World Journal of Biology Pharmacy and Health Sciences
eISSN: 2582-5542
Cross Ref DOI: 10.30574/wjbphs
Journal homepage: https://wjbphs.com/

(RESEARCH ARTICLE)

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Studies on optimization of pigment production of SB_2 isolate from the Saltern region of Marakanam (T.N)

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World Journal of Biology Pharmacy and Health Sciences, 2022, 12(01), 086–102

Publication history: Received on 03 September 2022; revised on 06 October 2022; accepted on 09 October 2022

Article DOI: https://doi.org/10.30574/wjbphs.2022.12.1.0152

Abstract

The marine environment plays a vital role in producing valuable natural products for biomedical and pharmaceutical research. The pigment is one of the bioactive molecules derived from marine bacteria. The strain SB_2 was isolated from the saltern areas of Marakanam to produce yellow pigment, round, and smooth colonies on selective media. The strain SB_2 was opted out for further studies based on its pigment production (1.68 mg l^{-1}). The incubation period, pH, and temperature are critical factors in increasing pigment production (7.81 mg l^{-1}) from the strain SB_2 using RSM (Response Surface Method) by Box Behnken Design (BBD). Similarly, the strain produced the highest pigmentation (7.67 mg l^{-1}) in a nutrient medium containing olive oil, molasses, and corn cob powder. Using BBD, various concentrations of methanol, ethyl ether, and ethanol solvents were optimised with the isolate SB_2 to yield maximum pigment (4.29 mg l^{-1}). The extracted pigment was effectively treated with bacterial pathogens to inhibit their growth when the isolate grew at pH 7, temperature 30ºC, and salt 2% concentration. Using Box Behnken Design (BBD), optimising nutrient sources, solvents, and environmental factors with the yellow-pigmented SB_2 strain recovered from seawater results in the highest carotenoid pigment production, and the pigment has antibacterial properties.

Keywords: SB_2 bacterial strain; Box Behnken design; Antibacterial activity; Yellow pigment; Quadratic model; Marine water

1. Introduction

The pigment is insoluble in water with a broad ecological niche which can be distributed everywhere from earth to the atmosphere and occurs in all the living organisms from various parts of plants, including photosynthetic plants and the microorganisms which play a critical role in the synthesis of the carotenoids, especially bacteria, fungi, microalgae, and yeast, to the great extent their availability produces various colours from red, yellow, and orange [1]. In recent years, natural pigments have been highly demanded to be used as colouring agents in food, fabrics, feed, printing ink, and cosmetics, which are non-polluted, eco-friendly, and less costly. Some compounds are synthesised from inorganic chemical substances, while flavonoids, tetrapyrroles, and carotenoids are naturally derived pigments [2]. An approximate estimation of the carotenoids market in the years 2018–2024 for beverages (9.2%), animal feed (34.8%), pharmaceuticals (6.5%), foods (26.1%), and cosmetics (23.5%) [3]. It was reported that beta-carotene fetched a market value of 309 million dollars in 2010 [4]. The market value of astaxanthin is expected to be around 1.5 billion dollars in 2020 [5]. RSM (Response Surface Method) is a recent technique used to approach a statistical methodology for increasing pigment production by optimising and screening several parameters of the production medium. These statistical design methods have recently optimised the carotenoid-producing microbes. It predicts the system's optimal condition by testing different parameters and interactive effects of various components, which can be applied to chemical, biological, and agriculture [6]. RSM also assessed the interaction between the variables and quadratic outcome. The Box Behnken design (BBD) employed three factors such as fermentation media, cultivation, and process

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conditions, and each changed at three levels (-1, 0, and +1) to optimize the bioprocesses for the experiment. The temperature, agitation, pH, carbon, nitrogen, and inorganic salts are the optimum conditions for culturing bacteria for pigment production [7]. In the optimization process compared to the conventional method, RSM is the preferred technique [8]. Beta-carotene and zeaxanthin production were increased using RSM [9]. The RSM combined with experimental design was applied to draw several input variables, and it also determined the output response to the input variables. The desired end product of the reaction could be estimated with the effect of various independent variables, and finally, we found out the best possible response value through an optimization technique [10]. The mutual interaction among the independent variables decides the shape of surface plots and the orientation of principal axes of contour plots [11]. RSM applied for production was increased by using a limited concentration of nutrients in the medium to be optimised [12]. Beta-carotene and zeaxanthin production were increased by using RSM [9]. Different nutrient factors were used in the media to obtain an optimum response output using the RSM for optimization [13]. Beta-carotene production was induced by *Dunaliella salina* using RSM [14]. The higher production of beta carotene was increased from molasses used in the medium and the temperature was maintained at 30ºC. [15] described the pigment activity as an antimicrobial activity isolated from *Micrococcus endophyticus* and *Salinococcus roseus*. Antimycobacterial activities were observed at a concentration of 64 µg ml^{-1} violacein from *C. violaceum* [16]. In this study, yellow-pigmented strains were isolated from marine water samples, optimization of the pigment by various environmental, solvents, and nutritional factors by the RSM method, and studies on antibacterial activity were conducted.

2. Material and methods

2.1. Collection of marine water samples

Water samples were collected from the marine surface at various places of Tamil Nadu and Puducherry from Marakanam (TN), the coastal region (Rock Beach), and the coastal area of Kalapet Puducherry, India. The marine water samples from the sea surface were collected using Teflon plates, dipped into water, lifted horizontally, and scrapped off the adhering surface film until the procedure was separated and the total volume was 30 to 50 ml of water sample was collected. The samples were stored at 4°C until the isolation was carried out within 24 h [17]. The salinity of the collected marine water samples was determined using the reported protocol [18].

2.2. Isolation of yellow-pigmented bacterial isolates from marinewater

Isolation of pigment producing colonies from the seawater sample (1 ml) were transferred into the 9 ml tube 1 to make 10^{-1} dilution. From 10^{-1} dilution one ml wastransferred into 2^{nd} tube to make 10^{-2} dilution and 1 ml was taken from 10^{-2} dilution transferred to tube 3 to make 10^{-3} were serially diluted up to 10^{-4}. The dilutions 10^{-3} and 10^{-4} were selected, the 100 µl of the diluted sample were transferred into petriplate by spread plate using selective Zobell marine agar medium. The sample was inoculated and the plates were incubated at 30°C for three days. The positive yellow strains in the plates were observed. The resulting colonies showing yellow pigmentation were sub-cultured for purification.

2.3. The isolates were cultivated for pigment production

To screened out the efficient pigment-producing isolates, prepare selective Zobell marine broth containing nystatic acid and nalidixic acid to avoid fungal and gram-negative bacterial contamination in the medium and sterilized at 121 ºC for 15 minutes. The strains MB_2, SB_2, MB_4, and MMB_8 were inoculated into the media separately and kept for incubation under a shaker at 30º*C* for one week. The maximum pigment production was observed among the isolates that were taken out and expressed in mg l^{-1} [19].

2.4. Optimization and production of pigment by various environmental parameters by using Response Surface Method (RSM) of SB_2 culture

The optimum conditions of the pigment production were affected by various parameters being evaluated by using the Box–Behnken design (BBD) of RSM [20]. In the present study, the pigment production by isolating SB_2 was optimized with different parameters to validate the process. The selected factors, temperature, pH, and incubation period, were used to optimize pigment production (mg l^{-1}) using BBD. The lowest to the highest pH, temperature, and incubation values were analyzed through (-1, 0, +1) three levels. The experimental data obtained were applied to the RSM using Design-Expert 13.0.0.0 (Stat-Ease, trial version). The data were analyzed by the quadratic model. Design-expert software clearly mentioned the statistical terms and their definition for interpreting experimental data for RSM [21]. The regression equation is obtained from experimental data, which is fitted with linear second-order polynomials [22]. For selecting the best model, ANOVA and fit statistics were used to be interpreted [23]. The significance of the models

was determined by ANOVA. The validity of the models confirms the triplicate values were done for the predicted values and verified for optimized conditions.

To analyze the process of pigment production (response Y), where Y depends ontemperature, pH, and incubation period, which indicate as X_1, X_2,…..X_n, the relationshipbetween pigment production and environmental parameters were represented by

$$Y = f(X_1, X_2, X_3\ \ ) + \varepsilon \ldots\ldots\ldots\ldots\ldots\ (1)$$

Where;

f- The real response function (unknown)
ε- The residual error

Since the correlation between pigment production and X_1, X_2,….X_n represents temperature, pH, and incubation period as a response surface and the relationships of these factors are called as RSM. In Eqn. (2), describe the quadratic response model, squared terms, interaction effects, and linear terms.

Where,

β0- Constant
βi- Slopes or linear effect
βij- Interaction of the quadratic effects (2)

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \ldots\ldots\ldots\ldots\ldots\ (2)$$

2.5. Various nutrients sources were used for optimization of pigment by using BBD of SB2 isolate

The pigment production was affected by different nutrient sources and evaluated and validated using the Box–Behnken design (BBD) of RSM. Molasses, olive oil, and corn cob powder were used to optimize pigment production (mg l^{-1}). Three different levels (-1, 0, +1) were used to analyze this model and the molasses, olive oil, and corn cob powder ranges were fixed from low to high. The experimental data obtained were applied to the RSM using the statistical software, Design-Expert 13.0.0.0 (Stat-Ease, trial version).

2.6. Optimization of pigment extraction using different solvents by BBD

The different solvents were used to optimize the pigment production by SB_2 to investigate and validate using Box–Behnken design (BBD) of RSM. Solvents, namely methanol, ethyl ether and ethanol, were used to optimize pigment production (mg l^{-1}). The lower to the higher value of methanol, ethyl ether and ethanol was analyzed through (-1, 0, +1) three various levels. The experimental data obtained were applied to the RSM using the statistical software, Design-Expert 13.0.0.0 (Stat-Ease, trial version). The data were analyzed by the quadratic model. Design-expert software clearly mentioned the statistical terms and their definition for interpreting experimental data for RSM. The regression equation confirmed the linear and second-order polynomials obtained from experimental data [24]. ANOVA and fit statistics were used for interpretation to select the best model. The values of R^2 and adjusted R^2 were used to evaluate the adequacy of the model and the significance of the models by Analysis of variance. The validity of the models confirms the triplicate values were done for predicted values and verified for optimized conditions [20].

2.7. Evaluating the performance of carotenoid pigment against antibacterial activity

The SB_2 bacterial cells of various salt concentrations (%), temperature and pH conditions were grown for seven days of culture. The crude pigment extracted from bacterial isolates the protocol described by [25]. The natural pigment was used to evaluate the antibacterial activity of the SB_2 yellow-pigmented isolate was studied for the maximum inhibition zone and dry weight observed at pH (4, 6, 10, 12 and 14), temperature (20, 30, 40, 50, and 60ºC) and salt (2, 4, 6, 8, 10, and 12%) concentration was evaluated as described by [26]. The bacterial pathogens were swabbed on the nutrient agar medium of each pathogen separately and kept for incubation. After the growth formed on each plate was made well, 20 µl of the pigment extract was added to each pathogenic plate. After 24 hrs incubation at 37ºC, the diameter of the inhibition zone (mm) and dry weight of the cell (µg ml^{-1}) was observed.

2.8. Statistical analysis

Experiments were carried out in triplicate in this study, and data were presented as the mean of triplicate values and analysed using a one-way ANOVA and Design of Expert software.

3. Results and discussion

3.1. Seawater samples collected from different regions

A marine water sample from various coastal regions of Puducherry and Tamil Nadu (India) was collected (Table 1). The salinity of the samples was Puducherry (35.3 psu), Kalapet (35.4 psu), and Marakanam (35.8 psu). The salinity is essential for marine water that could fluctuate during the summer and monsoon at the Marakkanam saltern [27]. Similarly, the Pichavaram mangrove forest salinity ranges from 0.6 to 36.6 psu [28]. Our results also showed the highest percentage of salt in water samples collected from the Marakanam coastal area, followed by the Kalapet region and Rock Beach in Puducherry for the isolation of pigment-producing bacteria. The pigments have a defense mechanism present in the isolates, providing the potential to survive in salinity stress, which may be exploited for human use.

Table 1 Collection of marine water sample

S. No.	Location	Source	DMS Latitude and Longitude	Salinity percentage (psu)
1	Coastal region, Marakanam	Marine water sample	12.1899° N, 79.9249° E	35.8
2	Coastal region (Rock beach), Puducherry	Marine water sample	11.9339° N, 79.8362° E	35.3
3	coastal region, Kalapet	Marine water sample	12.032311° N, 79.864624° E	35.4

(psu- practical salinity unit)

3.2. Isolation of bacteria produced pigment from the sea water samples

Twenty-four pigmented bacterial strains from marine water samples were isolated using a Zobell marine agar medium (Figure 1). The strains MB_2, SB_2, MB_4, MBS_6, and MMB_8 (MB- Marine Bacteria, MMB- Marakkanam Marine Bacteria, MBS- Marine Bacterial Strains & SB- Saline Bacteria) were selected, identified, and screened for yellow pigment production (Table 2). Among the isolates, SB_2 was chosen for further analysis of MMB_8, MB_4, MB_2, and SB_2, whose colonies were yellow, round, and smooth (Figure 2). A higher proportion of pigmented bacteria occurred in the seawater's surface layer due to the pigment's protecting metabolites [29]. A wide range of pigmented bacteria is present on the surface layer of seawater and its decided culture collection from the water sample. The diversity among marine bacteria is distributed abundantly in India [30]. *Actinomycetes* and pigmented strains of *Salinococcus roseus*, *Micrococcus endophyticus,* and *Streptomyces werraensis* were isolated from marine water [31]. [32] reported that diversity among the marine bacteria is distributed abundantly in India. *Actinomycetes* and pigmented strains of *Salinococcus roseus*, *Micrococcus endophyticus* and *Streptomyces werraensis* were isolated from deep-sea water [31]. Sometimes, pigmented bacteria were isolated from plates opened in the air [33]. Twenty-four bacterial strains were isolated from marine water using zobell marine agar and enrichment zobell marine broth to study their nature, growth characteristics, and pattern of pigment production for further studies. The colonies are yellow-pigmented, round and smooth. The isolate H1.7 (*Pseudoalteromonas piscicida)* produces yellow pigment [34]. A wide range of pigmented bacteria is present on the surface layer of seawater and its decided culture collection from the water sample. More nutrients were present in the artificial seawater. As a result, more culture growth was noticed than on low nutrient agar. *M. luteus* strain BAA2 also showed yellow colonies grown on nutrient agar at 37ºC for 3 days [35]. These yellow-pigmented marine bacteria contribute to carotenoids, especially zeaxanthin [36]. The first strain, *P. rubra* was isolated from seawater and identified with the capability to produce prodiginine [37]. The 3% sea salts inhibited the growth and red pigment production of terrestrial *Serratia marcescens* [38]. Bacteria capable of producing pigment with various colours are yellow, purple, golden yellow, pink-red, and creamy, developed by *Staphylococcus aureus, Serratia marcescens, Agrobacterium aurantiacum, Flavobacterium sp*, *Bacillus Spp,* and *Chromobacterium violaceum,* etc. [39].

Table 2 Screening of pigment-producing bacterial isolates

Location	Total no. of isolates	Screened pigment producing strains
Marakanam coastal region (12.1899° N, 79.9249° E)	MMB_7	MB_4 & SB_2
	MB_4	
	MMB_5	
	MMB_6	
	MBS_7	
	MBS_9	
	SB_2	
	SB_3	
Kalapet coastal region (12.032311° N, 79.864624° E)	MB_3	MMB_8
	MMB_8	
	MB_1	
	MB_2	
	MBS_5	
	MBS_8	
	SB_5	
	SB_7	
Coastal region (Rock beach) Puducherry (11.9339° N, 79.8362° E)	MB_8	MB_2
	MB_9	
	SB_9	
	SB_6	
	MBS_6	
	MBS_3	
	SB_4	
	SB_{10}	

(MB- Marine Bacteria, MMB- Marakkanam Marine Bacteria, MBS- Marine Bacterial Strains & SB- Saline Bacteria)

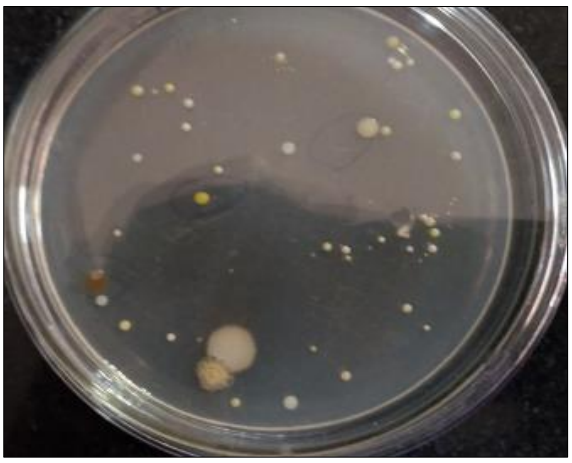


Figure 1 Isolation of pigment producing bacteria from marine source

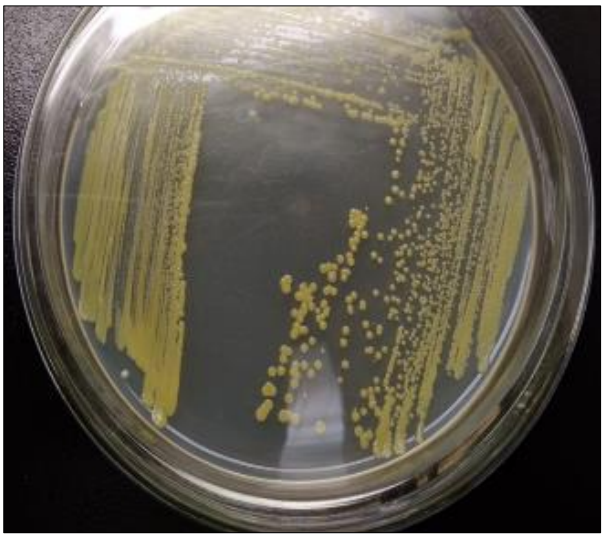


Figure 2 Yellow pigmented bacteria on Zobell's marine agar media

3.3. Strains were grown on selective medium

The strains MB_2, SB_2, MMB_8, and MB_4, were studied for pigment production using selective broth. Among the isolates, SB2 had the highest pigment production (1.68 mg l^{-1}) (Figure 3). The other isolates also produced better results, especially MB_4 and MB_2, and the lowest results were observed in MMB_8 isolates. We aim to get an increased yield of pigment. We opted out because the best-performed isolates were chosen for the entire research work. A modified method suggested by [40] uses selective marine agar to isolate and enumerate yellow-pigmented bacteria. Similarly, the strains MB_2, SB_2, MMB_8 and MB_4 were checked for pigment production using selective broth. Among the isolates, SB_2 recorded maximum production, and the Zobell marine broth containing kanamycin recovered yellow-pigmented colonies from marine water and sediment samples [41]. Based on the growth and pigment production, the SB_2 isolate was taken out for research studies.

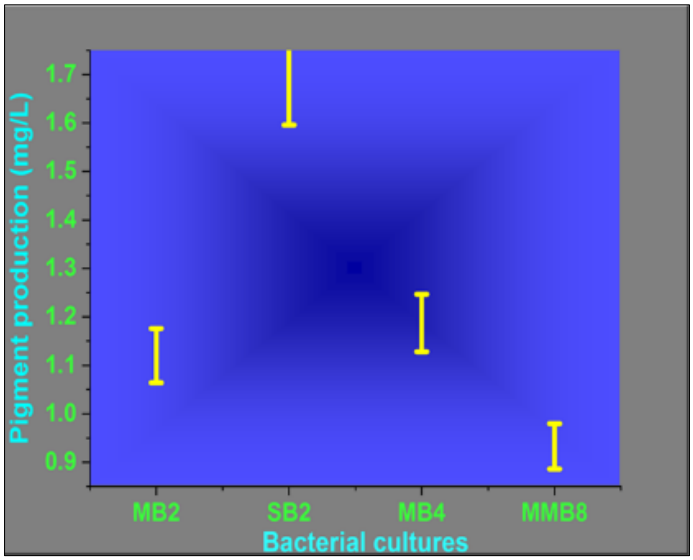


Figure 3 Screening of bacterial isolates for pigment production

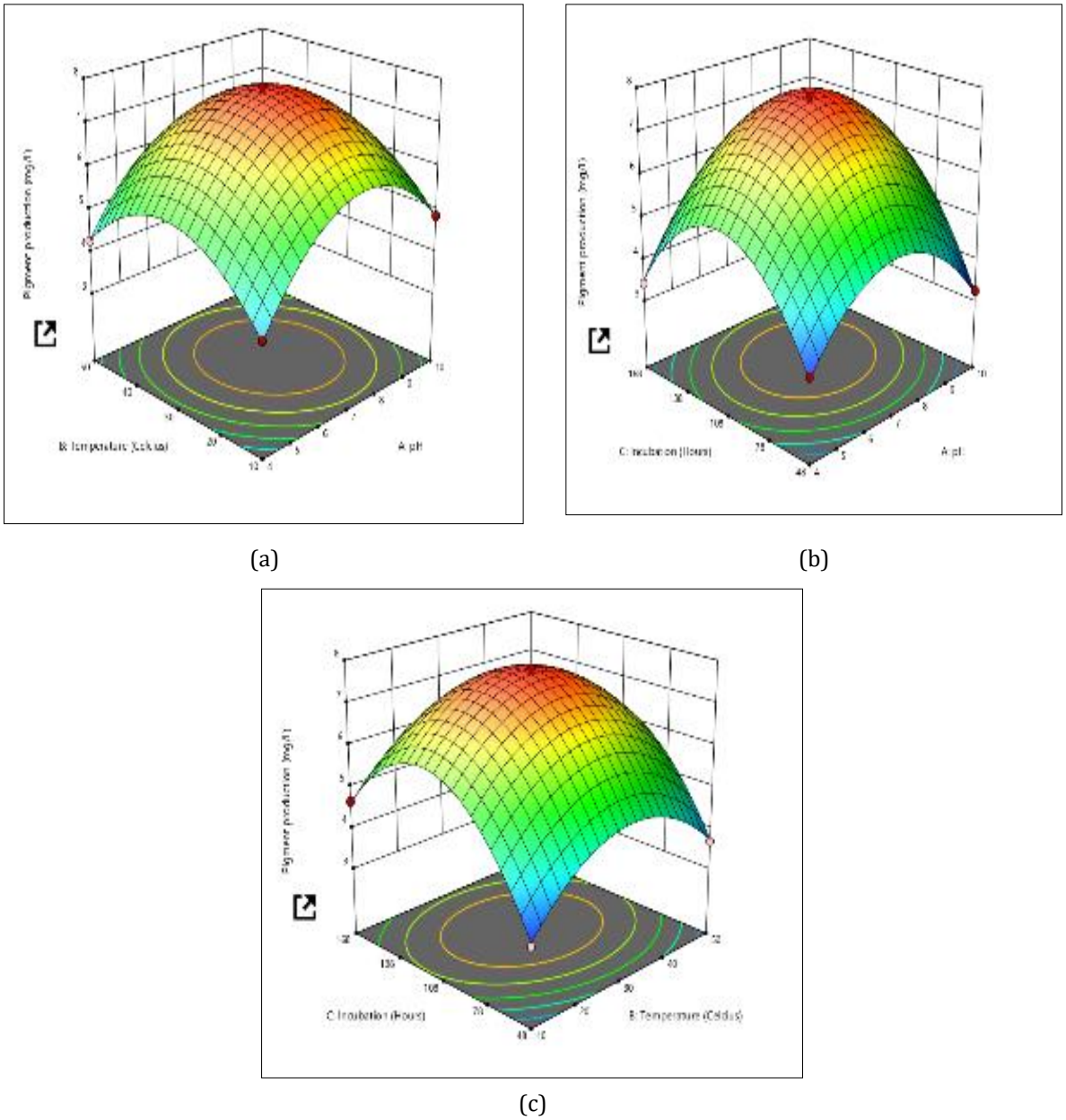
3.4. Pigment production from various environmental parameters for optimization by SB_2 culture using BBD

In the present study, BBD was evaluated from the interaction among various factors of pH, temperature, and incubation period to determine their optimal level of pigment production. Three factors were optimised for pigment production; *viz.,* pH, temperature, and incubation period from the lowest to highest ranges were analysed at 3 levels (-1, 0, and +1) in the model. A 17 experiment model was generated and performed in triplicate. The quadratic model was suggested from the analysed data for optimization. The predicted and observed response 1 (Pigment production mg l^{-1}) for each

experiment from the BBD experimental design was shown (Table 3). The result represents the statistical model of response 1 (pigment production mg l^{-1}) and the BBD design matrix and its corresponding findings. The three central points were involved for independent variables were standardised by BBD. Response 1 of pigment production (mg l^{-1}) was written as (Y) = +7.67+0.3625 *A+0.0775 * B+0.525 *C-0.1050 * AB+0.3950 * AC-0.1300 * BC- 1.76 * A^2-1.46 * B^2-2.19 * C^2 which is the second-order polynomial equation. All input variables' combined effects correspond to the pigment production (mg l^{-1}). The optimum levels of selected variables were obtained by analysing the response surface contour and solving the regression equation and surface plots. The result represents the result of ANOVA fitting of the second-order polynomial model. The determination coefficient (R^2= 0.9987) and adjusted R^2 (0.9971) values help to interpret the accuracy and adequacy of the model as indicated by 60.67 per cent of the response variability. The (P= 0.2752) value was not significantly lacking, indicating that the pigment production under all conditions was precise and reliable and confirmed the coefficient of variation (CV= 1.89 %). The 3-D response surface plots were obtained using the Design Expert software 13.0.0.3 (Figure 4). Some pigment stability has been affected by environmental factors [42]. The fermentation process can be optimised using *Exiguobacterium acetylicum* S01 to get higher biomass and beta-carotene [43]. Beta-carotene production was induced by *Dunaliella salina* using RSM [14]. The higher production of beta carotene was increased when molasses was used in the medium, and the temperature was maintained at 30ºC. Similar RSM optimization studies have been reported [44]. These factors could be applied to the regression equation, and the highest carotenoid production with predictable response was 7.64 mg l^{-1}, corresponding to the experiment response of 7.81 mg l^{-1}. The interactions between pH, temperature, and time in response to pigment production to illustrate the regression model were described by 3D response surface plots [45]. The interaction between temperature (ºC) and glucose (%), pH and temperature (ºC), incubation time (day), and pH is considered for pigment production. When the acidic pH is increased through submerged fermentation and the formation of metabolites, growth is optimal [46]. The highest pigment production was recorded at 30°C, which could be altered by changing the temperature [47]. The growth of microorganisms is affected by providing acidity or alkalinity in the media, which hinders pigment production. The mutual interactions among the variables were displayed in the corresponding contour plots [48]. The optimum pigment yield of 990.87 g g-1 was obtained using RSM at 25°C, pH 5, and 120 hr [11]. The *Paracoccus* sp strain LL1 was used for optimum pH 7.5 to achieve higher biomass and carotenoid production [49]. The salinity, temperature, and pH provide the optimum conditions for the growth of haloarchea for carotenoid production using the RSM method [50].

Table 3 Experimental design matrix for optimization of pigment production using environmental factors by SB_2 according to BBD

Std	Run	Factor 1	Factor 2	Factor 3	Response 1 Pigment production mg L^{-1}	
		A:pH	B:Temperature Celcius	C:Incubation Hours	Experimental	Predicted
11	1	7	10	168	4.65	4.61
17	2	7	30	108	7.64	7.67
6	3	10	30	48	3.24	3.17
15	4	7	30	108	7.65	7.67
1	5	4	10	108	3.94	3.91
2	6	10	10	108	4.86	4.84
16	7	7	30	108	7.59	7.67
9	8	7	10	48	3.21	3.30
14	9	7	30	108	7.81	7.67
12	10	7	50	168	4.59	4.50
5	11	4	30	48	3.29	3.23
13	12	7	30	108	7.67	7.67
3	13	4	50	108	4.26	4.27
8	14	10	30	168	4.95	5.01
4	15	10	50	108	4.76	4.79
7	16	4	30	168	3.42	3.49
10	17	7	50	48	3.67	3.71



(a) (b)

(c)

Figure 4 3D Interaction between different components were optimized to increase the pigment production by SB_2 whereas a) represents pH (A) vs temperature (B) (b) represents pH (A) vs Incubation (C) (c) represents temperature (B) vs Incubation (C)

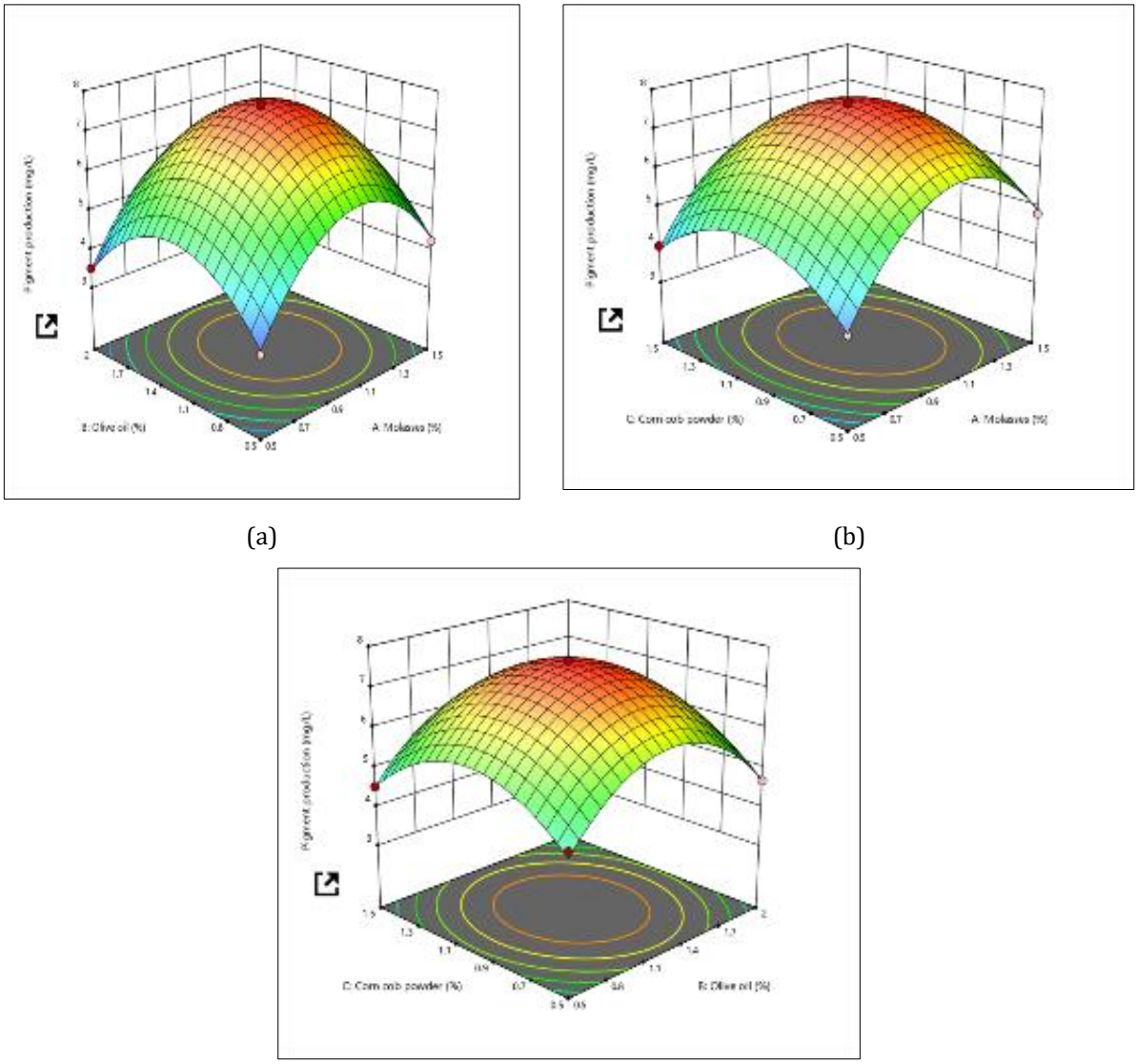
3.5. Nutrient parameters were optimized for pigment production by SB_2 isolate usingBBD

The interaction among the different concentrations of molasses, olive oil, and corn cob powder was used in the medium to determine their optimal level of pigment production. The production of pigment can be optimised by utilising different concentrations of molasses, olive oil, and corn cob powder from the lowest to highest ranges were analysed at 3 different levels (-1, 0, and +1) in the model. A 17 experiment model was generated and performed in triplicate. The quadratic model was suggested from the analysed data for optimization. The BBD experimental design summarised the predicted and observed response 1 (Pigment production mg l^{-1}) for each experiment (Table 4). The result represents the statistical model of response 1 (pigment production mg l-1) and the BBD design matrix and its corresponding findings. BBD standardised the three central points representing a response 1 of pigment production based on the independent variable, which was written as (Y) = +7.60+0.4838 *A+0.0550 * B+0.0384 *C+0.0775 *AB-0.1100 * AC+0.0325 * BC-2.00 * A2-1.65 * B2-1.31 * C2, which is a second-order polynomial equation. The combined effects of the input variables corresponded to the pigment production (mg l^{-1}). The optimum levels of selected variables are determined by surface plots, solving the regression equation and analysing the response surface contour. The result represents the result of ANOVA fitting the second-order polynomial model. The de-termination coefficient (R^2= 0.9968)

and adjusted R^2 (0.9926) values helped to determine the accuracy and adequacy of the model as indicated by 39.01 percent of the response variability. The (P= 0.3612) value was not significantly lacking, indicating that the pigment production under all conditions was precise and reliable and confirmed the coefficient of variation (CV= 2.64 %). The 3-D response surface plots were obtained (Figure 5) using the Design-Expert software 13.0.0.3. RSM applied for production was increased by using a limited concentration of nutrients in the medium to be optimised [12]. Different nutrient factors were used in the media to obtain an optimum response output using the RSM for optimization [13]. For large-scale production, medium optimization is an essential factor that is a more effective, robust, and economic advancement of current statistical and mathematical methods to meet market demand. Beta-carotene and zeaxanthin production were increased using RSM [9]. The cost of the bioprocess is determined by the substrates for the carotenoid production economically using corn cob, sugarcane bagasse, and corn steep liquor [51]. Various environmental conditions and nutrient sources are important factors that are subjective to bacterial growth and pigment production [52]. For the optimization of pigment production by BBD, three independent variables, molasses, olive oil, and corn cob powder. These factors can be applied to the regression equation, and the highest carotenoid production of predictable response was 7.60 mg l^{-1} consequently, the experiment response of 7.67 mg l^{-1} was obtained. The strain SB_2 was optimised with a (1%) molasses concentration, favouring higher pigment production yield. *E. acetylicum* S01 was achieved by a medium that could be optimised with glucose, and peptone increased the production of β-carotene by using RSM [43].

Table 4 Experimental design matrix for optimization of pigment production by SB_2 using various nutrients according to BBD

Std	Run	Factor 1	Factor 2	Factor 3	Response 1 Pigment production mg L^{-1}	
		A:Molasses	B:Olive oil	C:Corn cob powder	Experimental	Predicted
		%	%	%		
4	1	1.5	2	1	4.67	4.56
11	2	1	0.5	1.5	4.52	4.51
3	3	0.5	2	1	3.51	3.44
17	4	1	1.25	1	7.65	7.60
2	5	1.5	0.5	1	4.23	4.30
8	6	1.5	1.25	1.5	4.68	4.63
9	7	1	0.5	0.5	4.81	4.65
10	8	1	2	0.5	4.68	4.69
1	9	0.5	0.5	1	3.38	3.49
6	10	1.5	1.25	0.5	4.83	4.92
16	11	1	1.25	1	7.67	7.60
15	12	1	1.25	1	7.59	7.60
14	13	1	1.25	1	7.46	7.60
12	14	1	2	1.5	4.52	4.68
5	15	0.5	1.25	0.5	3.68	3.73
13	16	1	1.25	1	7.62	7.60
7	17	0.5	1.25	1.5	3.97	3.88



(a) (b)

(c)

Figure 5 3D Interaction between different components were optimized to increase the pigment production by SB_2 whereas a) represents Molasses (A) vs Olive oil (B) (b) represents Molasses (A) vs Corn cob powder (C) (c) represents Olive oil (B) vs Corn cob powder (C)

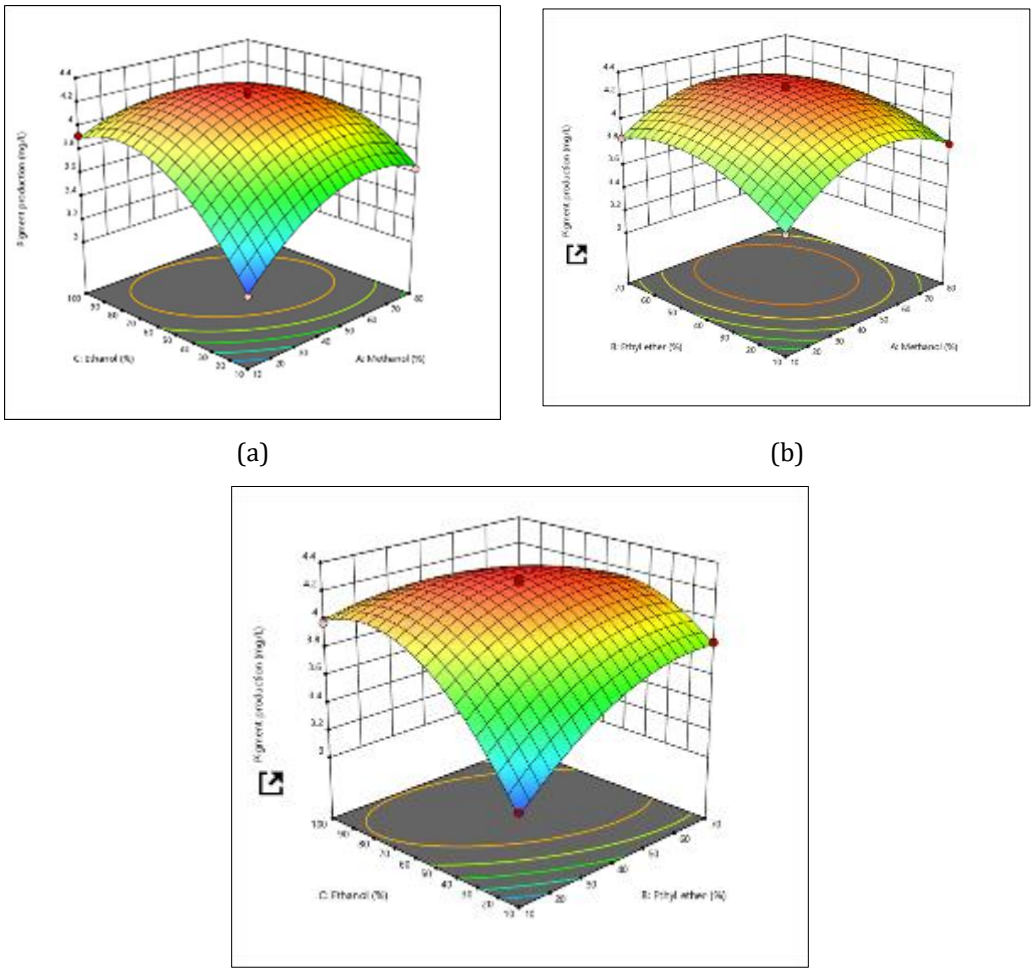
3.6. Pigment isolated from SB_2 optimized using solvent concentration by BBD

In the present study, BBD was evaluated from the interaction among various factors of different concentrations of methanol, ethyl ether, and ethanol to determine their optimal level of pigment production. The production of the pigment was optimised by utilising different concentrations of methanol, ethyl ether, and ethanol from the lowest to highest ranges were analysed at 3 different levels (-1, 0, +1) in the model. The 17 experiment model was generated and performed in triplicate. The quadratic model was suggested from the analysed data for optimization. Table 5 summarises the predicted and observed response 1 (Pigment production mg l-1) for each experiment from the BBD experimental design. The result was observed in a statistical model of response 1 (pigment production mg l^{-1}) and the BBD design matrix and its corresponding findings. The three central points for response 1 of pigment production (mg l^{-1}) by methanol, ethyl ether and ethanol were standardised by BBD. Response 1 of pigment production (mg l^{-1}) was written as (Y) = +4.26+0.0688 *A+0.0963 * B+0.1800 *C-0.0525 * AB-0.2000 * AC-0.2400 * BC-0.2928 * A^2-0.2028 * B^2-0.3852 * C^2 which is the second-order polynomial equation. The combined effects of all the independent variables were correlated to the production of pigment (mg l^{-1}). The optimum levels of selected variables are solved by analysing the response surface contour, the regression equation, and surface plots. The determination coefficient (R^2= 0.9961) and adjusted R^2 (0.9911) values help to determine the accuracy and adequacy of the model as indicated by 43.44 per cent of the response variability. The value (P= 0.0721) was not significant and lacked fit, indicating that the pigment production under all conditions was precise and reliable. The precise and reliable model confirmed the coefficient of

variation (CV= 0.8798 %). The result was presented in (Figure 6) 3-D response surface plots using Design-Expert software 13.0.0.3. The three independent variables were used for different concentrations of methanol, ethyl ether, and ethanol optimised for pigment production by BBD. The SB_2 was optimised with methanol (45%), ethyl ether (40%), and ethanol (55%) concentrations, which yielded higher production of carotenoid. [53] discovered that ethanol extracts carotenes (non-polar substances) more efficiently than xanthophyll (polar substances). The high yield of carotenoids obtained from the pummelo peel with ethanol at 50°C for 40 min [54]. The rapeseed meal was optimised for extraction of higher production of carotenoid by RSM [55]. A variety of solvents used for the production of carotenoid mostly organic [56]. The temperature and the solvent extraction time were protected for pigments which are not stable in high temperature [57].

Table 5 Experimental design matrix for optimization of pigment extraction of various solvents by SB_2 strain according to BBD

Std	Run	Factor 1	Factor 2	Factor 3	Response 1 Pigment extraction mg L^{-1}	
		A:Methanol	B:Ethyl ether	C:Ethanol	Experimental	Predicted
		%	%	%		
5	1	10	40	10	3.12	3.13
1	2	10	10	55	3.53	3.54
2	3	80	10	55	3.79	3.79
4	4	80	70	55	3.89	3.87
6	5	80	40	10	3.64	3.67
17	6	45	40	55	4.29	4.26
11	7	45	10	100	3.98	3.99
7	8	10	40	100	3.92	3.89
16	9	45	40	55	4.25	4.26
9	10	45	10	10	3.18	3.15
14	11	45	40	55	4.26	4.26
3	12	10	70	55	3.84	3.84
12	13	45	70	100	3.68	3.71
10	14	45	70	10	3.84	3.83
8	15	80	40	100	3.64	3.63
13	16	45	40	55	4.28	4.26
15	17	45	40	55	4.21	4.26



(a) (b)

(c)

Figure 6 3D interaction between different components were optimized to increase the pigment extraction by (SB_2) whereas a) represents Methanol (A) vs Ethyl ether (B) (b) represents Methanol (A) vs Ethanol (C) (c) represents Ethyl ether (B) vs Ethanol (C)

3.7. The performance of carotenoid yellow pigment against antibacterial activity from SB_2 strain

The yellow pigment (SB_2 isolate) recovered from the various concentrations of salt (%), temperature and pH conditions were studied for antibacterial activity. The maximum inhibition zone and dry weight were observed at pH (7), temperature (30ºC) and salt (2%) concentration. The results are presented in Figure 7. The carotenoid pigment's antimicrobial and antioxidant potential has already been reported [58]. The yellow pigment (SB_2 isolate) was recovered and studied for antibacterial activity. The maximum inhibition zone and dry weight were observed at pH (7), temperature (30ºC) and salt (2%) concentration. The zeaxanthin production was increased by media supplemented with 2% salt from marine bacteria [59]. [60] studied the *Micrococcus radiodurans* growing up to a concentration of 7% NaCl and synthesis pigment, which was also grown in media containing 3% NaCl and 5% NaCl concentration for maximum pigment production. The SB_2 isolate observed growth and pigment production from 2 to 12 per cent, notably a maximum of 2 per cent concentration in media. The microorganisms also grew at 10% salt [61]. Cycloprodigiosin was observed, and the concentration of pigment of 20 µg ml^{-1} controls the activity of *S. aureus* at its maximum inhibition zone formation (25.1 ± 0.55 mm) [62]. Similar studies recorded that the marine isolate *Serratia marcescens* IBRL USM 84 produces red pigment and evaluated 13 bacterial pathogens for antimicrobial activity out of 18 [63]. The pigments effectively control the growth and reproduction of gram-positive as compared to gram-negative for antimicrobial activity, which has rigid cell walls that may protect [64]. [65] pigments from *Zooshikella* sp S2.1 were separated from the south Andaman and capable of producing antibacterial activity with concentrations of 150 to 400 µg ml^{-1} which were also confirmed as red pigments from *Streptomyces* sp., BSE6.1. The antibacterial activity of the pigment was isolated from seawater by *M. luteus* [66]. [67] discovered that the marine pigmented *Micrococcus* sp. has antibacterial

properties by acting as an anti-inflammatory and wound healing agent. Carotenoid pigment inhibited bacterial pathogens [68]. The antagonistic activity was exhibited by prodigiosin isolated from *Chromobacterium prodgiosum* [69]. The antimicrobial activities are observed by the carotenoid production by *Halomonas* sp. [70]. Recently, [71] reported antimicrobial activity was tested with violacein and deoxyviolacein pigment. *Vibrio owensii* TNKJ.CR.24-7 was isolated from coral to produce the yellow pigment beta-carotene to inhibit the growth of *Klebsiella pneumonia,* MRSA, and *E. coli* [72].

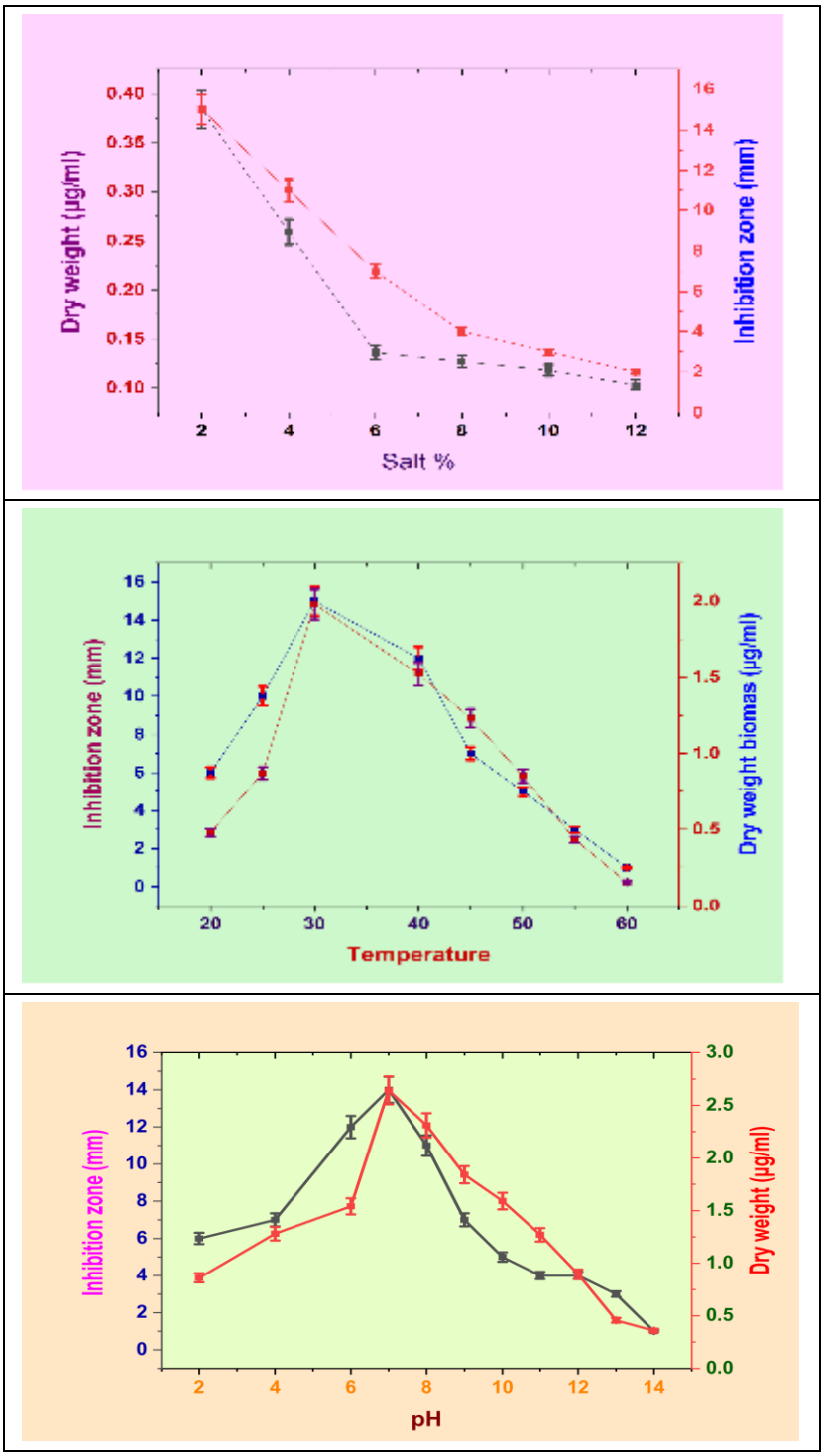


Figure 7 Antibacterial activity of Salt, Temperature and pH from the isolate

4. Conclusion

Twenty-four pigment-producing bacterial strains were isolated from the marine water samples observed for yellow pigment named MB_4, MMB_8, MB_2, and SB_2. The strain SB_2 was a yellow-colored colony, smooth and round with yellow pigmentation, taken for further studies. The highest pigment production was optimised with environmental factors, nutrients, and solvents using a quadratic model of BBD. The antibacterial activity of the extracted carotenoid pigment was demonstrated at pH 7, 30ºC, and 2% salt concentration.

Compliance with ethical standards

Acknowledgments

This study was supported by National Fellowship for Person with Disabilities (NFPWD), 2017-18, Grant number: F No. 01-01/2019-sch, dated 21st October 2019) funding provided for pursuing PhD. The author wishes grateful thanks to The University Grant Commission (UGC), New Delhi, India. The author was very much thankful to the Supervisor, Professor, Department of Microbiology, School of Life Sciences, Pondicherry University facilitated the research work.

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