ORIGINAL RESEARCH PAPER

Microbiology

OPTIMIZATION OF CAROTENOID PRODUCTION BY PARACOCCUS BEIBUENSIS USING PLACKETT-BURMAN DESIGN AND RESPONSE SURFACE TECHNIQUE.

KEY WORDS: *Paracoccus beibuensis*, Response surface method, Plackett-Burman Design, Carotenoids

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ABSTRACT

This work is aimed at evaluating the conditions for growth and carotenoids production by *Paracoccus beibuensis*. The sequential statistical methods were used to maximize carotenoid production. Initially, a Plackett-Burman design was used to optimize the variable i.e. nutritional components such as glucose, peptone, beef extract, meat extract and NaCl, out of which meat extract effects significantly for carotenoid production ($P < 0.05$). Further investigation of effect of meat extract using a Response Surface Methodology (second -order central composite design (CCD) with one factor analysis) was done to optimize carotenoid production, which was adequately approximated with a full quadratic equation obtained from a two-factor-2-level design. The analysis of quadratic surfaces showed that meat extract has significant effect on carotenoid production ($P < 0.05$) (R^2) 0.93 and adjusted R-squared 0.90. Validation of the experimental model was done where maximum carotenoid production (1112µg/l) was obtained with 15g/l of meat extract. The carotenoid production is more sensitive for presence of meat extract and its concentration. Maximum biomass production was achieved with 15g/l of meat extract.

1. Introduction

Carotenoids are functional molecules produced by variety of microorganisms and plants. Carotenoids have special properties that make them important for many applications in Food, Pharmaceutical (Schmidt-Dannert C. 2000), Neutraceuticals (Sandmann G., 2003), Cosmetics and as Feed. Some of these compounds are precursor of vitamin A, deficiency of which persist as a serious public health problem in developing countries (Irani R. M. *et al*; 2012). The intake of carotenoids can reduce many diseases such as degenerative diseases, cancer, cardiovascular problems, cataracts, macular degeneration and many neurodegenerative diseases (Schmidt-Dannert C. 2000). The increasing demand for consumption of natural carotenoids has raised interest in their bio-production. In present studies, an attempt was made to optimize the carotenoid production by using statistical approach.

Carotenogenesis by the bacteria depends on the strain which produce variable quantities of different carotenoids and also on culture conditions which affect the bacterial growth and carotenoid production. However, biotechnological production of carotenoids is limited by high cost, but this problem can be reduced by optimizing the process conditions (Irani R. M. *et al*; 2012).

Carotenoid biosynthesis is characteristic of many species of *Paracoccus*. The major carotenoid produced is Astaxanthin (3,3'-dihydroxy-β-β' carotene-4,4'-dione) (Danilo G. M., *et al*; 2004), β-carotene (β, β-carotene) (Irani R. M. *et al*; 2012), Zeaxanthin (3,3'-dihydroxy-β-β' carotene) (Alan B., *et al*; 2003), Canthaxanthin (4,4'-diketo-β-carotene) (Armgstrong GA 1994). Astaxanthin is an increasing carotenoid for commercialization purpose because of its high antioxidant activities and dark red to bright orange pigmentation. (Wollgast, J. and Anklam, E. 2003).

In the present work, optimization of nutritional components for carotenoid production by *Paracoccus beibuensis* which was previously isolated from Lonar Crater, Buldhana, Maharashtra, India (Dhere Deepti. D. *et al*; 2016) was done. For optimization of nutritional components, series of statistical experimental design was employed rather than one-factor-at-a-time approach. The main problem with one-factor-at-a-time approach is that possible interaction between factors will go unnoticed but interaction between factors are very common and important. Therefore, the effects of nutritional components including interactions of factors, could be simultaneously investigated by using statistical experimental methods.

2. Materials and methods

2.1 Micro-organisms and culture conditions

Paracoccus beibuensis was isolated previously from hypersaline - hyperalkaline environment of Lonar Crater, Buldhana, Maharashtra, India and was identified by 16s rRNA sequencing approach (Dhere Deepti. D. *et al*; 2016) was used through the study. The culture was maintained on nutrient Agar slant pH (9.5) at 4°C. Each inoculum was prepared by inoculating a loopful culture from slant into 25ml Nutrient broth (Himedia) in 100ml Erlenmeyer flask and incubating at 30°C with shaking at 120rpm for 24hours. Cells were separated after incubation by centrifugation and added to sterile saline (0.85% NaCl) and adjusted to 1 unit absorbance at 660nm and 1% of this was used as inoculum. Fermentation experiment was carried out in 100ml Erlenmeyer flask containing 25ml media prepared with different combinations of glucose, peptone, beef extract, meat extract and NaCl; (Himedia) and incubated for 3 days in white light with shaking 120rpm by using ORBITEK shaker.

2.2 Bio-pigment extraction

The *Paracoccus beibuensis* was inoculated (1%) in sterile Nutrient broth having initial pH 9.5 and incubated at 30°C for 72h with shaking condition (120 rpm) in white light. The extraction was done by slight modification of the procedure used by Manish R. Bhat *et al;* 2015. Then culture medium was centrifuged (REMI-cooling ultracentrifuge Model- cBL24) at 8000rpm for 15 min at 4°C to separate cells. Separated cells were washed twice with sterile distilled water by centrifugation at 8000rpm for 10min. The cell pellet was suspended in Acetone (MERK) and heated at 60°C water bath for 20 minutes with intermediate vortexing followed by centrifugation at 8000rpm for 10 min at 4°C. The absorbance of the pigmented extract was measured at its optical density at 480nm by using Agilent spectrophotometer (carry -60) and by using the standard graph the concentration of the carotenoid produced in g/25ml of media was calculated. Biomass was determined in terms of wet weight of the pellet in g/25ml of media.

2.3 Plackett-Burman Experimental Design

Glucose**,** peptone, beef extract, meat extract and NaCl were the five medium constituents selected for study. The selection of nutrients for Plackett-Burman Design was done by performing the optimization by one-at -a -time approach. The Plackett-Burman Design for five variables were used to evaluate the relative importance of various nutrients for carotenoid production in shake flask culture and experimental design was prepared by with the help of software MINITAB 14 trial. In table No. 1, each row represents an experiment and each column represents a different variable. For each nutrient variable two different concentration high (+) and low (-) was tested Table-2

2.4 Shake flask fermentation.
All experiment has been carried out in duplicates in 100ml Erlenmeyer Flask containing 25ml media as per experimental design. The medium pH was adjusted to 9.5 which is optimum pH for carotenoid production optimized by one factor-at-a-time analysis (Dhere Deepti D. *et al*; 2017) and flask was autoclaved at 10 psi for 40 minutes. Finally, each flask was inoculated with 1% inoculum Table 2.

2.5 Determination of total carotenoids
The absorbance of crude extract for each experiment was measured by using spectrophotometer. The concentration of total carotenoids was estimated by the absorbance at 480, using the equation given by Davis (1976). The coefficient of absorbance used was that equivalent to Carotenoid (Astaxanthin) $E^{1\%}$1cm=2306.6 for acetone Davis (1976)

$$\text{Total carotenoid content } (\mu g/l) = \frac{A(\text{Volume of sample})10^4}{E^{1\%}_{cm}\ (2306.6)\ \text{dry weight(g)}}$$

Concentration of carotenoids was expressed in terms of total carotenoids (µg/l) and specific of carotenoids (µg g/l).

2.6 Determination of Biomass
Cells were separated by centrifugation with 8000rpm at 4°C for 15 minutes. After extraction of carotenoids the cells were washed with distilled water and centrifuged and cell mass was quantified through drying at 80°C in hot air oven until a constant weight obtained.

2.7 Response surface method- Central composite design
CCD methodology was exploited by using one factor analysis of significant factor to optimize the effective concentration of significant media component. The basal medium i.e. glucose5g/l and NaCl 5g/l with 7 different concentrations of meat extract broth was prepared Table-3. The experimental results of the central composite design were fitted with a second order polynomial equation by a multiple regression technique. The quality of the fit of the second order model equation was expressed by determining the coefficient (R^2) and its statistical significance was determined in terms of P-Value. The response studied were in terms of Carotenoid production (µg/l) by using the standard graph. During this experiment, the biomass (g/l) and total carotenoid content of the cell was also determined separately.

2.8 Statistical analysis
Statistical analysis of the Plackett- Burman design and Response Surface Methodology was done by using statistical software Minitab15, 30 days' trial version and Design Expert 10 (Stat Ease USA) respectively. The statistical testing of the model was done by using ANOVA to test the significance and adequacy of the model. Regression analysis was used to obtain the coefficient of second order polynomial. The data reported is an average of three independent experiments. Significance of the experiment was expressed as $P< 0.05$.

3. Result and Discussion
3.1 Analysis of Plackett -Burman Design.
In the present experimental studies screening of important nutrient parameters influencing carotenoid production by *Paracoccus beibuensis* was analyzed by Plackett- Burman Design. The Plackett-Burman design analysis was aimed to know the significant media component involved in carotenoid production by shake flask fermentation. Maximum carotenoid production was found at 11th experimental trial i.e. 252µg/l, whereas least was found with the 12th experimental trial i.e. 222µg/l under shake flask fermentation (Table- 2). Out of all nutritional parameters under study i.e. Glucose, Beef extract, Peptone, Meat Extract and NaCl; only Meat Extract contributed significantly for carotenoid production, where P-Value for meat extract was found less than 0.05. (Figure-1). Similar studies were carried out by the Chaudhari *et al*;2013 where screening of significant media parameter was done by using Plackett-Burman design for carotenid production from *Planococcus matrimitus* AHJ_2 by shake flask fermentation. Bui Dhinh Hanh Dung *et al*; 2010 Optimized the carotenoid production by *Rhodotorula glutinis* where screening of nutritional parameter was done by using Plackett -Burman design. Many reports have proved the applicability of Plackett-Burman design in optimization of media components for various culture activities (Haque M.A. *et al*; 2012). Appling Plackett-Burman Design we have estimated the variables that caused the growth and carotenoid production by *Paracoccus beibuensis* are not the same. The result showed that meat extract was the most important factor affecting the carotenoid production. The Carotenoid production by *Paracoccus beibuensis* was increased by 2 folds than control medium. The regression equation is-

Carotenoids produced (µg/25ml) = 5.73 - 0.163 Glucose + 0.393 Meat Extract - 0.113 Peptone + 0.0833 Beef Extract - 0.107 NaCl

Table-1. Range of the factors studied in the Plackett-Burman Design and effect estimated for Carotenoid production from results of the Plackett-Burman Design.

Coded Factors	Name	Lower Level (-1)	Higher level (+1)	Effect	T-value	P-value
A	Glucose	0.5	1.5	-0.16333	-1.80	0.122
B	Meat Extract	1	2	0.39333	4.33	0.005
C	Peptone	1	2	-0.11333	-1.25	0.259
D	Beef Extract	1	2	0.08333	0.92	0.394
E	NaCl	0.5	1	-0.05333	-0.59	0.579

Factorial Design

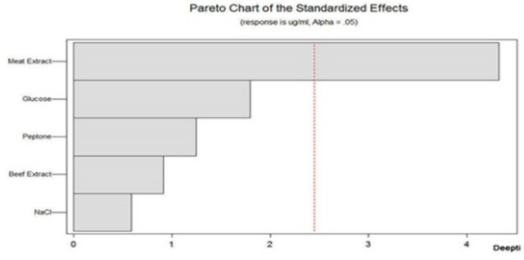
Plackett-Burman Design, Factors: 5, Replicates: 1
Design: 12, Runs:12, Center pts (total):

Table-2. Plackett-Burman Design matrix for screening medium composition of Carotenoid production by Paracoccus beibuensis.

	Carotenoid produced (µg/l)						
RUN	A	B	C	D	E	Observed	Predicted
1	+	+	+	-	+	247.6	240.8
2	-	-	-	+	+	238.8	239.6
3	-	+	+	-	+	243.2	247.2
4	-	-	-	+	+	237.6	234.8
5	-	+	+	+	-	250.4	252.8
6	+	+	-	+	-	250.8	250.8
7	+	-	+	-	-	227.6	227.2
8	-	-	-	-	-	246.8	238.4
9	+	-	+	+	-	228.0	230.4
10	-	+	-	-	-	250.8	254.0
11	+	+	-	+	+	252.4	248.8
12	+	-	-	-	+	222.0	229.6
					Maximum	252.4	254.0
					Minimum	222.0	227.2
					Mean	237.6	238.0

Legend- (+) high value, (-) low value

Figure-1. standardized Pareto Chart for Carotenoid Production using Paracoccus beibuensis



3.2 Response Surface methodology
This is best method for optimization of fermentation process is

response surface methodology (RSM). This process is helpful for demining the optimum concentration and also give the information necessary to design a process. The second order polynomial equation was used to correlate the independent process variable, A with Carotenoid production. In present study second order polynomial coefficient for each term of equation was determined through multiple regression analysis using the Design Expert 10 Trial Version (StatEase USA). The design of the experiment and respective experimental yields are given in Table-3. The results obtained was analyzed by using ANOVA i.e. analysis of variance suitable for experimental design. The results are shown in Table- 4. The model F- Value of implies that model is significant. There is only 0.20% chance that a "Model F-Value" to be large which could be due to noise. Model F-Value was calculated as ratio of mean square residual. Model P-Value (Prob >F) is very low (<0.05) This indicated the significance of model. The P-Value less than 0.05 indicates the significance of the model terms. The coefficient estimates and corresponding P-Values suggests that Meat Extract used in study A and A^2 are the significant model terms. Other interaction was found to insignificant. The corresponding second -order response model that was found after analysis for regression was-

Carotenoids produced (µg/l) =+281.43+1047.85*A-346.84*A^2

Where A= Meat extract and A^2 = Meat extract^2

Similar studies were done by Masoud Hamidi *et al*; (2014) for optimization of total carotenoid production by *Halorubrum* sp. TBZ126 where CCD method was applied to find optimal level of various factors to improve the cell growth and total carotenoid production. Maldonade (2003) evaluated the effects of the composition of the medium (glucose, yeast extract, $MgSO_4$, KH_2PO_4) in production of carotenoids by *Rhodotorula mucilaginosa* 137 where yeast extract was only variable that most influenced the total carotenoids with concentration 745µg/l with 15g/l yeast extract and 20g/l glucose. In recent study the significant media component is meat extract which influence significantly on carotenoid production. The optimum concentration of meat extraction for highest yield is 15g/l.

Table-3 RSM-CCD- one factor analysis for carotenoid production

Responses				
Run	Meat extract (%)	Biomass (g/l)	Carotenoids produced (µg/l)	Cellular Carotenoids (µg/l)
1	0.5	15	728	508
2	2.5	14	1008	508
3	1	9	728	788
4	1.5	14	1112	892
6	0.5	13	690	470
7	2.5	10	912	492

Table -4 Analysis of variance (ANOVA) for one factor analysis for carotenoid produced

Source	Sum of Squares	Df	MS	F-Value	P-Value	
Model for volumetric carotenoid produced						
Model	1.485	2	74233.40	28.03	0.0044	Significant
A-Meat Extract	242.00	1	242.00	0.091	0.7775	
A2	1.482	1	1.482	55.97	0.0017	
Residual	10594.06	4	2648.51			
Lack of fit	8947.56	2	4473.78	5.43	0.1554	Not Significant
Pure error	1646.50	2	823.25			
Total	1.591	6				

Table-5 RSM-CCD- one factor analysis for carotenoid produced

Std. Dev.	51.46	R-Squared	0.9334
Mean	849.86	Adj R-Squared	0.9001
C.V. %	6.06	Pred R-Squared	0.8200
PRESS	28636.26	Adeq Precision	10.512
-2 Log Likelihood	71.12	BIC	76.96
		AI Cc	85.12

3.3 Validation of experimental model

The statistical model was validated with respect to carotenoid production under the condition predicted by the model containing Meat extract (15g/l) as a significant component along with glucose (5g/l) and NaCl (5g/l) as a basal media. The media was inoculated with 1% inoculum and incubated in shaking incubator adjusted at 30^{0}C and 120rpm for three days in white light. The production of carotenoid obtained experimentally using the above medium was 1112±50 µg/l, which is in correlation with the predicted value of 1072.83 µg/l by RSM regression study. An overall 5-fold enhancement in carotenoid production was obtained due to optimization. Similar udies for Lutein production was done by Somnath D.S. *et al*; (2010)

4. Conclusion

The results obtained indicates an important side of optimization of carotenoid production process from bacteria *Paracoccus beibuensis* using statistical methods. The yield of carotenoid was increased significantly from average 237 µg/l to 1112±50 µg/l. The RSM was fairly accurate in predictive modeling and media optimization. The experimental studies and results obtained suggests there is a relation between media component and carotenoid yield can reasonably approximated by quadratic non-linearity. The fit of the model was also expressed by the coefficient of determination R^2 which was found to be 0.9356, indicating that 93.56% of variability in the response could be explained by the model. The "Pred R-squared" of 0.8298 is the reasonable agreement with "Adj R-Squared" of 0.9001 "Adeq Precision" measures the signal to noise ratio. A ratio greater the 4 is desirable. Ratio of 10.51 indicates an adequate signal. This model can be used to navigate the design space. Accordingly, graph was generated for meat extract as significant factor. Thus, the optimal concentration for a component as obtained from the maximum point of model was meat extract 15g/l. The production of carotenoid obtained using optimized medium was 1112µg/l. After RSM optimization 5-fold increase in Carotenoid yield was obtained

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