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Research Article

STATISTICAL OPTIMIZATION OF MEDIUM COMPONENTS FOR CAROTENOID PRODUCTION USING AN INDIGENOUS ISOLATE *RHODOCOCCUS KROPENSTEDTII*

Simran R. Lilwani[1], Sonal U. Patil[2], Parvathi J.R.[3], Madhavi R. Vernekar*[1]

[1]*School of Biotechnology and Bioinformatics, D.Y. Patil Deemed to be University, C.B.DBelapur, Navi Mumbai, Maharashtra, India*

[2]*Nilon's Enterprises Private Limited, Baner, Pune, Maharashtra, India*

[3]*Somaiya Institute for Research & Consultancy, SomaiyaVidyavihar University, Vidyavihar, Mumbai, Maharashtra, India*

**Corresponding author: madhavi.vernekar@dypatil.edu*

ABSTRACT

Carotenoid's functional and qualitative applicability in the food, cosmetic and pharmaceutical industries creates a demand for new carotenoid producers. In the present work, indigenously isolated *Rhodococcus kropenstedtii*, a non-photosynthetic bacteria producing a range of carotenoid pigments, was used. Statistical technique involving one-factor-at-a-time and response surface methodology were adopted to acquire the best medium for carotenoid production by *R. kropenstedtii*. The one-factor-at-a-time approach helped in the initial screening of media components while response surface methodology (RSM) involving four factors at three different levels determined the optimum values of the screened components for maximum carotenoid production.The optimal combination of media components included glycerol-8.75 g/L, beefextract-20g/L, ammonium sulphate- 2g/L and magnesium chloride-4g/L that gave maximum production of 0.38 µg/g of carotenoid, which was 2.1 fold more than the basal medium.

Keywords: Carotenoids, *Rhodococcus kropenstedtii*, Fermentation, Response Surface Methodology (RSM).

1. INTRODUCTION

Carotenoids are C40 isoprenoids ranging from colorless to yellow, orange and red, widely distributed in a number of bacteria, algae, fungi, and plants. They are bioactive molecules possessing several clinical properties like; antioxidant, antitumor, heart disease prevention agents, precursors of vitamin A and enhancers of *in vitro* antibody production [1]. Hence, they find wide usage as dyes and functional components in the food, pharmaceutical and cosmetic industries [2].

Even though the carotenoids' market is extremely fragmented, it has witnessed a significant advancement in the last few years, which is anticipated to remain in future [1]. Satisfying the market demand of carotenoids from plant sources can be challenging due to limitations like low concentration, instability, difficulty in extraction, etc. [2] thus, creating dependency on chemical synthesis [3]. Synthetic carotenoids have stability and operational advantages, but the growing awareness of their harmful effects as irritants and carcinogens [4-6] limits their use. Recently, microbial carotenoids are gaining a lot of importance as they are readily accessible and reproducible than plants, with no seasonal-dependence [1]. A more detailed inquisition to evaluate the real potential and availability of these alternative sources of carotenoids has thus become the need of the hour.

Most of the studies in the past have been focused on carotenoids from microorganisms such as Zeaxanthin by *Flavobacterium. sp.*[7], *Synechocystis. sp.*[8], *Microcystis aeruginosa* [9] and *Spirulina* [10], Astaxanthin by *Phaffia rhodozyma* [11], and *Haematococcus pluvalis* [12] Canthaxanthin by *Gordonia jacobeae*[13], β-cryptoxanthin by *Brevibacterium linens* [14] and β- carotene by *Blakeslea trispora* [15]. Owing to diverse microbial resources, there is a necessity for expanding the spectrum of carotenoid producers and tapping their carotenoid producing potential. The bacteria belonging to the genus *Rhodococcus* are non-photosynthetic and are known to produce different types of carotenoid pigments [16, 17]. However, only a few *Rhodococcus* species have been explored so far in terms of their carotenogenic potential. The production of carotenoids in these bacteria is influenced by various physio-chemical factors such as the

composition of the fermentation medium, temperature, agitation speed and aeration [18]. Hence, screening of the media components and their optimization is necessary to improve the carotenoid yield.

In the present work, *Rhodococcus kropenstedtii* was explored for its ability to produce natural high-value carotenoids. *R. kropenstedtii* is an orange-red pigmented, gram-positive actinobacterium which was reported in 2006 by Maliyaraj *et al*. [19]. Since then, the organism has remained unexplored, and there are very few published reports [20, 21] on this species of *Rhodococcus*. Hence, the main objective of the present work was to investigate the carotenogenic potential of *R. kropenstedtii* and improve its carotenoid production by optimizing the fermentation medium using one factor at a time and response surface methodology. Based on an extensive literature survey, the authors would like to highlight that optimizing fermentation medium using the statistical approach for carotenoid production by *R. kropenstedtii* has not been yet reported.

2. MATERIAL AND METHODS

2.1. Chemicals

All the chemicals were purchased from Sisco Research Industries (SRL) Pvt. Ltd and Hi-Media Laboratories Pvt. Ltd, Mumbai and were of the highest purity available.

2.2. Microorganism and Inoculum preparation

Rhodococcus kropenstedtii (Accession No. MH715196) used for the study was isolated from a sediment sample of Rajapur hotspring, Ratnagiri-Maharashtra, India. The spectral scanning and qualitative test of the reddish-orange pigment produced by this strain indicated the presence of carotenoids. The culture was maintained in Tryptic Soy Agar (TSA) medium at 4°C and sub-cultured regularly. For seed culture preparation, slants grown at 37± 2°C for 4 days were used for inoculation into a basal medium (Pancreatic digest of casein-17g/L, Peptone-3g/L, NaCl- 5g/L, K_2HPO_4-2.5g/L, and Dextrose-2.5g/L, pH-7.2). Shake flask cultures of organism was carried out at 37±2°C with continuous agitation at 110 rpm for 96 h in 100 ml Erlenmeyer flask containing 25 ml medium.

2.3. Fermentations

Experiments were performed in 100 ml Erlenmeyer flasks with 25 ml of basal medium. 5% $^v/v$ inoculum (O.D=1.0) was inoculated in 100 ml Erlenmeyer flask containing 25 ml basal medium incubated at 37±2°C with continuous agitation at 110 rpm for 96 h. The biomass obtained after 96h was used for extraction and quantification of carotenoids. After extraction, the cell pellet was dried at 37±2°C to obtain the dry biomass weight. All experiments were carried out in triplicates.

To study the growth and pigment production profile of *R. kropenstedtii,* 5% $^v/v$ inoculum (O.D=1.0) was transferred to 25 ml basal medium and incubated at 37 ± 2°C on a rotary shaker (110 rpm). After every 24 h, carotenoid yield (μg/g) and dry weight of the biomass (g) were estimated. The experiment was carried out in triplicates and was terminated at the death phase.

2.3.1. Screening of media components using one-factor-at-a-time (OFAT) approach

To investigate the effect of carbon source on carotenoid production, glucose was substituted with seven different carbon sources viz; glycerol, starch, lactose, maltose, cellulose, sucrose and sorbitol. The basal medium with glucose was used as control.

Combination of organic and inorganic nitrogen source are beneficial for increasing the biomass as well as carotenoid yield [22]. Hence for optimization of the nitrogen source, two studies were conducted. In the first study, casein hydrolysate from the basal medium was replaced with different organic nitrogen sources such as yeast extract, beef extract, soy peptone, and protease peptone. In the second study, peptone from the basal medium was replaced with inorganic nitrogen sources such as urea, ammonium nitrate, ammonium sulphate and sodium nitrate.

To study the effect of mineral salts, sodium chloride in the basal medium was replaced with different salts such as magnesium chloride, potassium chloride, and calcium chloride, while the medium with sodium chloride was considered as control.

2.3.2. Optimization of screened components by RSM

Design Expert 7.0 was employed for RSM. The complete experimental plan of D-optimal design was set up and measured in triplicates in 25 experimental trial runs. The experimental set up in the form of coded and uncoded levels of factors is depicted in table 1. The biomass yield (g) and carotenoid yield (μg/g) were studied as response variables. The resultant data was fitted into second order polynomial equation and the coefficients were calculated and analysed. After the analysis of data, additional experimental runs with optimum values of variables were performed to check the validity of model.

Table 1: Experimental layout employed in D-optimal mixture design

Run Order	Beef Extract (g/L)		Glycerol (g/L)		Ammonium sulphate (g/L)		Magnesium Chloride (g/L)	
	Coded value	Uncoded value	Coded value	Uncoded value	Coded value	Uncoded value	Coded value	Uncoded value
1	-1.000	5	1.000	10	1.000	4	-1.000	4
2	1.000	20	1.000	10	-1.000	2	-1.000	4
3	-1.000	5	1.000	10	1.000	4	-1.000	4
4	1.000	20	-1.000	2	1.000	4	1.000	6
5	-1.000	5	-1.000	2	-1.000	2	1.000	6
6	-1.000	5	0.223	6.89	0.204	3.2	0.250	5.25
7	1.000	20	-1.000	2	-1.000	2	0.025	5.02
8	0.007	12.55	1.000	10	0.020	3.02	-0.103	4.9
9	1.000	20	-1.000	2	1.000	4	1.000	6
10	-1.000	5	1.000	10	1.000	4	1.000	6
11	-1.000	5	1.000	10	-1.000	2	-0.117	4.88
12	0.501	16.25	-0.155	5.38	-0.143	2.86	-0.938	4.06
13	0.238	14.29	-0.243	5.03	-0.250	2.75	1.000	6
14	1.000	20	-1.000	2	1.000	4	-1.000	4
15	1.000	20	-1.000	2	-1.000	2	0.025	5.02
16	-1.000	5	-1.000	2	1.000	4	-0.117	4.88
17	1.000	20	1.000	10	-1.000	2	1.000	6
18	-1.000	5	-0.117	5.53	-0.117	2.88	-1.000	4
19	.0.411	9.42	-1.000	2	-1.000	2	-1.000	4
20	0.746	18.09	0.813	9.25	0.746	3.75	-1.000	4
21	-1.000	5	-1.000	2	-1.000	2	1.000	6
22	0.006	12.55	0.006	6.02	1.000	4	-0.019	4.98
23	1.000	20	1.000	10	-1.000	2	-1.000	4
24	-0.540	8.45	-0.969	9.88	-0.457	2.54	1.000	6
25	1.000	20	1.000	10	1.000	4	0.259	5.26

2.3.3. *Statistical analysis*

Commercial statistical package: Design Expert 7.0 (State-Ease Inc., Minneapolis, MN, USA) was used to perform Analysis of Variance (ANOVA) test to determine the significance at different levels. Response surface plots were generated using the same software.

2.4. Extraction and quantification of carotenoids

Extraction of carotenoids produced by *R. kropenstedtii* was carried out by solvent extraction method using ethanol as a solvent [23]. Culture broth (96 h old) was centrifuged at 4,500 rpm for 10 min. Supernatant was discarded and the cell pellet was washed twice with distilled water and centrifuged. For pigment extraction, the cell pellet was suspended in chilled ethanol and then centrifuged at 4,500 rpm for 10 min. The supernatant containing the pigment was collected and the pellet was re-suspended in ethanol, mixed and re-centrifuged. This procedure was repeated till the pellet was colorless. At the end of extraction, the colored supernatant was pooled and quantified at 450 nm using the formula [24]; Carotenoids content (μg/g) = $A \times V (mL) \times 10^4 / A^{1\%}_{1\,cm} \times P(g)$

Where, A = Absorbance; V = Total extract volume; P = sample dry weight; $A^{1\%}_{1cm}$=2620 (β-Carotene Extinction Coefficient in ethanol).

3. RESULTS AND DISCUSSION

An indigenous isolate *Rhodococcus kropenstedtii* (Accession No.MH715196) isolated from a sediment sample of hotspring was used in the present work. Initial studies were carried out to study the growth and pigment production profile of the *R. kropenstedtii* using the basal medium. In batch culture, cells exhibited a lag phase of 24 h, followed by exponential growth up to 48 h and a stationary phase, which extended until 96 h followed by a death phase (Fig. 1). The accumulation of biomass and increase in the carotenoid yield started after 48 h of

growth and maximum titre of 0.17µg/g was observed at 96 h.

3.1. Media Optimization Studies

3.1.1. Screening of media components using one-factor-at-a-time (OFAT) approach

The one factor at a time approach for media optimization consist of changing one independent variable while keeping the others fixed at certain levels. Carotenoid biosynthesis is influenced by carbon source, as acetyl co.A, a product of carbohydrate catabolism act as a precursor for isoprenoid synthesis which is the backbone for carotenoid structure [25]. In this study, seven different carbon sources i.e glycerol, mannose, lactose, sucrose, cellulose and sorbitol, were evaluated.

Fig. 2 illustrates the effect of different carbon sources on carotenoid production by *R. kropenstedtii*. Starch gave the lowest yield of 0.0005µg/g. This might be due to the lack of amylase resulting in poor utilization of starch by the organism. Glycerol was the best utilized carbon source and gave a maximum yield of 0.37µg/g carotenoid compared to the glucose (control) with 0.17µg/g of carotenoid yield. These results are in accordance to literature where other *Rhodococcus* species: *R. equi, R. rubroperctinctus, R. aichensis, R. sputi, R. chubuensis, R. obuensis, R. bornchialis, R. roseus, R. rhodocrous, R. rhodnii* and *R. terrae* have been reported to utilize glycerol as carbon source for the production of gamma carotene like substance [18].

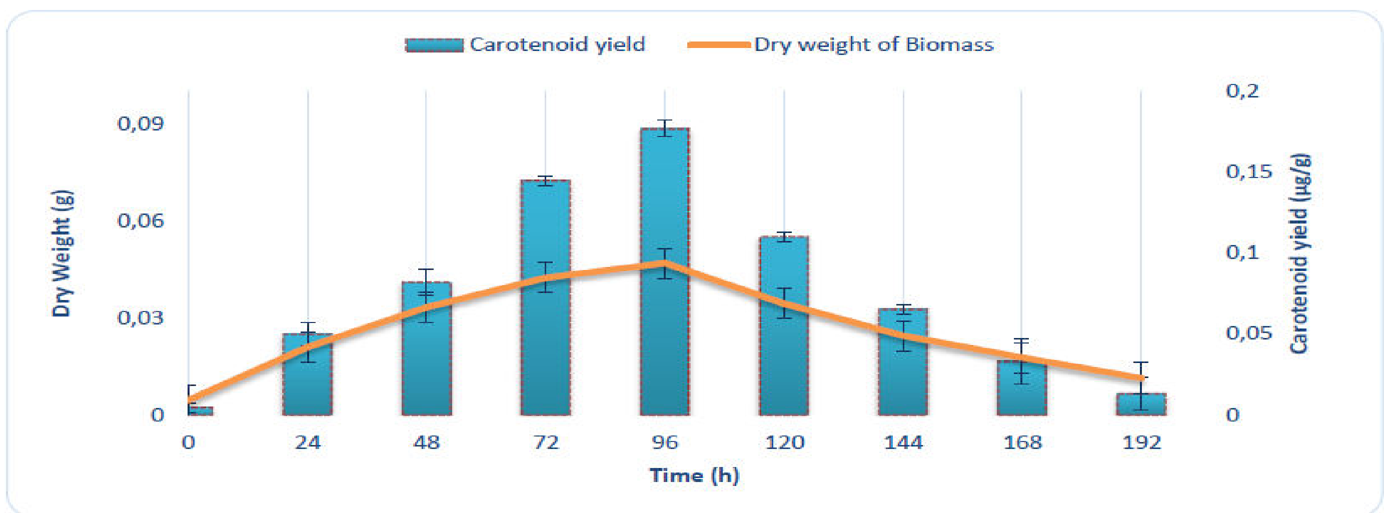

Fig. 1: Growth and pigment production profile of *Rhodococcus kropenstedtii*

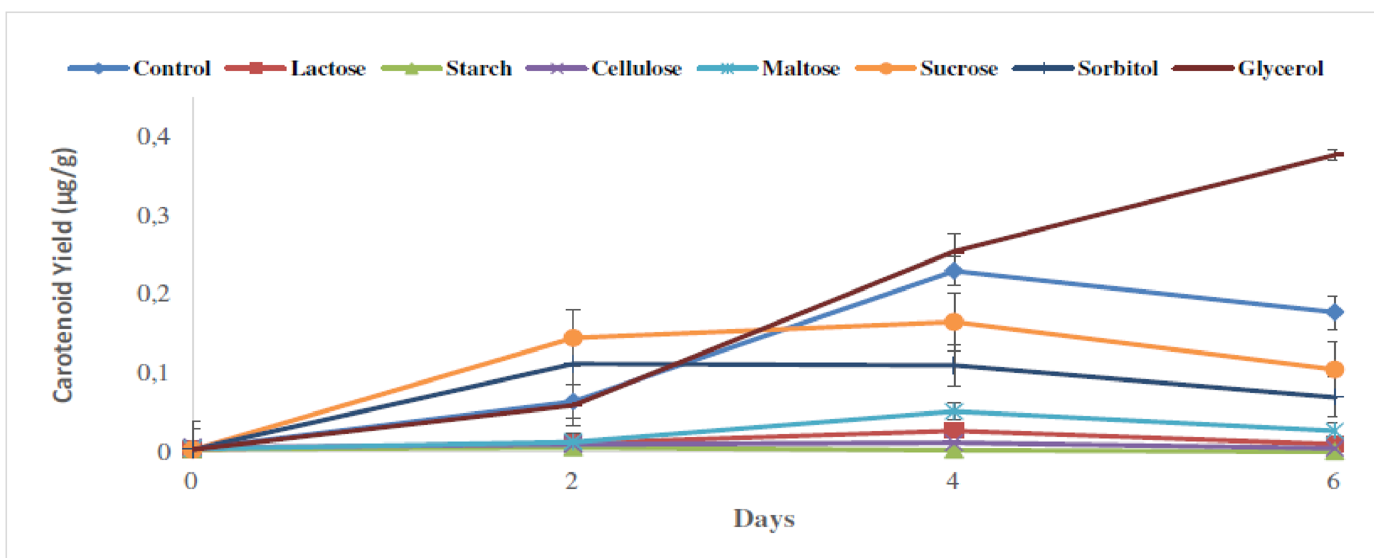

Fig. 2: Effect of carbon sources on carotenoid production by *Rhodococcus kropenstedtii*

Nitrogen source plays an important role in biomass production [22]. Therefore, it was thought that a combination of organic and inorganic nitrogen sources would be beneficial for increasing biomass and carotenoid yield. Fig. 3 and Fig. 4 depicts the results obtained using organic and inorganic nitrogen sources respectively on carotenoid production by *R. kropenstedtii*. Out of the five organic nitrogen sources investigated in this study, beef extract gave a maximum yield of 0.35μg/g of carotenoid as compared to control i.e, casein hydrolysate 0.18μg/g. While among the inorganic nitrogen sources used, ammonium sulphate gave higher yield of 0.26 μg/g of carotenoid compared to control 0.18μg/g. Hence, beef extract and ammonium sulphate were selected as the best nitrogen source. Macronutrients such as potassium, calcium, magnesium etc. are found to have several biological functions thereby affecting the growth of organism [26] and carotenoid production. Hence, the effect of different mineral salts on carotenoid production was assessed. Fig. 5 shows the effect of different mineral salts on carotenoid production. It was observed that the addition of magnesium chloride gave carotenoid yield of 0.33μg/g as compared to control i.e sodium chloride 0.17μg/g. Magnesium ions are known to be involved as cofactors for different enzymes and thus, might be responsible in increasing the carotenoid yield. The next step was to study the combined effect of the selected four independent variables viz; Glycerol, Beef extract, Ammonium Sulphate and Magnesium chloride using RSM.

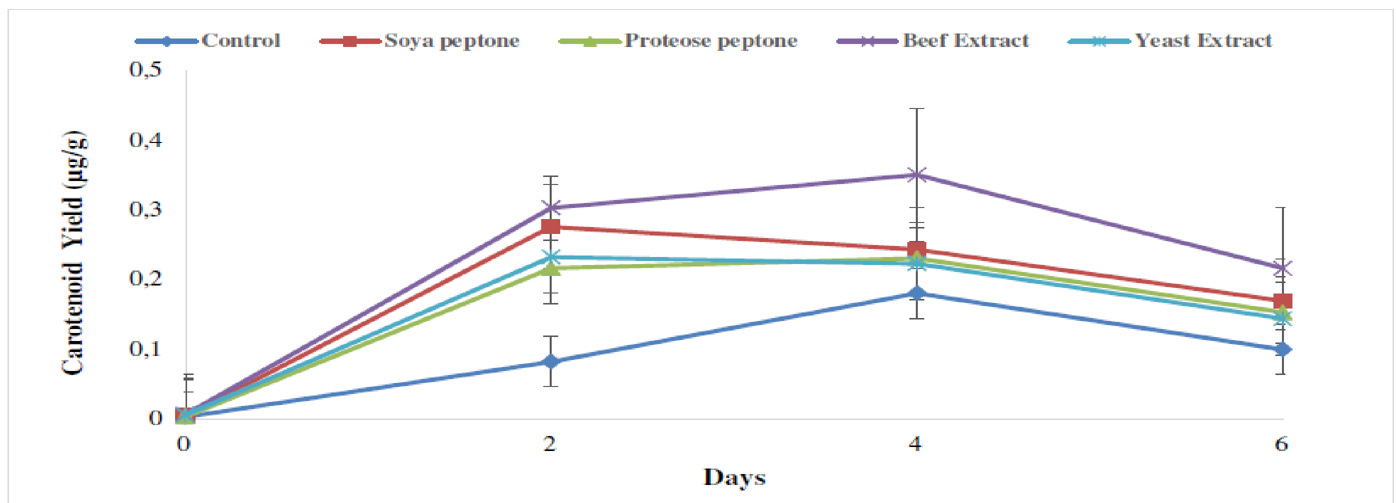

Fig. 3: Effect of organic nitrogen sources on carotenoid production by *Rhodococcuskropenstedtii*

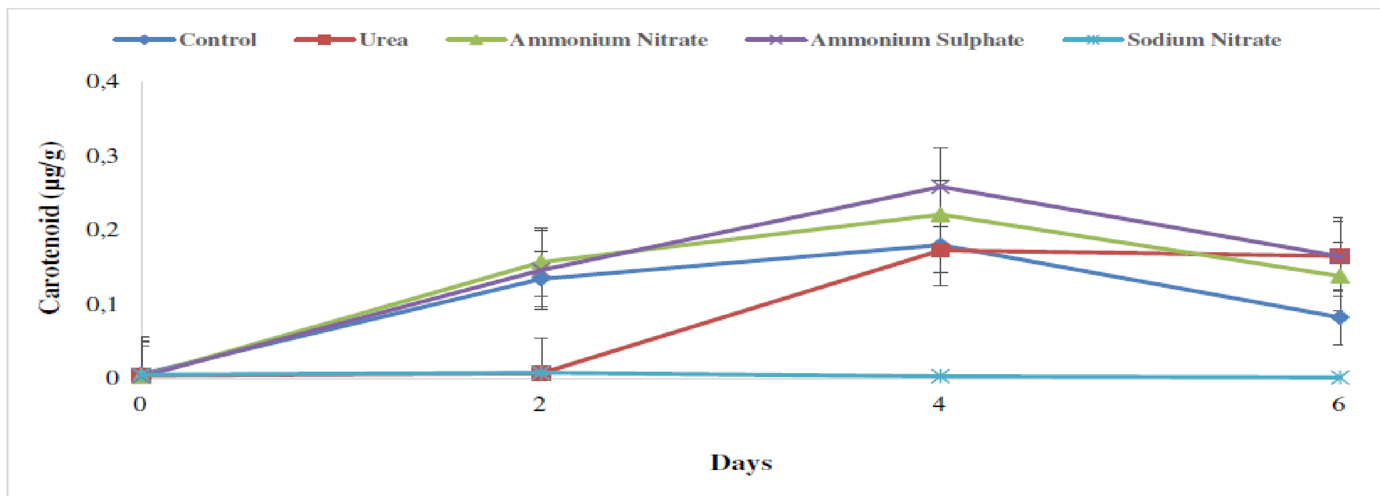

Fig. 4: Effect of inorganic nitrogen sources on carotenoid production by *Rhodococcuskropenstedtii*

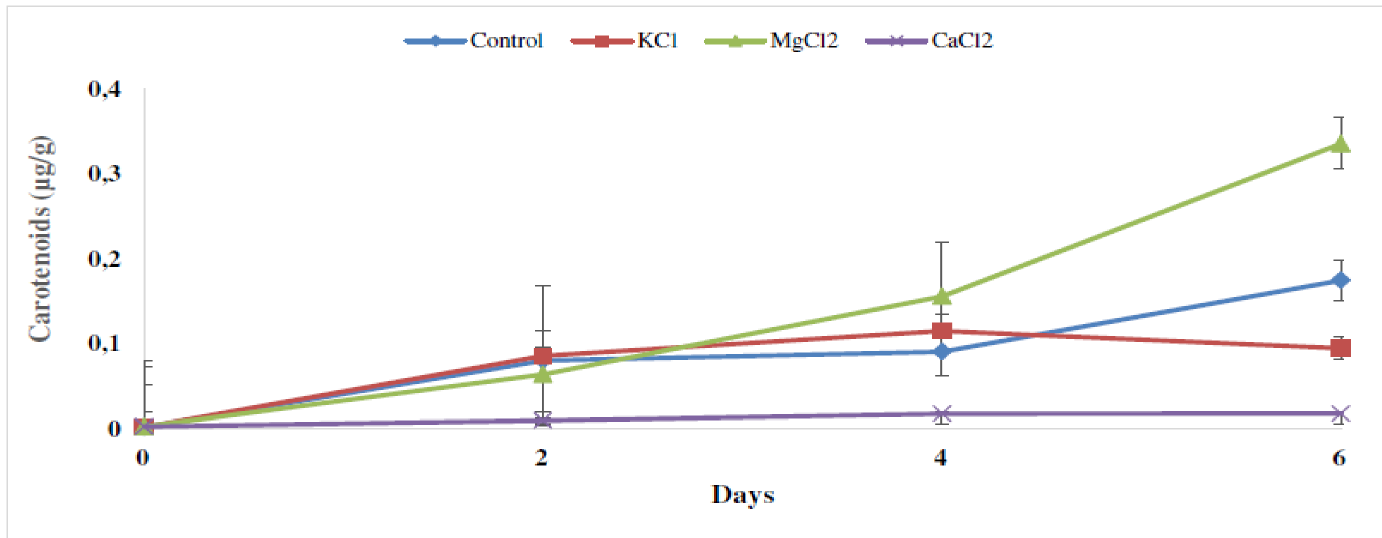

Fig. 5: Effect of mineral salts on carotenoid production *Rhodococcus kropenstedtii*

3.1.2. Optimization of screened components by RSM

RSM generates different combinations of experimental variables by changing them concurrently and thus helps in obtaining a suitable combination required for optimum production of desired product. In the present study, D-optimal RSM experimental design was used to generate responses in terms of biomass dry weight (g) and carotenoid yield (µg/g). The results were analysed by using ANOVA test applicable to the experimental design and the results are represented in table 2. The Model F-value of 19.94 for biomass yield and 22.22 for carotenoid yield suggests that the model is significant. The "Lack of Fit" value of 1.89 for biomass yield and 0.59 for carotenoid yield indicates the lack of fit is not significant relative to the pure error. Non-significant lack of fit is good and suggests that the model fits well. Apart from significant model F- value and non-significant lack of fit, the coefficient of determination R^2 value of 0.96 for both the responses also implies that the model is well fitted to the experimental data and the distance between the predicted and the experimental values would be less.

The prediction of biomass and carotenoid yield was calculated using the equation derived by design expert software. This equation in terms of coded factors was used to make predictions about biomass and carotenoid yield for any given level of each factor and is as depicted below; **Biomass Yield (g)** = + 0.016 + 3.345E-003 *A+4.812E-004 *B-9.226E-004 *C-1.541E-003*D+1.275E-003*A*B-8.895E-004*A*C-2.622E-004 *A * D-2.835E-004 *B*C-9.514E-004 *B*D-1.251E-004 *C*D+2.189E-003 $*A^2$-1.420E-003 $*B^2$+ 4.215E-003 $*C^2$+2.026E-003 $*D^2$

Carotenoid Yield (µg/g) = + 0.14+0.052 *A+ 0.013 *B-0.015*C-0.023 *D+0.023 *A*B-7.792E-003 *A* C-1.815E-003 *A*D-8.800E-003 *B*C-0.011 *B* D+4.953E-004 *C*D+0.028 $*A^2$- 0.034 $*B^2$+0.060 $*C^2$+0.032 $*D^2$

Where, A=Beef Extract B=Glycerol C=Ammonium Sulphate D=Magnesium Chloride

The application of RSM lead to the experiential relationship between biomass/carotenoid yield and media components. The effect of interaction of the four given variables on biomass and carotenoid yield was studied by means of response surface plots against any two independent variables while keeping the other two at their constant level. These response surface plots can thus be used to predict the optimal values for different test variables. The response surface plots for biomass yield (Fig. 6 a) suggest that biomass yield increases with an increase in glycerol and beef extract concentration, but glycerol at very high concentration slightly lowers the biomass yield. The increasing ammonium sulphate and magnesium chloride concentration had no impact on biomass yield (Fig. 6 b). Similar observations can be made for carotenoid yield (Fig. 7a & 7b), thus suggesting that the higher concentrations of beef extract and glycerol whereas lower concentrations of ammonium sulphate and magnesium chloride in combination would be most suitable for the increased carotenoid production.

Table 2: ANOVA terms for D-optimal mixture design

Analysis	Biomass Yield	Carotenoid Yield
F-value	19.94	22.22
Mean	0.021	0.21
Standard Deviation	0.001	0.020
Prob > F	< 0.0001 significant	< 0.0001 significant
Lack of Fit	1.87 not significant	0.59 not significant
R^2	0.9654	0.9689

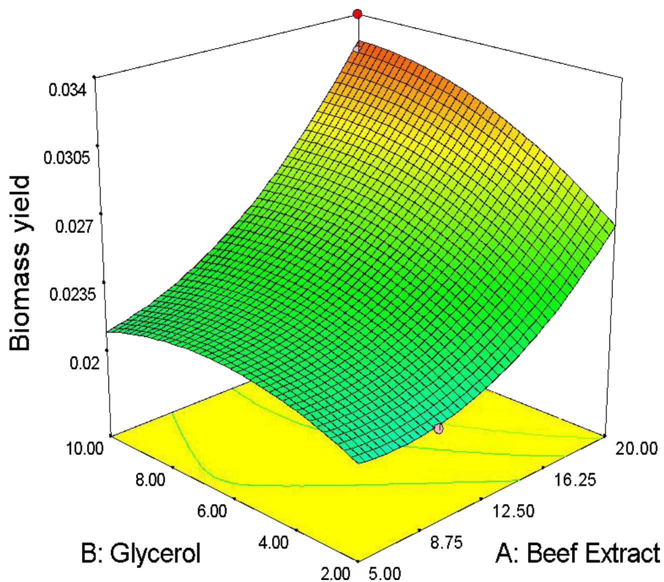

Fig. 6a: Three dimensional surface plot of Biomass yield (g) as a function of Beef Extract and Glycerol

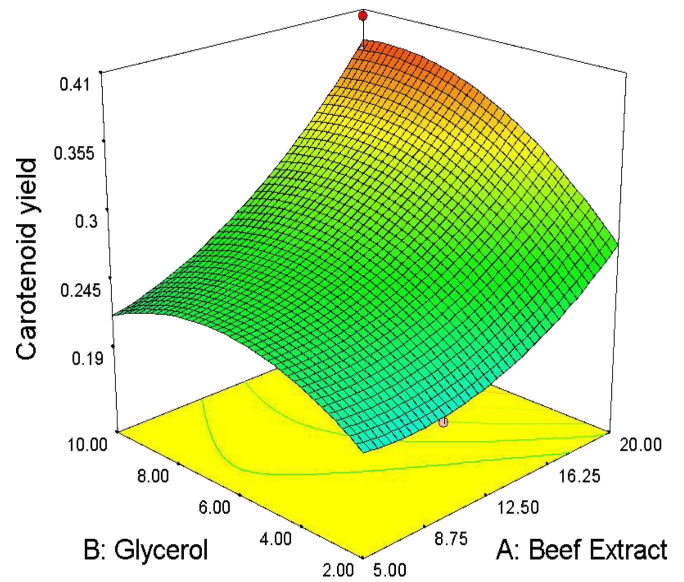

Fig. 7a: Three dimensional surface plot of Carotenoid yield (μg/g) as a function of Beef Extract and Glycerol

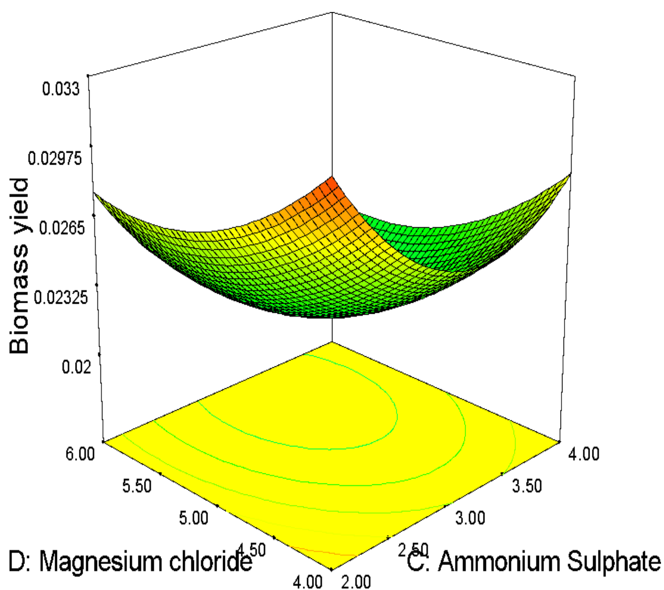

Fig. 6b: Three dimensional surface plot of Biomass yield (g) as a function of Magnesium Chloride and Ammonium Sulphate

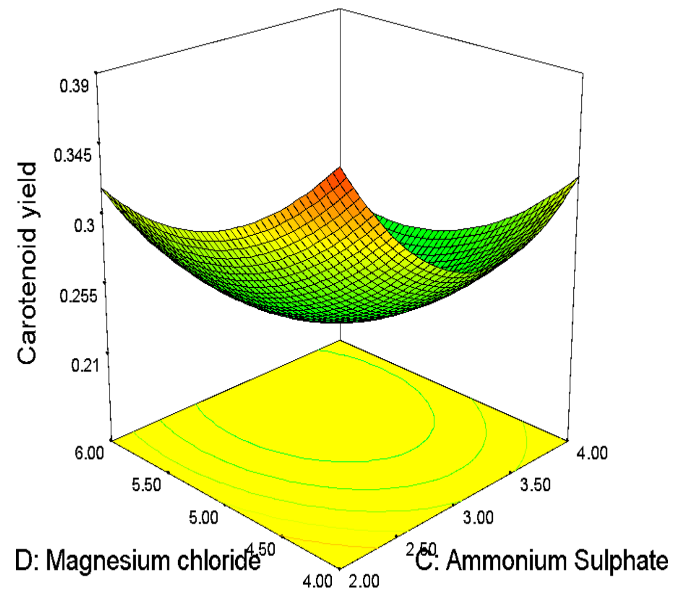

Fig. 7b: Three dimensional surface plot of Carotenoid yield (μg/g) as a function of Magnesium Chloride and Ammonium Sulphate

Validation of the experiment was carried out by conducting additional runs in triplicates under the conditions predicted by the statistical model (Table 3). The optimum concentrations used for the variables were: beef extract (20g/L), glycerol (8.75 g/L), ammonium sulphate (2g/L) and magnesium chloride (4g/L). The maximum production of carotenoids obtained experimentally using the optimized medium was 0.381±0.0057 μg/g which is in correlation with the predicted value of 0.384±0.0009 μg/g by the RSM regression study. The paired t-test with p-value of 0.359 indicate that there is no significant difference between the predicted and experimental values and thus verifying the validity of the model.

Thus, optimized media using RSM yielded the maximum carotenoid production of 0.38 μg/g which was about 2.11 fold higher than un-optimized medium with a carotenoid yield of 0.18μg/g.

Table 3: Optimization of constraints and validation of model

Constraints	Goal	Limits		Model Predicted value	Experimental value	T-test p value
		Lower	Upper			
Beef Extract	Is in range	5	20	0.384±0.0009	0.381±0.0057	0.359
Glycerol	Is in range	2	10			
Ammonium Sulphate	Is in range	2	4			
Magnesium Chloride	Is in range	4	6			
Carotenoid Yield	Maximize	0.105	0.404			

* *Experimental values are represented as mean± SD of three determination*

4. CONCLUSION

The present study aimed at using indigenously isolated *R. kropenstedtii* as a novel carotenoid producer. In the present study screening of media components for carotenoid production by *R. kropenstedtii* was done using one factor at a time approach. The media components influencing the carotenoid production were identified to be beef extract, glycerol, ammonium sulphate and magnesium chloride. Their optimum concentrations were determined using response surface methodology and 2.11 fold increase in carotenoid yield was achieved. Carotenoid yield using this microbial strain is significantly higher; thus this strain could be an attractive source for carotenoid production that has widespread industrial application.

5. ACKNOWLEDGEMENTS

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Conflict of Interest

The authors declare no conflict of interest

6. REFERENCES

1. Ram S, Mitra M, Shah F, Tirkey SR, Mishra S. *Journal of Functional Foods*, 2020; **67:**103867.
2. Saini RK, Keum YS. *Food Chemistry*, 2018; **240:**90-103.
3. Saini RK, Keum YS. *Journal ofIndustrial Microbiology &Biotechnology,* 2019; **46(5):** 657-674.
4. Rodriguez- Amaya DB. *Food Research International,* 2019; **124:**200-205.
5. Ye ZW, Jiang JG, Wu GH. *Biotechnol. Adv.*, 2008; **26:**352-360.
6. Woutersen RA, Wolterbeek APM, Appel MJ, van den Berg H, Goldbohm RA, Feron VJ. *Critical Reviews in Toxicology*, 1999; **29(6):**515-542.
7. Sajilata MG, Singhal RS, Kamat MY. *Comprehensive reviews in food science and foodsafety*,2008;**7(1):**29-49.
8. Lagarde D, Beuf L, Vermaas W. *Applied and environmental microbiology*, 2000; **66(1):**64-72.
9. Chen F, Li HB, Wong RNS, Ji B, Jiang Y. *Journal of Chromatography A*, 2005; **1064(2):**183-186.
10. Toyomizu M, Sato K, Taroda H, Kato T, Akiba Y. *British Poultry Science*, 2001; **42(2):**197-202.
11. Batghare AH, Singh N, Moholkar VS. *Bioresource technology*, 2018; **254:**166-173.
12. Christian D, Zhang J, Sawdon AJ, Peng CA. *Bioresource Technology*, 2018; **256:**548-551.
13. Veiga-Crespo P, Blasco L, Rosa dos Santos F, Poza M, Villa TG. *International Microbiology*, 2005; **8(1):**55-58.
14. Guyomarc'h F, Binet A, Dufossé L.*Journal of Industrial Microbiology and Biotechnology*, 2000; **24(1):** 64-70.
15. Choudhari S, Singhal R. *Bioresource Technology*, 2008; **99(4):** 722-730.
16. Takaichi S, Ishidsu JI, Seki T, Fukada S. *Agric. Biol. Chem.,* 1990; **54:**1931-1937.

17. Tao L, Cheng Q. *Mol. Gen. Genomics,* 2004; **272:** 530-537.
18. Cappelletti M, Presentato A, Piacenza E, Firrincieli A, Turner RJ, Zannoni D. *Applied Microbiology and Biotechnology*, 2020; **12:**1-28.
19. Mayilraj S, Krishnamurthi S, Saha P, Saini HS. *International journal of systematic and evolutionary microbiology*, 2006; **56(5):**979-982.
20. Kang Y, Chen Y, Zhang Z, Shen H, Zhou W, Wu C. *BMC Infectious Diseases*, 2021; **21(1):**1-6.
21. Madhukar CV. *World*, 2021; **10(1):**29-34.
22. Voaides C, Dima R. *Romanian Biotechnol. Lett.* 2012; **17(5):**7570-7576.
23. Handung LKEVM, Surya NPAPA, Radjasa WOK. *Int. Aquat. Res.,* 2017; **9:**61-69.
24. deCarvalho LMJ, Gomes PB, de Oliveira Godoy RL, Pacheco S, do Monte PHF, de Carvalho JL V*et al. Food Research International*, 2012; **47(2):**337-340.
25. Yimyoo T, Yongmanitchai W, Limtong S. *Agriculture and Natural Resources*, 2011; **45(1):**90-100.
26. Bautista-Gallego J, Arroyo-Lopez FN, Durán-Quintana MC, Garrido-Fernandez A. *Journal of Food Protection*, 2008; **71(7):**1412-1421.