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Evaluation of nutritional parameters for the enhanced production of carotenoid pigment by *Planococcus maritius* using one factor at- a- time methodology

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Abstract

The purpose of the present research work was to determine the influence of various nutritional parameters on the biomass and pigment production in *Planococcus maritimus*. A novel strain of *Planococcus maritimus* was found to produce an intense orange carotenoid pigment. This study revealed that among different physical parameters the maximum growth and pigment production in *Planococcus* was achieved when inoculated with 3% inoculum at 37°C, pH 7 with shaking at 120-150 rpm for 48 hours. Among the various carbon and nitrogen sources studied, maximum biomass and pigment production rate was found with sugar sucrose, yeast extract as the inorganic and ammonium per sulphate among the organic nitrogen source. Among the various metal ions $MgSO_4$ and $CaCl_2$, while K_2HPO_4 proved to be the best phosphate source for biomass and pigment production in isolate. When shake flask experiment containing optimized media was studied it revealed that two fold elevation in pigment production was achieved under optimum nutritional parameters.

Colour of a food substance is important to indicate its freshness and safety. Various synthetic colours and dyes are extensively used in many fields but these dyes can cause considerable non aesthetic pollution and serious health-hazards including harmful side effects[23]. In the recent years, coloring of food with pigments produced from natural sources is of worldwide interest and is gaining importance[14]. 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 do not have any side effects with the substrate to which they are added[17]. Bio-pigments obtained from the microorganisms have been preferred over those from plants because of their stability[7],[10] and the availability of their cultivation technology[11] throughout the year. Microorganisms produce various pigments like carotenoids, melanins, flavins, monascins, violacein and indigo[20]. Carotenoid pigments

have an important function to act as protective agents against oxidative damage[12]. There are over 750 different types of carotenoid produced by a variety of organisms. *Planococcus maritimus* is one of the antioxidative Glyco-C_{30}-carotenoic acid producing strain isolated by Shindo *et al*[25]. 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 *Planococcus maritimus* isolated from distillery effluent.

Microorganism :

Intense orange color carotenoid pigment producing bacterial culture of *Planococcus maritimus* was isolated on Luria- Bertani agar from distillery spent wash of Shri Satpuda Tapi Sahakari sugar factory and distillery section, Shahada. Isolate was purified and identified from Institute of Microbial Technology (IMTECH), Chandigarh, India. For every experiment performed during optimization, 100 µl of 24 to 30 hours pre-grown culture of isolate in Luria Bertani broth was used as inoculum.

Optimization of bioprocess variables :

In order to boost the pigment and biomass production, various bioprocess parameters were optimized. The parameters mainly studied were included nutritional media components such as different carbon sources, organic and inorganic nitrogen sources, phosphates, metal ions and physical parameters such as 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[1, 15]. Each optimization experiment was carried out in triplicates and the mean values were expressed in results.

Optimization of physical parameters :

The influence of various physical parameters such as different incubation temperatures 25°C, 30°C, 37°C, 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 (60,120,180 rpm) and static conditions on growth and pigment production were studied separately and the growth and pigment production was determined in each experiment.

Optimization of nutritional media components:

The influence of various sugars1% (w/v) (viz. maltose, sucrose, mannitol, glucose, fructose, starch and lactose) as a source of carbon on the growth and pigment production were investigated. Similarly, various types of economically feasible inorganic viz. urea, ammonium per sulphate, ammonium chloride and ammonium sulphate1 % (w/v) and organic nitrogen sources beef extract, malt extract, peptone, soya peptone 1 % (w/v) were used to investigate their effect on pigment production.

Influence of various metal ions viz. $MgSO_4$, $MnSO_4$, $CaCl_2$, $FeSO_4$ and $ZnSO_4$ in trace quantities and various phosphate sources 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 effect on the growth and pigment production. In each experiment basal growth medium was maintained as a control.

Growth and pigment production profile :

Shake flask experiment for maximum production of pigment production was designed with optimum physical and nutritional conditions. Aliquot of culture was withdrawn aseptically after every 4 hour and absorbance, biomass as well as pigment production was determined.

Estimation of biomass and pigment concentration :

Culture broth (100 ml) 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[8]. Dry weight of cell mass was expressed as g/100ml growth media[6]. Dry biomass obtained after every optimization experiment was subjected to extraction procedure[5]. Pigment was expressed as $mg.g^{-1}$ of biomass.

Pigments produced by microbes possess numerous efficacious medicinally important products. Carotenoid pigments are of considerable interest in nutrition because of their role as antioxidants and potentials for preventing or delaying degenerative diseases and for enhancing immune responses and antioxidant compounds in food play an important role as a health protecting factor. Intense orange pigment produced by *Planococcus maritimus* belongs to 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 isolate.

Optimization of physical parameters
Influence of incubation temperature :

Incubation temperature is one of the important parameters influencing the growth and pigment production by organisms. Effect of temperature on the growth and pigment production revealed (Table-1) that maximum growth and pigment production was observed at temperature 30°C followed by at 37°C, and again rise in the temperature up to 45°C growth and pigment production decreased. According to literature, incubation temperature which favoured the best growth and pigment production is 30-37°C[13]. Reduction in the pigment production at elevated temperatures is well documented by Pastrana *et al.*,[19] and Rekha *et al.*,[24].

Table-1. Influence of temperature on the pigment production rate by *P. maritius*

Temperature	Cell dry weight (g/100ml)	Carotenoid Pigment (mg/100ml)	Production rate mg/gm
25	0.243	0.203	0.835
30	0.315	0.272	0.863
37	0.338	0.300	0.887
45	0.212	0.131	0.617

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

Influence of pH on the growth and pigment production :

The pH of the growth medium exhibits pronounced effect on the biomass and pigment production by organisms[21]. The results of the effect of different pH on growth and pigment production revealed that,with raising pH 3.0 to 7.0 pigment production and growth increases but maximum growth and pigment formation occurs at pH 7.0 (0.44g/100ml, 0.77 mg/gm) Further increase of the pH resulted in reduction of growth and pigment production by isolate.

Influence of incubation time on pigment production :

Influence of incubation time on the growth and pigment production revealed that prominent orange pigment production and growth in *Planococcus maritius* was observed at the end of 48 hours to 72 h (0.262gm/100ml, 0.954mg/g).

Influence of aeration and agitation :

Aeration of the growth medium is important for successful growth of aerobic organism. The influence of aeration on the biomass and pigment production by the isolate when examined by varying speeds of aeration (Table-2) revealed that maximum growth and pigment production (25gm/100ml and 0.8mg/gm) was obtained at 120 rpm. Thus increase in the aeration rate after 120 rpm to 180 rpm, resulted in the decrease in the biomass while very minute growth was observed at static condition.

Table-2. Influence of aeration on the pigment production rate by *P. maritius*

Time	Cell dry weight (g/100ml)	Carotenoid Pigment (mg/100ml)	Production rate mg/gm
0	0.08	0.03	0.375
60	0.09	0.04	0.44
120	0.25	0.20	0.8
150	0.23	0.153	0.66
180	0.182	0.11	0.60

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

Influence of inoculum concentrations on the growth and pigment production:

Effective inoculum development is one of the key programmed during industrial fermentations. Optimum level of inoculum concentration that supports maximum growth, biomass and pigment production was found to be 3% (0.36gm/100ml, 0.916mg/g),inoculum size was found to be directly proportional to biomass formation in *Planococci*.

Optimization of nutritional parameters
Influence of carbon on the pigment production:

The influence of various sugars revealed that among the various tested carbon sources,

Table-3. Influence of various sugars on the pigment production rate by *P. maritius*

Sugars	Cell dry weight (g/100ml)	Carotenoid Pigment (mg/100ml)	Production rate mg/gm
Maltose	0.309	0.211	0.682
Sucrose	0.425	0.398	0.936
Mannitol	0.239	0.16	0.669
Fructose	0.20	0.12	0.60
Lactose	0.09	0.05	0.555
Starch	0.13	0.08	0.615
Glucose	0.31	0.22	0.706

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

sucrose, maltose, glucose and mannitol enhanced the biomass, carotenoid pigmentproduction rate in *P. maritius*.. Moderate level of pigment production was observable in starch and fructose, whereas addition of lactose in the growth medium reduces biomass formation and pigment production rate in *Planococcus*.

Studies of the effect of various carbon sources on the growth, biomass and pigment production indicated that preferably sucrose followed by glucose, maltose and mannitol exhibited increased pigment production in organisms while rest of the sugars exhibited moderate to poor pigment production.

Influence of nitrogen source on the pigment production :

Among the various organic nitrogen sources used in the media, biomass and pigment production was found to be maximum in the presence of beef extract and yeast extract. The moderate level of pigment production was observable in malt extract. The presence of peptone, soya peptone in the medium did not exhibit any remarkable increase in pigment production. Among the various inorganic nitrogen sources maximum pigment production was observable in the presence of ammonium persulphate, and urea and poor pigment production was observable in the presence of ammonium chloride and ammonium sulphate. It has been reported that organic nitrogen sources gave better yield than inorganic nitrogen sources by *Monascus* sp.[18]. It has been also observed that the pigment production varies with nitrogen supplementation. The organisms like *Serratia marcescens* failed to grow in the media supplemented with inorganic nitrogen possibly indicating the toxicity of ammonium salts towards the organism[4], while yeast extract and beef extract exhibited maximum biomass and pigment accumulation in *Monascus*[6].

Influence of metal ions on the pigment production :

Data obtained on the studies of influence of various metal ions revealed that among the various trace metals magnesium sulphate and calcium chloride exhibited an enhancing effect on growth and pigment production in*Planococcus* and found to be very

Table 4. Influence of organic and inorganic nitrogen source on the pigment production by *P. maritius.*

Nitrogen source	Cell dry weight(g/100ml)	Carotenoid Pigment (mg/100ml)	Production rate mg/gm
Yeast extract	0.335	0.3	0.895
Beef extract	0.412	0.365	0.885
Malt extract	0.286	0.204	0.713
Peptone	0.05	0.01	0.2
Soy peptone	0.029	0.01	0.344
Ammonium chloride	0.025	0.001	0.04
Ammonium Sulphate	0.125	0.082	0.656
Ammonium persulphate	0.149	0.12	0.805
Urea	0.205	0.182	0.887

Table-5. Influence of trace metals on the pigment production rate by *P. maritius.*

Metal ions	Cell dry weight (g/100ml)	Carotenoid Pigment (mg/100ml)	Production rate mg/gm
$MgSO_4$	0.4	0.336	0.84
$CaCl_2$	0.26	0.203	0.780
$MnSO_4$	0.379	0.286	0.754
$FeSO_4$	0.042	0.003	0.0714

crucial components in the pigment production. It has been reported[5]that in presence of calcium chloride maximum pigment formation occurs in *P. sinclairii.* Trace-elements are one of the important factors affecting pigment production in several micro-organisms[2,16].

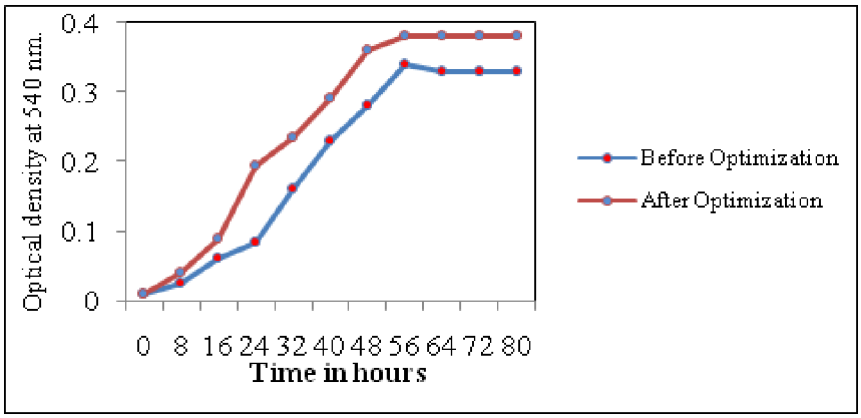
Influence of phosphate source :

Phosphates are one of the very essential components required for the synthesis of nucleic acids in bacteria. Data obtained of the studies revealed that phosphates exerted a nearly equivalent effect on growth and pigment production in isolate *Planococci*. The effect of phosphates were additive for growth as well as pigment production. However studies revealed that presence of dipotassium hydrogen phosphate is required for pigment production in *Pseudomonas*[23].

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}, sucrose 1.5; 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 3% inoculum of *Planococcus*. Flasks were incubated in rotary shaker 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 *Planococcus* commenced after 16 hours (0.09 OD) of incubation and continued thereafter till 56 hours. Maximum biomass and pigment production started from 16h to 56 h. Further incubation after 56 h resulted in steady state in the growth and pigment production. An overall two fold increase in pigment production was achieved after experimental optimization of media components for pigment production at shake flask level.



Pigment profile in optimized medium and optical density of broth at 540 nm

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