivity in our experiments closely matched the values anticipated by the model (Table 2). The effectiveness and adequacy of the carotenoid yield and biomass were evaluated using the ANOVA. Tables 3 and 4 displayed the coefficients of regression for the quadratic and linear interaction of all variables, as well as p-values at the <95% ($p < 0.05$) confidence levels. The model demonstrated an extremely low p-value (0.0001), indicating a high significance. The statistical significance of every coefficient and the pattern of reciprocal relationships among the examined variables were determined using the p-values. The estimated coefficient and the related p-values show that among the study's examined variables A (lactose), B (yeast extract), A (lactose) × B (yeast extract) provided significant model terms. The following regression analysis [Eq. (3), and (4)] showed the related second-order response model.

Biomass = 10.92 + (0.992 * A) + (1.16 * B) – (0.2628 * C) + (0.0197 * AB) + (0.2594 * AC) +(0.2906*BC)–(0.4687*A^2)–(0.4341*B^2)–(1.56*C^2) Eq.3

Pigment wt. = 88 – (2.30 * A) – (3.11 * B) – (0.5915 *C) + (0.7556 * AB) + (0.4750 * AC) +(0.1650*BC)+(0.1229*A^2)+(0.4504*B^2)+(3.28*C^2) Eq.4

The R^2 values 0.9520 and 0.8878 for biomass and total carotenoid, respectively, supported the fit statistic of the model and showed that it explained 95.20% and 95.00% of the variability in the responses, respectively. As a result, the three-dimensional response surface plot demonstrated the interactive effect of lactose vs. yeast extract (Fig. 3a), lactose vs. $MgSO_4$ (Fig. 3b), yeast extract vs. $MgSO_4$ (Fig. 3c), and predicted vs. actual plot for pigment wt. in *B.*

infantis (Fig. 3d). Similarly, lactose vs. yeast extract (Fig. 4a), lactose vs. $MgSO_4$ (Fig. 4b), yeast extract vs. $MgSO_4$ (Fig. 4c), and predicted vs. actual plot for biomass in *B. infantis* (Fig. 4d). According to the findings, the highest biomass production in a medium containing lactose (10 g/l), yeast extract (10 g/l), and $MgSO_4$ (7.5 g/l) was reported to be 12.58 g/l, while the expected value was 12.20 g/l. The highest pigment weight of 147.79 mg/l was found in a medium containing the lowest concentrations of lactose (5 g/l), yeast extract (5 g/l), and $MgSO_4$ (5 g/l), whereas the predicted response value was 147.79 mg/l.

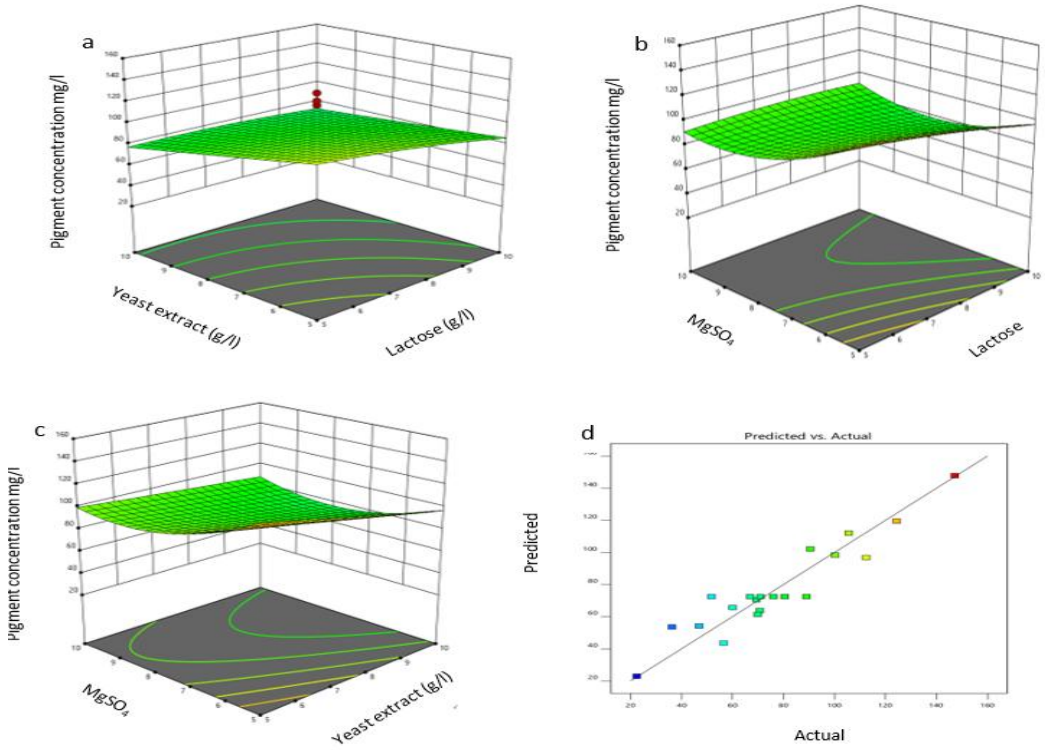

Fig 3 The 3D response surface plots depicting the interactive effect of the two variables on pigment wt. (mg/l) in *B. infantis* has grown in the RSM-CCD. The 3-D surface plots of lactose and yeast extract (a), lactose and $MgSO_4$ (b), yeast extract and $MgSO_4$ (c), and predicted vs. actual response plot (d) for pigment wt. in *B. infantis*.

The 3D surface response plots were created for the response (Biomass and Pigment wt.) at any two independent variables while maintaining the others at their 0 levels. The effect of lactose and yeast extract on pigment and biomass production was shown in Fig 3a and 4a respectively. The 3D surface plot demonstrated that, at a fixed lactose concentration of 5 g/l, the pigment yield decreased from 109.37 mg/l to 77.71 mg/l as the yeast extract concentration increased from 5 g/l to 10 g/l (Fig. 3a). In contrast, the biomass yield increased from 7.89 g/l to 10.16 g/l (Fig. 4a). However, as the lactose concentration increased, the yield of pigment decreased from 109.37mg/l to 86.64 mg/l at a fixed concentration of yeast extract 5 g/l while the biomass increased from 7.89 g/l to 9.86 g/l. Hence, in summary, increased concentrations of carbon and nitrogen sources increased biomass production while limiting pigment synthesis.

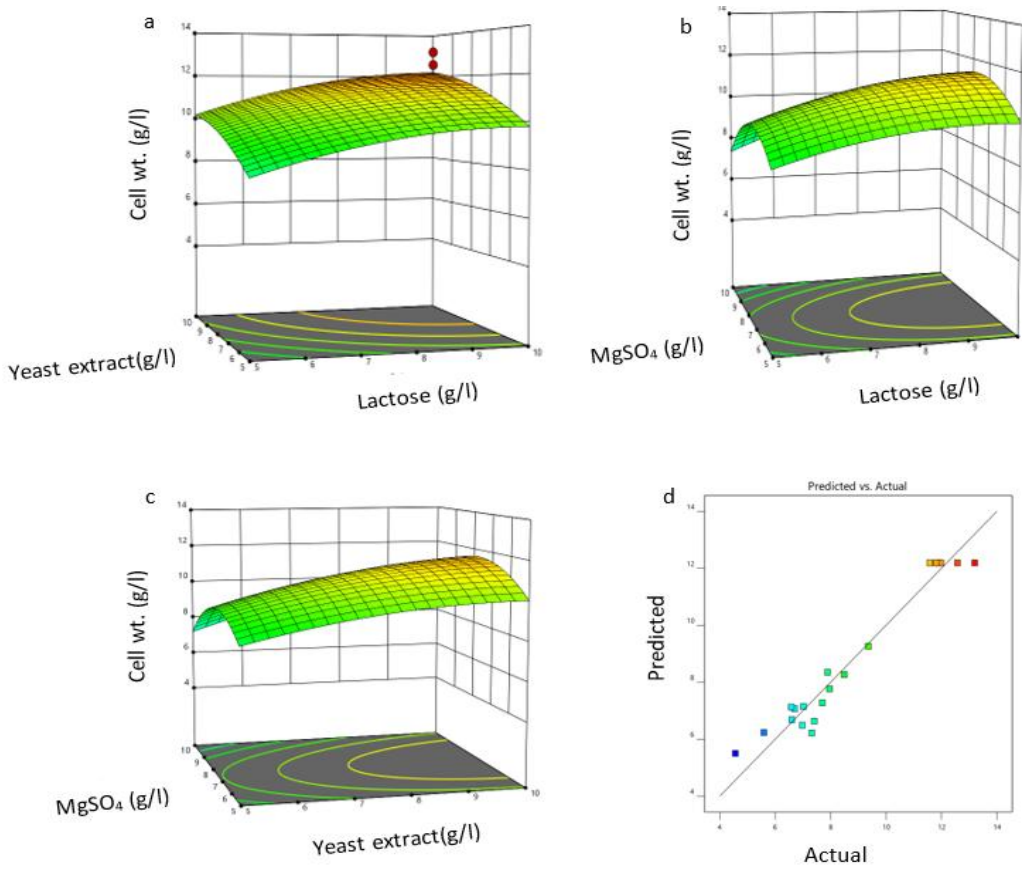

Fig 4 The 3D response surface plots depicting the interactive effect of the two variables on biomass (g/l) in *B. infantis* has grown in the RSM-CCD. The 3-D surface plots of lactose and yeast extract (a), lactose and $MgSO_4$ (b), yeast extract and $MgSO_4$ (c), and predicted vs. actual response plot (d) for biomass production in *B. infantis*.

The combined effects of lactose and $MgSO_4$ on pigment and biomass yield are shown in Figs. 3b and 4b, respectively. The yield of pigment was not significantly increased at fixed lactose concentrations, but biomass reduced from 8.48 g/l to 7.50 g/l as $MgSO_4$ concentration increased from 5 g/l to 10 g/l. Conversely, at a specific concentration of MgSO4, the pigment synthesis dropped from 105.08 mg/l to 97.28 mg/l, but there was no noticeable rise in the production of biomass was observed with the increasing concentration of lactose.

Figs. 3c and 4c, respectively, show that yeast extract and $MgSO_4$ affected the production of pigment and biomass. The synthesis of pigment decreased from 109.30 mg/l to 97.21 mg/l and biomass increased from 8.43 to 10.16 g/l at a fixed concentration of $MgSO_4$ and increasing concentration of yeast extract from 5 g/l to 10 g/l. On the other hand, at a fixed concentration of the yeast extract biomass declined from 8.42 to 7.25 g/l while the yield of pigment was not affected by the increasing concentration of $MgSO_4$.

4. Discussion

This study examined the production of orange carotenoid by the marine isolate *Bacillus infantis*. Carotenoids have been employed in several medicinal applications, such as anti-diabetic, anti-inflammatory, and anticancer drugs [27, 28] and also as a natural UV protector [29]. The generation of distinctive and varied secondary metabolites is a well-known characteristic of marine microorganisms. Numerous studies have examined the optimization of media components and physical factors to increase the production of carotenoids from

marine organisms. Such as optimization of carotenoid production from marine *Rhodotorula* sp. RY1801 [30], *Kocuria* sp. RAM1 [31], *Paracoccus* sp. OC1 [13], *Haloferax mediterranei* [32]. Carotenoid production is facilitated by several enzymes, mostly through the polyketide and mevalonate pathways [33, 34]. Due to its comparatively untapped biodiversity in comparison to terrestrial areas, the marine environment has become a focus for natural products. The groundbreaking research done in the 1950s by Bergmann established the possibility of using marine natural compounds as medicines [35]. A large-scale production employing available microorganisms has been facilitated by the growing market need for carotenoids from natural sources. Additionally, novel sites are being explored to locate outsources for this valuable commodity at a lower cost of production.

The one-factor-at-a-time optimization results in the current study revealed that nutrient broth was a well-suited medium for the synthesis of orange pigment by *B. infantis* whereas biomass production was slightly low compared to the marine broth. Ratnakaran et al. also discovered that bacteria isolated from garbage produce the maximum pigment in nutrient broth [36]. The organic carbon and nitrogen sources found in nutrient broth, peptone, and meat extract, respectively, supply the organism with the vital growth components and vitamins required for its reproduction [37]. Marine broth is formulated with additions of 12 different mineral salts, which are absent in the nutrient broth. Therefore, the starvation of these salts in the nutrient broth may support the maximum production of carotenoids [13].

In comparison to shaking, a higher synthesis of carotenoids (42.59 ± 4.01 mg/l) was noted under static conditions. It was noted that the color of the pigmented pellet was paler under shaking conditions than it was under static ones. So, primarily in shaking conditions aeration may accelerate the route up to the xanthophyll class and cause it to expand by introducing oxygen molecules into the backbone of carotenoid molecules which might then be oxidatively degraded. A similar result was obtained by Tailor and Davies they found the carotenoid degraded product in the highly aerated culture of *Streptococcus faecium* UNH564P [38]. In contrast, agitation and aeration promote the highest amount of biomass formation in *B. infantis* following a 24-hour incubation period. Aeration is an important component in the growth of *Rhodotorula mucilaginosa* MTCC 1403 as the organism's growth increases in the shaking condition [16].

Temperature and pH must always be optimized to obtain the highest pigment production because they have an impact on the metabolic growth and physiological activities of organisms. There was not a significant temperature difference as maximum pigment synthesis was measured at 30 °C and maximum biomass production was measured at 27 °C. Although *B. infantis* was shown to grow at temperatures ranging from 27 to 45 °C, pigment production was not seen at higher temperatures (45 °C). The denaturation of enzyme structure at 45°C may be the cause of inhibition in the synthesis of carotenoids. This strain was isolated from the Gulf of Khambhat, which had a temperature of about 25 to 30°C. This temperature may be necessary for the growth and stimulation of many enzymes that contribute to the biosynthetic pathway of carotenoids [13]. An earlier investigation found that 30 °C was the optimum temperature for the production of zeaxanthin from *Sphingomonas sp*. isolated from Sponges in the Gulf of Thailand [39]. The maximum biomass and carotenoid production was observed at 37 °C in *Hfx. Alexandrines* while the highest carotenoid production from *Halorubrum* sp.SS-12 isolated from solar saltern of Mulund Mumbai was recorded at 35 °C [40]. This amply demonstrates the strain-specific impact of temperature on the synthesis of carotenoids. The amount of carotenoid produced by microorganisms can be modified by temperature through changes in the concentration of enzymes involved in the process [41]. The generation of pigment is known to decrease at higher temperatures, even though bacteria can grow in a wide range of temperatures [42]. Growth and pigment production occur from neutral to alkaline (pH 7 to pH 9) conditions, while growth and pigment production are inhibited in acidic (pH 5) conditions.

The optimum pH for carotenoid production in *B. infantis* was alkaline, with nearly the same growth and pigment production seen from pH 7 to 9, and pH 8- 9 respectively could be attributed to organism adaption in seawater with alkaline pH. The highest β-carotene production of *Paracoccus* sp. OC1 at pH 8 was reported by [13]. Shatila and colleagues discovered that *E. aurantiacum* FH produced a total cellular pigment of 534.51 µg/g dry cell weight when cultivated in LB medium (pH 7) in a shaking condition [43]. For the halophilic *Halorubrum* sp. TBZ126 isolated from Urmia Lake, the ideal pH values were 7.51 and 7.94 for growth and pigment production respectively.

The effects of carbon and nitrogen on biomass and pigment synthesis were investigated. The amount of carotenoid and biomass was reduced when additional carbon sources were added to the nutrient broth. Lactose yields the highest production of carotenoid and biomass when compared to the carbon sources that were evaluated. Based on the obtained results, it was determined that the growth of *B. infantis* was reduced by an additional 1 % carbon source. Although glucose is typically a good carbon source for growth, it prevents numerous secondary metabolites from being produced [44]. Patel et al. also published a supportive finding indicating that the addition of glucose, maltose, and sucrose caused a decrease in the synthesis of carotenoids [13]. The effect of glucose on the generation of carotenoids by *Halobacterium* Sp. was examined by Gochnauer et al. It has been found that a higher concentration of glucose (4%) in the medium inhibited the process of pigment synthesis [7]. Likewise, B. *infantis's* ability to produce pigment could not be improved by any additional nitrogen source in the nutrient broth. Yeast extract was proved to be effective for the synthesis of carotenoids and biomass in *B. infantis* among the nitrogen sources investigated in this study. The production of bioactive compounds from *Streptomyces* sp. KB1 was inhibited in the presence of yeast extract-malt extract-dextrose-containing medium [45]. As a whole, both carbon and nitrogen are unable to enhance the production of carotenoids from *B. infantis*.

The current research also examined the potential impacts of different trace elements on carotenoid synthesis. In all studied salts, carotenoid, and biomass synthesis was seen, but it was relatively low in calcium chloride. The maximum amount of pigment and biomass synthesis were noted in $MgSO_4$ and $MgCl_2$, respectively. However, the concentration of pigment and biomass was not greater than that of nutrient broth alone. Magnesium chloride (5g/l) increased β-carotene synthesis (12.34 $\pm$1.31 mg/l) in *E. acetylicum* S01 in comparision with other examined elements [46]. It might be made clear by speculating on a feasible mechanism of assessed metal ion inhibition and activation on specific enzymes involved in carotenoid production [47].

The economic success of the fermentation process is attributed to the optimal concentration of specific variables, which are determined by constructing models and analysing the effects of various variables. Statistical optimization of the medium used for fermentation is a comprehensive and significant tool for designing experiments. The results of this study showed that statistical experimental design may be a useful tool for improving the fermentation medium to obtain greater biomass and carotenoid production from *B. infantis*. Among the variables investigated by the OFAT experiment, lactose, yeast extract, and $MgSO_4$ are recognized as important constituents for biomass and pigment synthesis. Applying the CCD of RSM, the ideal values of each of these variables were further substantiated. The highest levels of biomass (12.58 g/l) and carotenoid (147 mg/l) production were attained in runs 15 and 16, respectively. The results of biomass and pigment production were analyzed by ANOVA. The primary effects of lactose and $MgSO_4$ were found to be not significant, whereas the main effect of yeast extract was determined to be the most significant component determining the formation of pigment. The 3D surface plots were created to illustrate the relationship between the studied variables as well as the ideal combination of carotenoid production. The highest carotenoid production (147 mg/l) was achieved at the -1code level, where the concentration of lactose, yeast extract,

and $MgSO_4$ was 5 g/l while the highest biomass production (12.58 g/l) was attained at 0 code level of the components. The highest carotenoid production at the lowest concentration of the constituents may indicate starvation stress. According to Patel et al. (2019), starvation of nutrient components enhances carotenoid production [13]. The ideal circumstances for maximizing the production of total carotenoids are not the same as those for maximizing biomass production. Likewise, Hamidi et al. discovered that *Halorubrum* sp. TBZ126 had different optimal conditions for biomass and total carotenoid synthesis [48]. In comparison to the unoptimized medium, we found that the optimized fermentation medium facilitated increased production of carotenoid (3.45-fold) and biomass (1.97-fold) by *B. infantis.* RSM has also been effectively used in *Halorubrum* sp. TBZ126 in ideal conditions, which include a pH range of 7.51 to 7.94 and a temperature range of 31 °C to 32 °C. Under these circumstances, biomass and total carotenoid yields increased by 18.33% and 20.55%, respectively [48]. Using shake flask culture with RSM-optimized media, Abdelhafez et al. observed an elevated yield of β-carotene 2.24 mg/l for *S. marcescens* [49]. A maximum β- carotene production of 139 ± 1 mg/l was reported when *B. trispora* was cultivated in RSM optimized medium under submerged fermentation conditions [50]. A study conducted revealed that growing *Sporidiobolus salmonicolor* in RSM optimized submerged fermentation medium resulted in increased total carotenoid production of 1,019 µg/l [51]. As far as we are aware, this is the first publication detailing improved carotenoid and biomass production by RSM-CCD-utilizing *B. infantis*. The validation trials' outcomes, showed that using this optimum fermentation medium, maximized the synthesis of carotenoid from *B. infantis*. These findings collectively imply that ideal fermentation conditions for the synthesis of biomass and carotenoids by *B. infantis* could be established for use in industry as a commercial microbe that produces carotenoids.

Validation of model

Under ideal circumstances, the regression equation and statistical model were validated to verify the expected outcome based on the medium composition acquired. It was found that total carotenoid production was increased and found maximum on the 6^{th} day, 154.58 ± 2.48 mg/l, which was near the predicted value of 147.79 mg/l with a 3.45-fold increase of carotenoids, and indicated that the model was validated for significant response near the predicted value.

5. Conclusion

To conclude, we present here for the first time the improved yield of carotenoid (3.45-fold) and biomass (1.97-fold) in *B. infantis* under RSM-optimized medium conditions. While there are differences between the ideal conditions for maximum biomass and maximum carotenoid production. This finding implies that a statistical analysis helped regulate the fermentation process toward the accumulation of desired metabolites by identifying the most important medium components and their ideal concentrations. The development of a well-defined bioprocess for increased production of the targeted pigment will be possible with a comprehensive understanding of the regulation and pathway of pigment formation.

Acknowledgment Authors are thankful to the P.D Patel Institute of Applied Sciences for the laboratory facility. Nidhi Soni is thankful to the Charotar University of Science and Technology for providing a doctoral fellowship.

Conflict of interest The authors declare that they have no conflict of interest

6. References

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