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Research Article

ASSESSMENT OF BIO-PROCESS VARIABLES FOR THE ENHANCED PRODUCTION OF CAROTENOID PIGMENT BY SOIL ISOLATE *RHODOCOCCUS KROPPENSTEDTII*

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ABSTRACT. Natural pigments like carotenoids are known for possessing an extensive range of health promoting antioxidant, antibacterial and anticancer properties. *Rhodococcus kroppenstedtii* has the potential to produce an intense red carotenoid pigment. Various factors influencing the pigment production includes nutrient media composition, pH, temperature, aeration, incubation time and inoculum concentration. This study aimed at improving the medium composition for efficient and economical production of pigment. The present research work revealed the influence of various physical parameters such as temperature, pH, inoculum %, aeration, and incubation time as well as nutritional parameters such as sugars, organic-inorganic nitrogen, metal ions and phosphates on the biomass, pigment and pigment production rate in *Rhodococcus kroppenstedtii*. In the present study, optimum cultural parameters were determined to obtain the enhanced production of carotenoid pigment. This research study focuses that the maximum growth and pigment production in *Rhodococcus* was achieved when inoculated with 2% inoculum at 37°C, pH 7.0 with shaking at 120-150 rpm up to 48 hours. Among the various sugars studied as a carbon source maximum biomass and pigment production rate was found with sugar sucrose and mannitol. Studies on the influence of various organic and inorganic nitrogen sources revealed that yeast extract and beef extract among the organic nitrogen and ammonium chloride as an inorganic nitrogen source was found to boost the pigment production in *Rhodococcus kroppenstedtii*. Out of the various metal ions $MgSO_4$ and $CaCl_2$ were found to enhance the pigment production while K_2HPO_4 as the best phosphate source for biomass and pigment production in isolate. When shake flask experiment containing optimized media was conducted, it revealed that two-fold increase in pigment production achieved in optimum physico-chemical nutritional parameters as compared to growth of isolate in basal medium.

Keywords: *Carotenoid pigment, Rhodococcus kroppenstedtii, physico-chemical parameters, optimization.*

INTRODUCTION

Color is the most pleasing attribute of any article. Color is associated with quality and sensory properties of food. Color determines the acceptance of food [1]. Color of the food substance is important to indicate its freshness and safety that are also indices of good aesthetic and sensorial values. In recent years, coloring of food with pigment produced from natural sources is of worldwide interest and is gaining importance [2]. These pigments are looked upon for their safe use as a natural food dye in replacement of synthetic ones because of undesirable market. Various synthetic colors and dyes are extensively used in many fields e.g. in the textile industry, in-the leather, tanning industry, in paper production, in food technology, in agricultural research, in hair coloring, in

pharmaceutical industries but these dyes can cause considerable non aesthetic pollution and serious health-hazards including harmful side effects [3].

It is therefore, essential to explore various natural sources of food grade colorants and their potentials. Natural colors are available in a wide range of colors and are not have any side effects with the substrate in which they are added [4]. These natural pigments can be obtained from two major sources, plants [5] and microorganisms [6]. Bio pigments from the micro-organisms have been preferred over those from plants because of their stability [7] and the availability of their cultivation technology [8, 9] throughout the year. There is worldwide interest generated for the production of pigments from natural sources such as microorganisms [10, 11].

Microorganisms are known to produce a variety of pigments; therefore, they are promising source of food colorants [12, 13]. Microorganism produces pigment like carotenoids, melanin, flavins, monascins, violacein and indigo [14]. Carotenoids are a group of bioactive compounds and are responsible for bright yellow, orange, red pigments of various plants, microorganisms and animals [15] and are widely distributed in nature [16]. These pigments have an important function to act as protective agents against oxidative damage [17]. Recently carotenoids have attracted greater attention due to the beneficial role on human health. There are numerous 750 different types of carotenoids produces by variety of organisms. *Rhodococcus* spp. are one of the antioxidative, antimicrobial carotenoid producing strain as previously reported [18]. There is very little literature available on large scale production of microbial carotenoids and optimum growth conditions to increase the yield.

The present study focuses on the optimization of cultural parameters to achieve the enhanced production of carotenoids from *Rhodococcus kroppenstedtii* isolated from local soil.

MATERIALS AND METHODS

Microorganism

Intense red carotenoid pigment producing bacterial isolate, identified as *R. kroppenstedtii* was isolated from soil sample collected from soyabean field of surrounding local area of Shahada, Maharashtra. Isolate was found to be producing prominent red color pigment on yeast extract mannitol agar plate (YEMA) within 48 hours. Stock culture was maintained on YEMA slant at 4°C and sub-cultured after every 30 days.

Inoculum

For every experiment performed during optimization, 100 µl of 24 to 48 hours pre-grown culture of isolate in yeast extract mannitol broth was used as inoculum. This quantity corresponded to 0.01 absorbance at 620 nm.

Optimization of bioprocess variables

In order to boost or enhance the production of pigment and biomass, various physico-chemical bioprocess parameters, medium composition that influences pigment production were optimized. The parameters mainly studied included nutritional media component such as -various carbon sources, organic and inorganic nitrogen sources [19],

-phosphates, metal ions and physical parameters viz. pH of the medium, incubation temperature, inoculum concentration, aeration, agitation and incubation time.

Strategy adapted for the optimization was to investigate individually the effect of different parameters, through "one variable at a time approach", on pigment production. In each variable, pigment was extracted, absorption was measured spectrophotometerically [20].

Optimization of physical parameters

The effect of various physical parameters viz. different incubation temperature (25^{o}C, 30^{o}C, 37°C and 45°C), pH (3, 5, 7, 9, and 11), incubation time (24, 48, 72, 96 h), inoculum concentration (1, 2, 3, 4, 5 % v/v), Shaking (60, 120, 150, 180 rpm) and static conditions, on growth and pigment production were studied separately by inoculating bacterial culture of *R. kroppenstedtii* for maximum pigment production. The biomass, pigment and pigment production rate were determined separately.

Optimization of nutritional media components

Optimization of nutritional media components [21] was done to standardize the conditions favouring the maximal production of pigment by *R. kroppenstedtii*. Influence of various sugars as a source of carbon on the growth and pigment production was investigated, as carbon rich conditions give rise to more accumulation of pigments. Organism was inoculated in the 100 ml of growth medium containing 1% (w/v) of various sugars viz. maltose, sucrose, mannitol, glucose, fructose, cellobiose, starch, lactose, sorbitol in 500 ml Erlenmeyer flask.

The accumulation of pigment is greatly influenced by level of nitrogen supplemented in production media. Taking into consideration the importance of nitrogen for cell growth, different type of economically feasible inorganic (urea, ammonium per sulphate, ammonium chloride, ammonium sulphate, and ammonium oxalate 1% [w/v]) and organic nitrogen (beef extract, malt extract, peptone, and soya peptone 1% [w/v]) were used to investigate their effect separately on biomass and pigment production.

Influence of various metal ions such as -$MgSO_4$, $MnSO_4$, $CaCl_2$, $FeSO_4$, $ZnSO_4$ and $CuSO_4$ in trace-quantities and various phosphates viz. K_2HPO_4, KH_2PO_4, Na_2HPO_4 and NaH_2PO_4 in a concentration of 0.5 % were supplemented separately for the investigation of their effect on the growth and pigment production by isolate *R. kroppenstedtii*. In each experiment basal growth medium was maintained as a control.

Scale-up in shake flask

Shake flask experiment was designed with optimum physical and nutritional conditions to investigate the biomass and pigment production. Initially scale-up was carried out in 2 L capacity flask containing 500 ml of the optimized growth medium obtained after studying individual parameter. Aliquot of culture was withdrawn aseptically after every 4 hour and absorbance, biomass as well as pigment production were determined.

Analytical methods

Estimation of growth, biomass/Dry weight and pigment production

For growth measurement, absorbance was measured at 620 nm on UV-Vis spectrophotometer (Shimadzu, UV mini 1240) against uninoculated broth as a control. For estimation of biomass, 100 ml culture broth was centrifuged at 10,000 x g for 20 minutes; pellet obtained was washed twice with sterile distilled water and allowed to dry to remove complete moisture till constant weight was obtained [22]. Dry weight of cell mass was expressed as g/100 ml growth media [23]. Dry biomass obtained after every optimization experiment was subjected to extraction procedure [24] pigment was expressed as $mg.g^{-1}$ of biomass. Pigment production rate was calculated from biomass and total pigment. Each optimization experiment was carried out in triplicates and the mean values with SD were expressed in results.

RESULTS AND DISCUSSION

The intense red pigment produced by *R. kroppenstedtii* belongs from the group of carotenoids having potential antioxidant and antibacterial activity. The present research work focussed on the formulation of production medium for maximum production of carotenoid pigment by the isolate.

Optimization of physical parameters

Influence of pH on the growth and pigment production.

The pH of the growth medium exhibit pronounced effect on the biomass and pigment production of organisms [25]. Initial pH of the media plays a very crucial role in the synthesis of secondary metabolites like pigment production by the organisms [26]. The results of the effect of different pH on growth and pigment production in *Rhodococcus* (Table 1) revealed that, with raising pH, pigment production and growth increases but maximum growth, and pigment production rate was found to be produced at pH 7.0 (0.51g/100ml, 0.381 $mg.g^{-1}$ 0.747 $mg.g^{-1}$). Further increase of the pH resulted in reduction of growth and pigment production by isolate. Acidic and alkaline conditions were found to be not suitable for pigment production.

Table 1. *Influence of pH on pigment production rate*

pH	Cell dry weight (g/100ml)	Carotenoid Pigment ($mg.g^{-1}$)	Pigment production rate ($mg.g^{-1}$)
3	0.18	0.09	0.428
5	0.202	0.12	0.632
7	0.51	0.381	0.747
9	0.332	0.21	0.594
11	0.28	0.12	0.428

Values are the means of triplicates. Standard deviation 2-3%

Influence of incubation temperature

Incubation temperature is one of the important parameters influencing the growth and pigment production in organisms. Temperature of the growth medium exerts considerable effect on pigment formation in organism. The data obtainable on the effect of temperature revealed that, maximum growth and pigment production was observed at temperature 30^{o}C followed by at 37°C, and again rise in the temperature up to 45^{0}C growth and pigment production decreases (Table 2).

According to the literature, although the bacteria grow over a broad range of temperature, but incubation temperature which favoured the best growth and pigment production is 30-37^{0}C [27, 28]. Reduction in the pigment production at elevated temperatures is well documented previously by Sundaramoorthy *et al.*, 2009 [29, 30].

Table 2. *Influence of Temperature on the pigment production rate*

Temperature	Cell dry weight (g/100ml)	Carotenoid Pigment ($mg.g^{-1}$)	Pigment production rate ($mg.g^{-1}$)
25	0.337	0.23	0.682
30	0.58	0.402	0.693
37	0.589	0.418	0.709
45	0.31	0.19	0.612

Values are the means of triplicates. Standard deviation 2-4%

Influence of incubation time on pigment production

Results of the influence of incubation time, shown in Table 3 revealed that cell biomass and prominent red pigment production, in *Rhodococcus* was observed at the end of 48 hours. The yield obtained was 0.40g/100ml and pigment 0.35 $mg.g^{-1}$ and pigment production rate was found to be 0.875 $mg.g^{-1}$ of cell mass.

Table 3. *Influence of incubation time on the pigment production rate*

Incubation time	Cell dry weight (g/100ml)	Carotenoid Pigment ($mg.g^{-1}$)	Pigment production rate ($mg.g^{-1}$)
24	0.231	0.162	0.701
48	0.242	0.212	0.876
72	0.40	0.35	0.875
96	0.42	0.3	0.714

Values are the means of triplicates. Standard deviation 3-4%

Influence of aeration on pigment production

Microorganisms can improve transfer of substrates and oxygen in aerobic conditions [31]; hence aeration of the growth medium is important for successful growth of aerobic organism. The results of the influence of aeration on the biomass and pigment production revealed that maximum growth and pigment production rate (Table 4) obtainable at 120 rpm was 0.866 $mg.g^{-1}$ followed by at 150 rpm 0.857 mg g^{-1}. Increase in the aeration rate

after 150 to 180 rpm, resulted in the decrease in the biomass and pigment production in isolate. Vigorous aeration was found to be inhibitory for the growth of isolate. Similarly very minute pigment production rate (0.5 $mg.g^{-1}$) was observed at static condition. Conclusively it can be stated that maximum growth of the organisms can be achieved at 120-150 rpm and aerobic conditions yield more growth as compared to static condition.

Table 4. *Influence of aeration on the pigment production rate*

Aeration RPM	**Cell dry weight (g/100ml)**	**Carotenoid Pigment ($mg.g^{-1}$)**	**Pigment production rate ($mg.g^{-1}$)**
0	0.05	0.025	0.5
60	0.1	0.05	0.7
120	0.30	0.26	0.866
150	0.28	0.24	0.857
180	0.23	0.15	0.652

Values are the means of triplicates. Standard deviation 4-6%

Influence of inoculum concentrations on the growth and pigment production.

Effective inoculum development is one of the key programmed during industrial fermentations. Properly developed inoculum provide contamination free homogeneous environment for the production of fermentation products. Optimum level of inoculum concentration that supports maximum growth, biomass and pigment production rate was found to be 2% (0.251gm/100ml, 0.72 $mg.g^{-1}$). Inoculum size was found to be directly proportional to biomass formation in *Rhodococci* (Table 5). It was reported previously that, 2% inoculum yield best pigment production in *Salinicoccus* sp. M KJ997975 [32] and *Monascus purpureus* [33, 34]. High inoculum sizes increase biomass but decreases pigment production, due to the inhibition of critical components of culture medium by increased bacterial biomass.

Table 5. *Influence of inoculum concentration on the pigment production rate*

Inoculum conc.	**Cell dry weight ($g.100ml^{-1}$)**	**Carotenoid Pigment ($mg.g^{-1}$)**	**Pigment production rate ($mg.g^{-1}$)**
1	0.26	0.16	0.615
2	0.251	0.181	0.72
3	0.19	0.11	0.578
4	0.12	0.06	0.5
5	0.09	0.05	0.555

Values are the means of triplicates. Standard deviation 2-4%

Optimization of nutritional parameters

Influence of carbon on the pigment production

The influence of various sugars such as maltose, sucrose, mannitol, glucose, fructose, cellobiose, starch, lactose, and sorbitol as a source of carbon on the growth and pigment production were investigated. Among the various tested carbon sources (Table. 6), sucrose, mannitol, maltose and lactose boosted or enhanced the biomass, carotenoid pigment and pigment production rate in *Rhodococcus*. Moderate level of pigment production was observable in fructose, whereas addition of starch, sorbitol, cellobiose and glucose in the growth medium reduces biomass formation and pigment production rate in *Rhodococcus*.

Table 6. *Influence of various sugars on the pigment production rate*

Sugars	Cell dry weight (g/100ml)	Carotenoid Pigment ($mg.g^{-1}$)	Pigment production rate ($mg.g^{-1}$)
Maltose	0.41	0.33	0.804
Sucrose	0.319	0.29	0.909
Mannitol	0.225	0.197	0.875
Fructose	0.28	0.173	0.617
Cellobiose	0.12	0.09	0.75
Lactose	0.217	0.185	0.852
Starch	0.1	0.09	0.9
Glucose	0.13	0.112	0.861
Sorbitol	0.15	0.05	0.333

Values are the means of triplicates. Standard deviation 2-3%

Influence of nitrogen source on the pigment production

The influence of various organic nitrogen sources viz. yeast extract, beef extract, malt extract, peptone, soya peptone, and inorganic nitrogen sources viz. ammonium per sulphate, ammonium chloride, ammonium sulphate, ammonium oxalate, and urea were used for investigating their influence on growth and pigment production in *Rhodococcus*.

Among the various organic nitrogen sources (Table 7) amended in the media, maximum biomass and pigment production was observable in presence of yeast extract (0.910 $mg.g^{-1}$) and beef extract (0.995 $mg.g^{-1}$) as organic nitrogen source and ammonium chloride (0.862 $mg.g^{-1}$) as an inorganic nitrogen source (Table 8). Malt extract, peptone, soya peptone, urea, ammonium sulphate support the moderate growth and pigment production in *Rhodococcus* and presence of ammonium per sulphate and ammonium oxalate exhibit very poor pigment formation.

Literature survey suggested that though the studies on the use of nitrogen sources for microbial pigment production is very scarce, yet it has been reported that organic nitrogen sources gave better yield than inorganic nitrogen sources by *Monascus* sp. [35] It has been also observed that the pigment production varies with nitrogen supplementation. Yeast extract and beef extract exhibited maximum biomass and pigment accumulation in *Monascus* [36, 37].

Table 7. *Influence of organic nitrogen on the pigment production rate*

Nitrogen source	Cell dry weight (g/100ml)	Carotenoid Pigment ($mg.g^{-1}$)	Pigment production rate ($mg.g^{-1}$)
Yeast extract	0.492	0.448	0.910
Beef extract	0.427	0.425	0.995
Malt extract	0.126	0.112	0.888
Peptone	0.112	0.10	0.892
Soya peptone	0.096	0.076	0.791

Values are the means of triplicates. Standard deviation 4-6%

Table 8. *Influence of inorganic nitrogen on the pigment production rate*

Nitrogen source	Cell dry weight (g/100ml)	Carotenoid Pigment ($mg.g^{-1}$)	Pigment production rate ($mg.g^{-1}$)
Ammonium chloride	0.35	0.302	0.862
Ammonium sulphate	0.281	0.18	0.640
Ammonium per sulphate	0.052	0.04	0.768
Urea	0.15	0.09	0.6
Ammonium oxalate	0.06	0.03	0.5

Values are the means of triplicates. Standard deviation 4-6%

Influence of metal ions on the pigment production

Trace metals have an important effect on secondary metabolism and pigment production [38]. Data obtained on the influence of various metal ions revealed that magnesium sulphate (Table 9) and calcium chloride exhibited an enhancing effect on growth and pigment production in *Rhodococcus,* and found to be very crucial components in the pigment production. It has been reported that in presence of calcium chloride maximum pigment formation occurs in *P. sinclairii* while growth and pigment formation were very poor in ferrous sulphate, zinc sulphate and copper sulphate [39, 40, 41, and 42].

Table 9. *Influence of trace metals on the pigment production rate*

Metal ions	Cell dry weight (g/100ml)	Carotenoid Pigment ($mg.g^{-1}$)	Pigment production rate ($mg.g^{-1}$)
$MgSO_4$	0.357	0.298	0.834
$CaCl_2$	0.123	0.092	0.747
$MnSO_4$	0.289	0.183	0.633
$FeSO_4$	0.04	0.001	0.025
$ZnSO_4$	0.052	0.019	0.365

Values are the means of triplicates. Standard deviation 2-4%

Influence of phosphate source

Phosphates are one of the very essential components required for the synthesis of nucleic acids in bacteria. Phosphate concentration affects the cell growth and rate of cell division. The influence of varying source of phosphates (Table 10) revealed that phosphates exerted a nearly equivalent effect on growth and pigment production in isolate *Rhodococci*. The effect of phosphate was additive for growth as well as pigment production. However, studies revealed the presence of di-potassium hydrogen phosphate is required for pigment production in *Pseudomonas* [43].

Table 10. *Influence of phosphates on the pigment production rate*

Phosphates source	Cell dry weight (g/100ml)	Carotenoid Pigment ($mg.g^{-1}$)	Pigment production rate ($mg.g^{-1}$)
K_2HPO_4	0.405	0.33	0.814
KH_2PO_4	0.26	0.23	0.884
Na_2HPO_4	0.256	0.21	0.820
NaH_2PO_4	0.387	0.2302	0.780

Values are the means of triplicates. Standard deviation 4-6%

Growth and pigment production under the optimized conditions at shake flask level

In order to increase the pigment production and lower the production cost the optimized medium protocol was studied at shake flask level. Shake flask experiments for pigment production was carried out with a scale-up in 2 L capacity flasks containing 500 ml of the optimized growth medium contained gL^{-1}, sucrose1.5; yeast extract, 1.0; $MgSO_4.7H_2O$, 0.01; KH_2PO_4, 0.5; NaCl, 0.1: pH 7.5 ± 0.2 and at 2% inoculum of *Rhodococcus,* incubated at 120 rpm for 48-72 h at 37^0C. Data obtained for this experiment conducted with the optimized conditions revealed that exponential phase of the isolate *Rhodococcus* was commenced after 16 h of incubation and continued thereafter till 56 hours. Maximum biomass and pigment production was started from 20 h to 56 h (Fig. 1). While in basal growth medium isolate enters in exponential phase after 24 h. An overall two fold increase in pigment production was achieved after experimental optimization of media components for pigment production at shake flask level. The standard protocol showed a marked increase in pigment production by isolate.

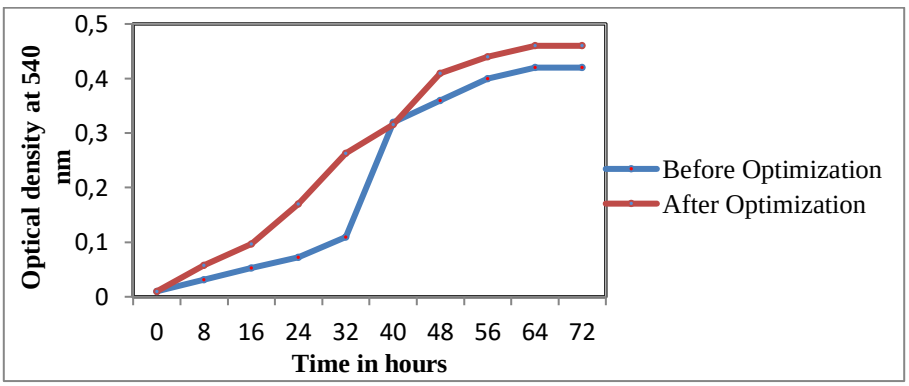

Fig. 1. *Pigment production in optimized medium.*

CONCLUSION

On the basis of the results obtained in the present study it can be concluded that bioprocess variables play an important role in the pigment production by *R. kroppenstedtii*. Studies on the influence of various nutritional parameters revealed that sucrose was found to be the best carbon source while yeast extract in the organic and ammonium chloride among the inorganic nitrogen source was found to boost the pigment production. Influence of various metal ions revealed that $MgSO_4$ enhance the pigment production and K_2HPO_4 was found as the best phosphate source for biomass and pigment production in isolate.

When shake flask experiment containing optimized media was studied with 2% inoculum at 37°C and pH 7.0 with shaking at 120-150 rpm for 72 h., it revealed that two-fold increase in the pigment production was achieved in optimum physico-chemical nutritional parameters.

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