Carotenoid Production by *Rhodotorula sp*. on Fruit Waste Extract As a Sole Carbon Source and Optimization of Key Parameters

Tarangini, Korumilli; Mishra, Susmita*[+]

Department of Chemical Engineering, National Institute of Technology, Rourkela–769 008, Orissa, INDIA

ABSTRACT: *Fruit waste extract was selected as the sole substrate for carotenoid production and the effect of parameters like pH, temperature and agitation have been studied. A two-step simple sequential strategy was employed for the optimization of carotenoid production by Rhodotorula rubra. In the first step, one factor at a time was employed to evaluate the impact of pH on carotenoid production. The outcome revealed that pH has noteworthy influence on pigment production at ambient conditions with constant temperature and agitation. The produced carotenoid was characterized and confirmed by UV-Visible and FT-IR spectroscopic analyses. A Box–Behnken design was then applied in the second step to optimize the pH, temperature and agitation to obtain high pigment yield. The statistical experimental design predicted the high yield conditions of different responses. The interaction between pH and temperature stood vital for improved carotenoid production (2.98 ± 0.28 mg/L) with biomass yield of 7.83 mg/mL by the optimization of significant parameters. The optimum conditions followed for high yield carotenoids are pH (7.0), temperature (28.2 °C), and agitation (150 rpm). Along with the above, biomass growth conditions were also studied and optimum parameter values were reported. The present work shows the effectiveness of abundantly available cheaper fruit waste extract as a sole substrate in obtaining carotenoids in significant amount by R. rubra.*

KEY WORDS: *Rhodotorula rubra, Fruit waste extract, Carotenoid; Box-Behnken design.*

INTRODUCTION

Pigments are of great commercial interest and have received considerable attention because of their potential beneficial effects on human health besides pigmenting properties [1]. In recent years there is an increasing demand for microbial pigments as promising alternatives for synthetic pigments that are widely used in food industries [2]. The pigments from microorganisms are nature selected and have advantages over plant and animal derived pigments with no seasonal variations, no geographic inconsistency in the production, high productivity and are easily manipulated in the processing schemes [3-4].

Among microbial derived pigments, carotenoids are fat soluble diverse class of yellow, orange, red and purple natural pigments which are unanimously produced by a wide range of microorganisms and plants. Due to the recent discovery of anticancer and antioxidant properties

** To whom correspondence should be addressed.*

+ E-mail: smishra1234@gmail.com , smishra@nitrkl.ac.in

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of carotenoid pigments, their use in pharmaceuticals and nutraceuticals is expected along with their applications in the fields of food, cosmetics, chemicals and so on [5-7]. Globally carotenoids are estimated to supersede USD S 280 million in 2015 [8]. In order to improve the yield of carotenoid pigments and to decrease the cost of production, various studies have been performed using several microorganisms using numerous different substrates [9-10]. Amongst different microorganisms *Rhodotorula sp*. stood as a potential and scientifically favorable candidate for high yield carotenoids production [11]. The yeast *Rhodotorula sp.* is one of very few types of yeast which is able to produce a large number of carotenoid pigments which are mainly β - carotene, torulene, torularhodin [12]. Moreover, many investigations have been done using this genus to make the process economical [3,6, 13-14]. Key process parameters like type of substrate, nutrients availability, pH, temperature and so on are optimized in various processes [15]. However, there are still extensive investigations focused on this versatile genus *Rhodotorula* to produce carotenoids in more economical ways.

The cost of the substrate has an important contribution to the overall pigment production cost, and it can be minimized by using cheaper organic waste. Substrate composition, typically carbon source has a significant effect on carotenoid production and its cost. Many substrates have been considered as potential substrates for carotenoid production [16]. Using a low cost or cheap substrate for the production of high value products may help to make the process economical. Till now, several cheaper substrates have been used (e.g. sugar cane juice, peat extract, whey, grape must, beet molasses, hydrolyzed mug bean waste flour, soyabean and corn flour extract and sugar cane molasses etc.) for the production of carotenoids [17]. In this study, we have used Fruit Waste Extract (FWE) as the substrate for the production of carotenoids. In general, fruit processing units dispose the wastes which are rich in soluble sugars and micronutrients that support the microbial growth. Nowadays, fruit waste disposal is one of the problems that the fruit processing industries are facing. Eyeing on this, we have chosen fruit waste extract as potential substrate for pigment production which can reduce the production cost and make the product economical. Using FWE as a suitable sole substrate; we examined the ability of *Rhodotorula rubra* for carotenoids production.

In recent years statistical design has been successfully employed to identify the optimum level of various parameters involved in the process. Statistical design is a powerful tool that accounts for the main as well as interactive influences of the parameters on the process performance. The disadvantages of other classical methods are that they are time consuming, laborious and expensive. In contrast, the use of statistical tools such as RSM methodology provides a great amount of information based on only a small number of experiments [18]. In this study a combination of traditional non statistical and statistical method based experimental design has been employed to optimize the biomass and carotenoid production. Primarily, one factor at a time, a classical approach was practiced that involves various levels of one factor when the other factors are constant. Using this method, the key parameters which influenced the pigment production significantly, were identified. Box–Behnken experimental design method is useful for rapidly optimizing the process with limited number of experiments. Hence the objective of this study is to explore the effectiveness of the sole substrate (mixed fruit extract) on pigment production using the Box-Behnken statistical tool. Optimization of process parameters such as pH, temperature, and agitation for high yield of biomass and pigment production was experimented.

EXPERIMENTAL SECTION

Reagents and chemicals

Methanol, acetone, HCL, NaCl, and diethyl ether were procured from Merck, Mumbai, India., malt yeast extract medium, malt yeast extract agar were purchased from HiMedia, India. All other chemicals used were of analytical reagent grade throughout the study. Double distilled water was used through the study and aseptic conditions were maintained wherever necessary.

Microorganism and its maintenance

The microorganism *Rhodotorula rubra* (MTCC no: 1446) used in this study was obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. The microorganism was grown and maintained on malt yeast extract medium and stock cultures were preserved on malt yeast extract agar slants at 4 °C and sub-cultured at monthly intervals.

Table 1: Coded and actual levels of the three variables.

Variable	Symbol	Coded and actual levels		
		-1	0	1
pH	x1	6	6.5	7
Temperature	x2	25	27.5	30
agitation	x3	90	120	250

Substrate preparation

Fruit waste typically containing pineapple, pomegranate and orange is obtained from a fruit juice shop of a local market. It includes extracted carpels of oranges, core of pineapples, and crushed seeds along with arils of pomegranate. The soluble sugars are extracted from 1 kilogram of fruit waste by adding 2 liters of distilled water at 100 ^{0}C for 30 minutes. The resultant straw colored fruit waste extract (FWE) is filtered and stored at 4 ^{0}C for further experimentation.

Pigment production and extraction

The biomass (5 mg) was activated in malt yeast extract broth (100 mL) at 30 °C for 24 hrs. 1mL of the activated culture was inoculated in 250 ml conical flasks containing 50 mL autoclaved FWE medium maintained at different pH's (4.3, 5, 6, 7, 8). Until significant color appearance the incubation at 30 ^{O}C was continued. The pigmented biomass collected after centrifugation (9000 rpm, 10 min) was washed repeatedly (thrice) with distilled water and subsequently treated with 1 N HCl (60 °C, 10 min). The treated cells were further washed in sterile distilled water and subjected to repeated solvent washes to extract the intracellular pigment. Methanol: acetone (1:1) was used as solvent for the microbial pellet wash and washing step continued till colorless pellet was achieved. The washed colored solvent was collected separately and extracted with equal parts of diethyl ether and NaCl (10 %) as a part of further purification step of the carotenoid pigment. And the total carotenoid concentration (measured as β - carotene) in diethyl ether extract was determined using standard method [13]. It was estimated according to the absorbance at 448 nm using spectrophotometer (UV-3600 Shimadzu). The pigment quantity is estimated using absorption coefficient $E^{1\%}_{1cm}$ = 2659 via spectral analysis. To obtain purified pigment in solid form, the collected ether layer subjected to vacuum drying.

Analysis

Cell dry weight was measured by harvesting the cells after centrifugation of the growth medium at 5000 rpm for 10 minutes and subsequent washing thrice with distilled water. The cells were dried at 105 ^{0}C till constant weight was attained. Reducing sugars were estimated during experimentation by DNS assay method [19]. Elemental analyses were determined on a Vario EL Cube CHNS Analyzer for FWE, before and after carotenoid production at pH -7.

Experimental design

One factor at a time and Box-Behnken design were used for optimization of carotenoid production. The significant effect of pH towards carotenoid and biomass yield was determined by one factor at a time method. Knowing the safe operating zones of other production parameters like temperature and agitation, a Box-Behnken design (BBD) was selected for optimization of the mentioned key process parameters (Table 1) for carotenoids production by *R. rubra*. The experimental design and statistical analysis of the data were carried out using the Minitab (14.0) statistical software package. BBD with three factors and 15 runs was chosen; a model was developed and also validated. Table 1 shows the variables and experimental design levels for response surface and Table 2 shows the design matrix of BBD.

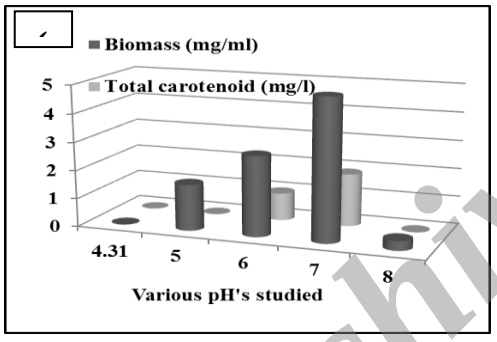
RESULTS AND DISCUSSION

Effect of pH on biomass and carotenoid yield

Using first approach the significant parameters that affected carotenoid production were identified as pH, temperature and agitation. Effect of pH on biomass growth and carotenoid production is illustrated in Fig. 1(a). The figure shows native FEW's pH (4.31) including different values studied. For evaluating the influence of pH on biomass and pigment production, *R. rubra* was cultivated in FWE medium at 30 ^{0}C for 7 days.

Table 2: Experimental design matrix for the Box-Behnken design.

Run	pH (x1)	Temperature (x2)	Agitation (x3)	Biomass-mg/ml	Pigment-mg/l
1	0	-	-	3.33	1.33
2	0	-	+	4.01	1.74
3	+	0	-	5.3	2.12
4	+	-	0	6.31	2.51
5	+	+	0	7.21	2.77
6	-	-	0	2.74	1.09
7	0	0	0	6.44	2.47
8	0	0	0	6.76	2.71
9	-	0	+	4.99	1.98
10	0	0	0	6.54	2.62
11	-	+	0	5.2	2.09
12	+	0	+	7.75	3.1
13	-	0	-	3.87	1.55
14	0	+	+	7.08	2.72
15	0	+	-	4.8	1.92



(b)

(c)

Sample	N%	C%	H%	S%
FWE-before	4.18	62.35	4.17	1.08
FEW- after	2.374	38.952	1.05	0.87

Fig. 1 (a) Growth of biomass and carotenoid yield at various pHs and at room temperature (30 ^{0}C). Prepared Fruit Waste Extract (FEW) – left, and FEW with R. rubra growth with intracellular carotenoid (right) upon incubation (b). CHNS analysis of FWE before and after fermentation; upon separation of R. rubra by centrifugation (c).

The microbial culture is inoculated in the medium with adjusted pH's 5, 6, 7, 8, and 9 along with initial pH 4.31. Among various pHs, biomass growth was identified at values of 5 to 9 and pigment production was observed only at 6 and 7. So, the results suggest that pigment production was highly dependent on pH irrespective of biomass growth with high yield caused at pH 7. The prepared FWE and fermented FWE with the yeast *R. rubra* are shown in Fig. 1(b).

The reducing sugar content in FWE was identified to be 19.90 mg/ml before experimentation. CHNS analysis was performed for the prepared FWE so as to estimate the presence of major and minor nutrients. CHNS analysis was done before and after carotenoid production (Fig. 1(c)). The values stated suggests that significant utilization of macro and micro nutrients by the microorganism followed the following order of H > N > C > S.

Biomass growth, pigment yield and glucose utilization

Fig. 2A presents the individual values of cell dry weight (mg/ml), total carotenoid content (mg/L) and glucose concentration (mg/ml) as a function of time in days. Pattern from the figure shows that carotenoid content paralleled the cell dry weight and substrate concentration decreased linearly till 3 days (72 h). Fig. 2A represents the growth of biomass and production of carotenoid with time. Initially the rate of growth of biomass superseded the carotenoid production. However after 3 days the biomass yield parallel to carotenoid production rate. On the contrary the substrate utilization rate also diminished after 3 days. This graphical representation suggests that maximum carotenoid production was achieved at the end of log phase at the expense of substrate utilization. Thereafter with the approaching stationary phase, the carotenoid production capacity was constant which is typical of any production process in a batch reactor. The observed condition shows the biomass's pigment production ability irrespective of quantitative substrate utilization. However maximum pigment can be produced within 4 days of incubation using FWE as sole substrate.

The UV-visible spectrum of the pigment produced was illustrated in Fig. 2B. The observed absorption maximum at 507 nm towards the higher wavelength states that the produced compound resembles Torularhodin type carotenoid, which is usually produced by *Rhodotorula* in significant quantities [5]. The conformation study showing structural details using FTIR analysis was carried out on the produced pigment along with the reference β - carotene (Fig. 2C-a, b).

The FTIR spectrum of extracted carotenoid is shown in the Fig. 2C-a. The peak descriptions can be described [20] as follows: the broad peak at 3351 cm^{-1} (which is in between 2900 to 3500 cm^{-1}) is due to hydrogen bonded O-H; peak at 2873 cm^{-1} is due to methyne C-H stretch; peaks at 1692, 1636 cm^{-1} is for alkenyl C=C and aryl substituted C=C in the compound; peak at 1429 cm^{-1} attribute to methyl C-H asymmetric band; peak at 1231 cm^{-1} is for C-O stretch and 1081 cm^{-1} is for C-C skeletal vibrations. The peak bands of 3351 along with peak at 1231 cm^{-1} confirms the presence of carboxylic group in the purified carotenoid.

Moreover on comparison with the purchased β - carotene's spectrum (Fig. 2C-b), the obtained carotenoid

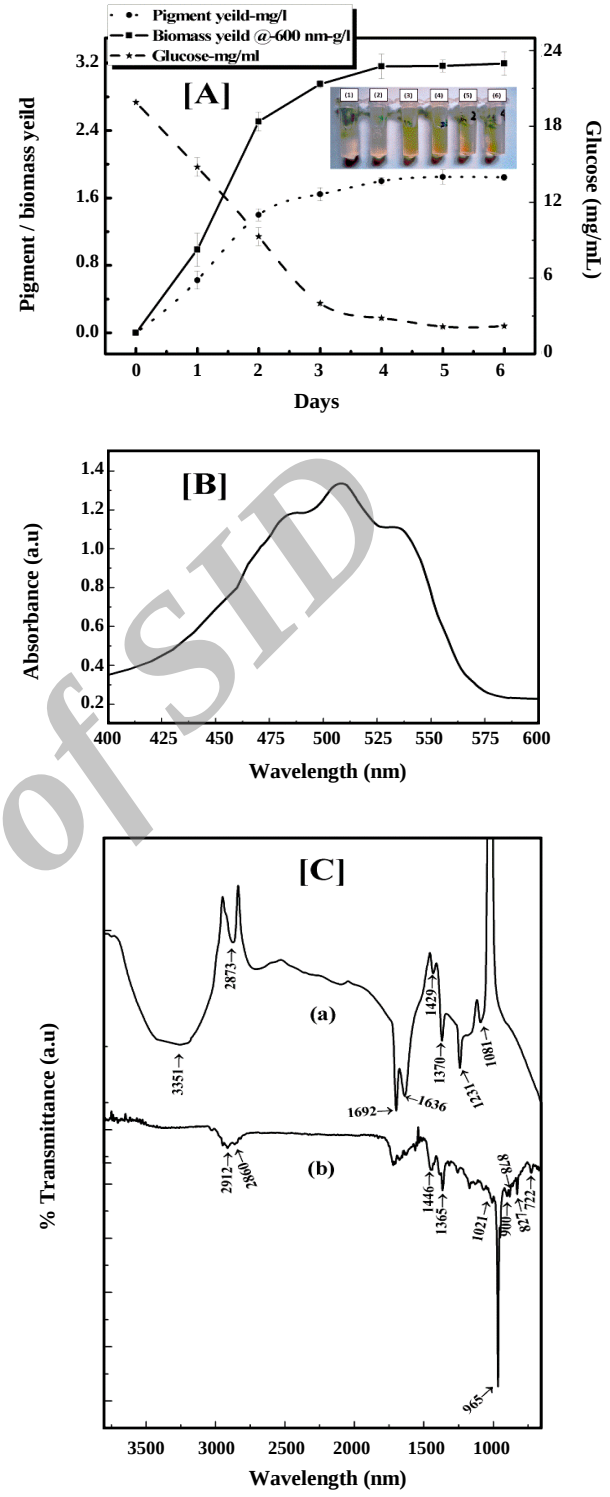


Fig. 2: Carotenoid production (mg/l) and biomass yield (mg/ml) along with glucose utilization (mg/ml) given in [A]. The picture insert shows the colored biomass from 1 to 6 days. UV-visible spectrum of the purified carotenoid in methanol medium [B]. FTIR spectra of the synthesized carotenoid (a) and the purchased β-carotene (b) pigment.

spectrum has some similarities and some variations. The spectrum of β-carotene displayed major peaks at 2912 and 2860 cm^{-1} (for asymmetric and symmetric vibrations of the CH_2 and CH_3); peak at 1446 cm^{-1} (due to CH_2 group); peak at 1365 cm^{-1} (for splitting due to dimethyl; group); peaks at 1021 cm^{-1} (is for in plane –CH-) and 965 cm^{-1} (is for trans conjugated alkene -CH=CH- out-of-plane deformation). Further peaks at 700 to 900 cm^{-1} are for skeletal vibration of C-C stretch. The IR analysis along with UV spectrum of the obtained carotenoid closely resembles the structures of torularhodin [14] foremost, and has some similarities with β-carotene (as shown in Fig. 2C-b). Also this spectral pattern is obvious as the mentioned pigments are predominantly obtained by *Rhodotoula sp* [5].

Box–Behnken design

The influence of pH on pigment productivity was identified as described in the above section, this indicates a slight deviation of pH values from 6 to 7 resulted in negative effects with low/negligible pigment yield. The above synthesis conditions were at static temperature 30 °C and agitation (100 rpm). The Box–Behnken design was employed to study the interactions among the significant factors and also determine their optimal levels. This methodology is essentially an assortment of statistical and regression techniques. The first step involves framing a statistically significant empirical model capable of describing the effect of multiple factors on a response. A commonly used empirical model of the response surface analysis is a quadratic polynomial of the type:

$$y = b_0 + \sum_i \left(b_i x_i\right) + \sum_i \left(b_{ii} x_i^2\right) + \sum_i + \sum_j \left(b_{ij} x_i x_j\right) \qquad (1)$$

where y is the predicted response, $x_i x_j$ are input variables which influence the response variable y; b_0 is the offset term; b_i is the i^{th} linear coefficient; b_{ii} the i^{th} quadratic coefficient and b_{ij} is the ij^{th} interaction coefficient.

Once a suitable model is obtained, it can be used for optimization which involves finding an optimum combination of factors that will maximize or minimize a response. The average yields of biomass and pigment are found to be 5.488 mg/ml and 2.181 mg/l from Table 2. By using multiple regression analysis, the experimental responses shown in Table 2 were correlated with two significant factors according to Equation (1).

$$\text{Biomass}\left(\frac{\text{mg}}{\text{mL}}\right) = 6.58 + 1.2213\left(x_1\right) + 0.9875\left(x_2\right) - \qquad (2)$$
$$0.8162\left(x_3\right) - 0.9438\left(x_2 \times x_2\right) - 0.8313\left(x_3 \times x_3\right) -$$
$$0.39\left(x_1 \times x_2\right) + 0.3325\left(x_1 \times x_3\right) + 0.4\left(x_2 \times x_3\right)$$

$$\text{Carotenoid production}\left(\frac{\text{mg}}{\text{L}}\right) = 2.6 + 0.4737\left(x_1\right) + \qquad (3)$$
$$0.3537\left(x_2\right) + 0.3275\left(x_3\right) - 0.3725\left(x_2 \times x_2\right) -$$
$$0.3\left(x_3 \times x_3\right) - 0.185\left(x_1 \times x_2\right) + 0.1375\left(x_1 \times x_3\right)$$

The factors x_1 and x_2 are indicated in their coded units (shown in Table 1). The goodness of fit of the quadratic polynomials is expressed by the coefficient of determination (R^2- which is a measure of how well the model can be made to fit the raw data). The closer the value of R^2 is to 1, the better is the correlation among the observed and predicted values. The R^2 values for Eq. (2) and (3) are 0.956 and 0.960 respectively; indicating that about ~ 95 % of the variations in biomass and carotenoid yield can be explained by the quadratic polynomials. This means that Equations (2) and (3) are adequate for correlating the experimental results. Moreover regression equations (2 and 3) were evaluated by the F-test for analysis of variance (ANOVA). Responses for biomass and pigment production are shown in Table 4. Prob > F value for the model is less than 0.05 infers that the model terms are statistically significant.

The actual and predicted values of responses for biomass and carotenoid concentrations versus the corresponding values calculated by regression models are shown in Fig. S1a,b respectively. Actual values are the measured values for a particular experiment, whereas predicted values are generated by using the approximating functions. The line of perfect fit is also shown in these figures and visualization of the two regression models provides an accurate description of the experimental data. In addition the values of R^2 and adjusted R^2 (Table 3) have advocated a high correlation between actual and predicted values. The response surface and contour plots were constructed using

Table 3: Statistical significance obtained for the regression coefficients in Eq's .(1), (2).

Model term	For Biomass		For Pigment	
	Coef	p	Coef	p
intercept	6.5800	0.000	2.6000	0.000
x1	1.2213	0.000	0.4737	0.000
x2	0.9875	0.000	0.3537	0.000
x3	0.8162	0.001	0.3275	0.000
x1*x1	-0.2713	0.166	-0.1125	0.122
x2*x2	-0.9438	0.002	-0.3725	0.002
x3*x3	-0.8313	0.004	-0.3000	0.004
x1*x2	-0.3900	0.059	-0.1850	0.024
x1*x3	0.3325	0.093	0.1375	0.064
x2*x3	0.4000	0.055	0.0975	0.154
	R-Sq = 98.4% R-Sq(adj) = 95.6%		R-Sq = 98.6% R-Sq(adj) = 96.0%	

Table 4 ANOVA results for biomass and pigment yields.

ANOVA					
For Biomass-mg/ml					
Source	DF	Sum of squares	Mean square	F	P
Regression	9	32.2579	3.58421	34.75	0.001
Linear	3	25.063	8.35433	81	0
Square	3	5.5043	1.83477	17.79	0.004
Interaction	3	1.6906	0.56354	5.46	0.049
Residual Error	5	0.5157	0.10314		
Lack-of-Fit	3	0.4621	0.15403	5.75	0.152
Pure Error	2	0.0536	0.0268		
Total	14	32.7736			
For Pigment-mg/l					
Source	DF	Sum of squares	Mean square	F	P
Regression	9	4.70655	0.52295	38.78	0
Linear	3	3.65468	1.21823	90.34	0
Square	3	0.80132	0.26711	19.81	0.003
Interaction	3	0.25055	0.08352	6.19	0.039
Residual Error	5	0.06742	0.01348		
Lack-of-Fit	3	0.03802	0.01267	0.86	0.576
Pure Error	2	0.0294	0.0147		
Total	14	4.77397			

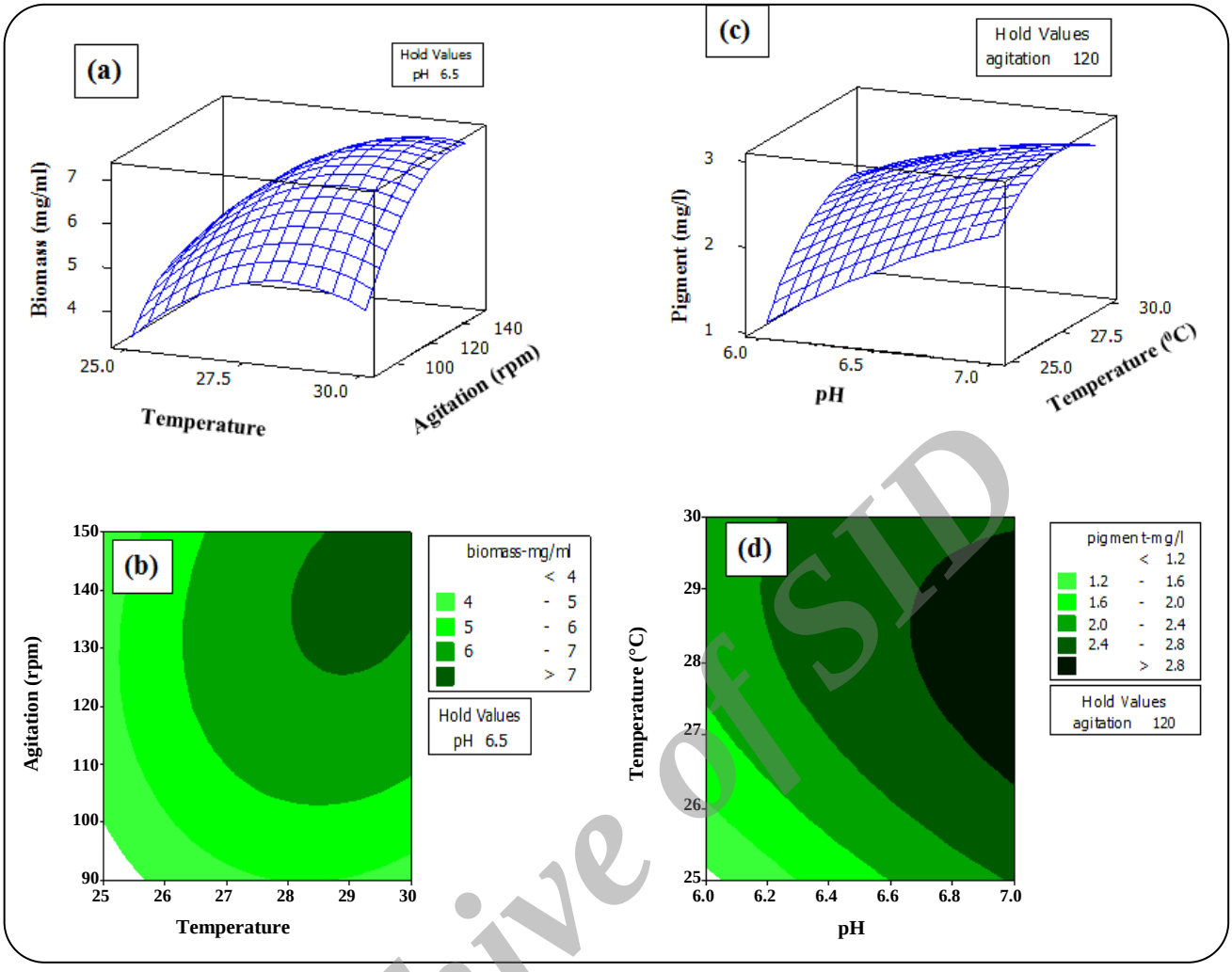


Fig. 3: Response surface and contour plots obtained from Equation (2) and (3) showing the effect of the temperature, agitation and their mutual interaction on biomass concentration (a), (b); while it is effect of pH and temperature and their mutual interaction on caroteniod pigment concentration (c), (d). The displayed units of all the graphs are in natural units.

the regression models and represented in Fig. 3a-d. Fig. 3a depicts the interactive effect of the agitation and temperature on the biomass concentration. At low to moderate agitation values, increases in temperature led to increased production of biomass initially up to centre point and then decreased. At low to moderate temperature values, increases in agitation values showed increase in biomass yield. Similar trends were not observed for pigment yield as a function of pH and temperature (Fig.3c). At high pH values, increases in temperature showed significant increase in pigment yield up to the centre point and decreased thereafter. While with increase in temperature values, increase in pH led to increased pigment production. The contour plots in Fig-3b,c indicates that a local optimum exists in the area experimentally studied; a set of values on the two factors that leads to maximum biomass or pigment production. The location of these optimal points can be obtained by differentiating Eqs. (2) and (3). Eq. (3) was used to derive the most efficient combination of x_1 to x_3 to produce carotenoids from *Rhodotura sp*. According to Eq. (3), a maximum carotenoid recovery of 3.17 mg/L could be attained with pH (7.0), temperature (28.2 °C), and agitation (150 rpm). Experimental validation of the optimum x_1-x_3 combination gave a carotenoid yield of 3.298 ± 0.28 mg/L (with 7.83 mg/mL biomass concentration). Equation (2) depicted that at pH (7.0), temperature (30 °C), and agitation (150 rpm) maximum yield of 7.97 mg/mL biomass could be achieved. The experimental validation resulted in biomass yield of

8.27 ± 0.33 mg/mL with 2.43 mg/L carotenoid production. The obtained results are in good agreement with the predicted values of pigment and biomass production with ~ 4 % and ~ 3.6 % deviations. Therefore the results are in agreement with the effectiveness of the response surface approach described here. Furthermore, the obtained pigment yield was found to be in comparison with the earlier reports which employed sugar cane molasses [6] and mug bean wastes [13]. In addition to the higher yield, the economic aspects (simple methodology, cheap substrate) suggested that the pigment production can be scaled up to the industrial level without any special conditions. Till date, most of the pigment production was associated with the special conditions like acid hydrolysis of their substrate which may hinder the scale up to industrial level.

CONCLUSIONS

In summary, the results obtained from the *R. rubra* in this study will be useful for efficient carotenoids production by utilizing the FWE as a sole substrate. The study also screened out key vital parameter like pH and optimized other imperative parameters such as temperature and agitation along with the pH using a response surface methodology. The simultaneous optimization of parameters yielded maximum carotenoid production conditions in an economical way, for its large scale production using a cheaper substrate (FWE). Our results demonstrated that FWE could be profitably used as a suitable substrate without any additional growth supplement for noteworthy carotenoid production. The response optimization of parameters pH (7), temperature (28.2 °C) and agitation (150 rpm) by Box-Behnken design resulted in 51.2 % more enhancement of the mean carotenoid production. The produced carotenoid pigment is having potential antioxidant applications in food industries. This can be valued for industrial scale utilization of FWE to generate high value carotenoids using *R. rubra* and opens up scope for exploring other high value microbial pigments.

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