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Research Article

OPTIMIZATION OF PIGMENT PRODUCTION USING PLACKET BURMAN AND SURFACE RESPONSE METHODOLOGY

Naziya M.A.Rehman and Prashant P. Dixit*

Dr.Babasaheb Ambedkar Marathwada University, Subcampus, Osmanabad, India (MS)

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ABSTRACT

Research on microbial pigments has been an important and interesting field, as microbial pigments have medicinal, nutritive and other beneficial characters. Many microbial pigments have shown good antibacterial, antifungal, antioxidant, antitumor, anticancer activities. Microbial pigments are considered as good alternatives to the artificial colorants, as many microbes produce intense pigments. Artificial colorants are harmful for human, animal and environmental health. Microbial pigments might be involved in many processes which are not known till now. They might act as drug or might possess some unique pharmacological properties. Many reports tell us that there is a great effect of environmental factors and media components on microbial pigment production, thus there is a need to optimize the media components for microbial pigment production, if we want to produce them at large industrial level.

Present research study was aimed to optimize the media components for the maximum pigment production. A Placket Burman design was used to screen main media variables which have a great influence on pigment production. The significant variables were optimized by using response surface Box Behenken methodology. Peptone, soybean meal and pH were screened as main significant factors by these optimization methodology.1% peptone, 1%soyabean meal and pH 7 was found as optimum for increased pigment production.

INTRODUCTION

Microorganisms have been used for a long time for production of molecules as diverse as antibiotics, enzymes, vitamins, texturizing agents and some other beneficial products. Many microorganisms are able to produce pigments. Many microbes produce pigments and many of them have been used in different industrial applications. The major pigment producing microbes are. *Monascus* species, *Pseudomonas* species, *Serratia* species, *Micrococcus*species, *Streptomyces* speciesetc. Microorganisms produce various pigments like carotenoids, melanins, flavins, quinones, prodigiosins and more specifically monascins, violacein or indigo (Dufosse, 2006).

Microorganisms are known to produce a variety of pigments; therefore they are promising source of food colorants (Aberoumand, 2011 and Ahmad, 2012). Now a day many industries are using artificial chemical dyes and colors .Since many kinds of synthetic dyestuffs have been found to be hazardous to human health, only limited kinds of such dyestuffs are allowed to be used in food and in other industries such as cosmetic, pharmaceutical .Therefore, there is a need to develop alternative sources of natural colorants. Natural colorants could be obtained from plants and animals. Plants are the biggest source of natural colorants now a days. Researchers are interested more in microbial pigments because of their friendly nature with humans and other plants and animal. They are advantageous over plant pigments because microbial pigments have shown antioxidant, anti-cancerous, anti-tumorous activities.

Many reports had shown that microbial pigments are involved in anticancerous, antitumour, antioxidative, immunosuppre - ssive activities so there is a need to isolate pigment producing microbe and to check the properties of these pigments as pharmacological agent. As there is a big effect of media components on microbial pigment production; optimization of media components is necessary for efficacy of the process and for economy. Optimization of media components plays an important role in the development of any process. Designing an appropriate nutritive medium is of great importance to improve efficacy and productivity of interested compound. In general, the traditional method of one variable at a time (OVAT) approach is laborious and time consuming and it decreases accuracy of the effects of interacting factors and might lead to wrong output ; conversely, the statistical planned experiments

Corresponding author:* **Naziya M.A.Rehman
Dr.Babasaheb Ambedkar Marathwada University, Subcampus, Osmanabad, India (MS)

are better to minimize error. In optimized medium, the interested compounds production is maximum which is essential for commercial point of view; hence the present research study was aimed to optimize the media components for maximum pigment production and to identify the most influential components and interactive effects of components on pigment production by statistical Placket Burman and Response Surface experimental design.

MATERIALS AND METHODS

Production and estimation of pigment- The bacterium NR[1] was inoculated in 250ml nutrient broth flask and it was kept for incubation at 40^0C for 3 days. The amount of pigment was estimated by using spectrophotometer. The produced pigment showed highest absorption at 484nm.

Effect of carbon and nitrogen sources on pigment production- Effects of different carbon and nitrogen sources was examined by using one variable at a time (OVAT) method. To detect effect of different carbon and nitrogen sources on pigment production, 1% of different carbon and nitrogen source was added in different 250ml nutrient broth flasks. Those flasks were inoculated with 5ml broth from 24 hours old culture flasks. Glucose, sucrose, maltose, pectin, glycerol, chitin, lactose, xylose, fructose, dextrose, galactose were used as carbon sources. Casein, tryptone, aspargine, beef extract, yeast extract, ammonium chloride, meat extract, sodium nitrate, sodium nitrite, ammonium sulphate, potassium nitrate, ammonium acetate, urea, ammonium phosphate, malt extract, soya bean meal were used as nitrogen sources. To determine pigment production, an uninoculated medium of each carbon source and nitrogen source was used as control and absorption was recorded at 382nm as the pigmented broth showed maximum absorption at this wavelength.

Placket Burman experimental Design to Screen Significant Variables- Initially, the carbon and nitrogen sources were screened and among them the best carbon and nitrogen sources were selected along with the basal nutrient medium components for further optimization using Plackett-Burman design. Placket Burman design is an effective method for screening for significant medium components that influence pigment production. The Placket Burman designs allow for the screening of main factors from a large number of process variables and designs are thus quite useful in preliminary studies in which the main objective is to select variables that can be fixed or eliminated in further optimization.

Table 1 Different variable and their levels in Placket Burman experimental Design

Sr.no.	Variable	Low level(-1) (%)	High level(+1) (%)
1	Yeast Extract	0.1	0.5
2	Peptone	0.5	1
3	NaCl	0.5	1
4	Casein	0	1
5	Soyabean Meal	0	1
6	Malt extract	0	1
7	Pectin	0	1
8	Sucrose	0	1
9	Lactose	0	1
10	pH	7	8
11	Beef extract	0.1	0.5

This experimental design allows the evaluation of N variables in the N+1experiments. In the present investigation, the significance of 11 variables (media components) on production of pigment was screened by Placket Burman design with 12 run. Total 11 factors were selected for the statistical experimental design. Every variable is examined at two levels; +1 and -1. Table 1 indicates the selected variables and their levels.

Optimization By Response Surface Methodology- Response surface methodology is an efficient experimental strategy to determine optimal conditions for multivariable system. For the optimization study, Design Expert Software package (version 9) was used where response surface methodology based on a three variables, three level Box Behenken Design was employed.

RESULTS

Effect of carbon and nitrogen sources on pigment production- Among the tested carbon and nitrogen sources, some sources showed positive and some showed less effect on pigment production. Sucrose, pectin, lactose were found to be best carbon sources (figure 1).

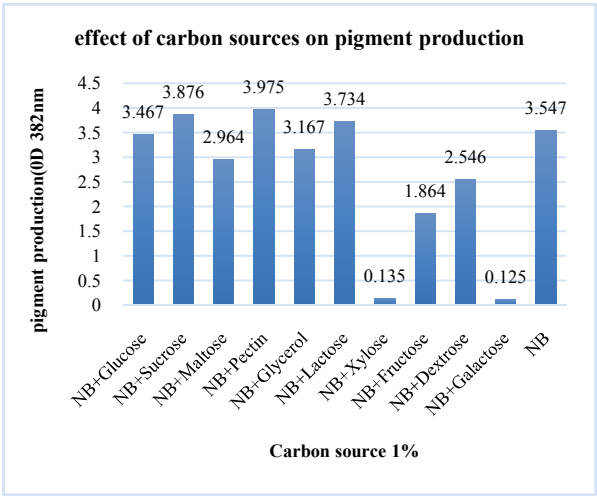

Figure 1 Effect of carbon sources on pigment production. (1% of each source were added in separate nutrient broth flasks(NB) flasks).

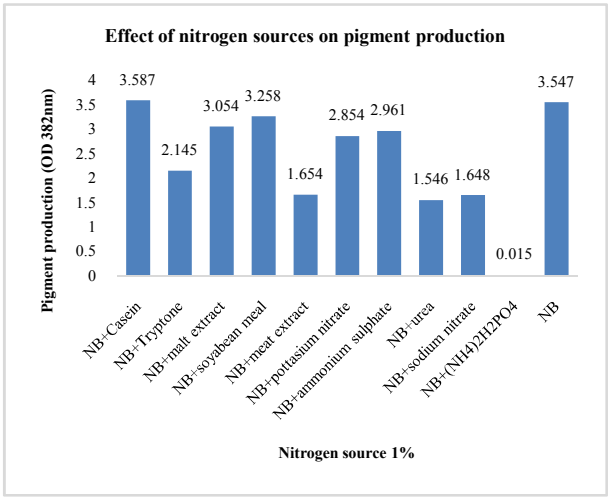

Figure 2 Effect of nitrogen sources on pigment production. (1% of each source were added in separate nutrient broth flask(NB) flasks).

The pigment production in presence of dextrose, glucose, fructose, glycerol was also good. Xylose and galactose showed less effect on pigment production. Casein, malt extract, soya bean meal (figure 2) were found as best nitrogen sources for pigment production. In presence of urea, and (NH4)2H2PO4 minimum pigment production was observed. Tryptone, potassium nitrate and ammonium sulphate also showed comparable effect on pigment.

Placket Burman experimental Design-The results of Placket Burman experiment can easily be interpreted by Pareto chart. The Pareto chart enable us to identify and select significant factors in the system very easily. Figure 3 depicts Pareto chart. According to this chart peptone, pH and soya bean meal showed significant effect on pigment production. Table 2 depicts the Placket Burman design matrix along with the obtained response.

Table 2 The Placket Burman experimental design matrix for screening of process and nutritional parametres for pigment production

		Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9	Factor 10	Factor 11	Response 1
Std	Run	A:yeast extract %	B:peptone %	C:NaCl %	D:Casein %	E:Soyabean meal %	F:Malt extract %	G:pectin %	H:sucrose %	J:lactose %	K:pH %	L:Beef extract %	pigment production(OD 382nm) nm
10	1	0.1	1	1	1	0	0	0	1	0	8	0.5	2.056
8	2	0.5	1	0.5	0	0	1	0	1	1	7	0.5	2.387
4	3	0.1	1	0.5	1	1	0	1	1	1	7	0.1	2.591
6	4	0.1	0.5	0.5	1	0	1	1	0	1	8	0.5	2.468
1	5	0.5	1	0.5	1	1	1	0	0	0	8	0.1	2.935
9	6	0.5	1	1	0	0	0	1	0	1	8	0.1	2.154
5	7	0.1	0.5	1	0	1	1	0	1	1	8	0.1	2.765
3	8	0.5	0.5	1	1	0	1	1	1	0	7	0.1	3.265
12	9	0.1	0.5	0.5	0	0	0	0	0	0	7	0.1	3.642
1	10	0.5	0.5	1	1	1	0	0	0	1	7	0.5	4.269
2	11	0.1	1	1	0	1	1	1	0	0	7	0.5	3.019
7	12	0.5	0.5	0.5	0	1	0	1	1	0	8	0.5	3.008

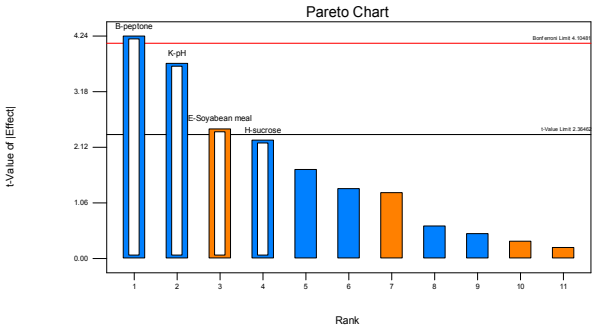

Figure 3 Pareto chart for Placket Burman data.

Table 3 ANOVA for Placket Burman.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
	3.15	4	0.79	10.76	0.0041	
B-peptone	1.32	1	1.32	17.99	0.0038	
E-Soyabean meal	0.45	1	0.45	6.10	0.0428	
H-sucrose	0.37	1	0.37	5.09	0.0585	Significant
K-pH	1.01	1	1.01	13.84	0.0074	
Residual	0.51	7	0.073			
Cor Total	3.66	11				

The data obtained was analyzed by ANOVA. A p value less than 0.05 for the three variables (peptone, pH and soya bean meal) showed that these are significant factors. The ANOVA indicates that the three individual factors were significantly influencing the process. The lowest p value 0.003 for peptone suggested that this is the most important parameter for pigment production. The model F value of 10.76 implies that the model is significant. There is only a 0.41% chance that the F value this large could occur due to noise. The low Coefficient of Variation i.e 9.48 in the present case denoted that the experiments performed were highly reliable.

Optimization By Response Surface Methodology -Total 17 experimental runs were designed for the optimization study. The significant factors selected in Placket Burman design were chose for the optimization. Three variables peptone, soyabean meal and pH were selected for optimization. Each variable were set at three different levels. The absorption at 382 nm was selected as a response in this experimental design. The 17 experimental runs and the observed responses are shown in table 4.

Table 4 Box Behenken Design matrix with response values

Std	Run	Factor 1 A:peptone %	Factor 2 B:soyabean meal %	Factor 3 C:pH	Response 1 Pigment roduction (OD 382) Nm
5	1	0.5	2	7	2.956
9	2	1	1	7	4.701
14	3	1	2	7.5	2.486
4	4	1.5	3	7.5	2.845
15	5	1	2	7.5	3.005
3	6	0.5	3	7.5	2.009
7	7	0.5	2	8	2.119
16	8	1	2	7.5	3.185
6	9	1.5	2	7	3.295
17	10	1	2	7.5	3.246
8	11	1.5	2	8	2.456
11	12	1	1	8	2.934
10	13	1	3	7	3.214
13	14	1	2	7.5	2.854
2	15	1.5	1	7.5	2.563

Statistical analysis is made by ANOVA. The result of ANOVA is given in table 5.

From ANOVA results, it is clear that model is significant. The model F value of 5.84 implies that the model is significant. In this case variable C, A^2, C^2 are significant as their p values is less than 0.005. From the data result, it is clear that there is no significant effect of interaction of factor on pigment production. The optimum conditions for maximum pigment productions are obtained from the statistical experimental design. pH 7,1% soya bean meal and 1% peptone found as optimum concentration in media for maximum pigment

production. The surface response 3D plots were drawn by using software and those graphs are given in figure 4, 5, 6.

Table 5 ANOVA results of Box Behenken design.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	4.31	9	0.47	8.27	0.005	
A-peptone	0.29	1	0.29	5.08	0.058	
B-soyabean meal	4.050E-003	1	4.050E-003	0.070	0.798	
C-pH	1.73	1	1.73	30.03	0.0009	
AB	0.16	1	0.16	2.86	0.134	Significant
AC	1.000E-006	1	1.000E-006	1.72	0.9967	
BC	1.600E-005	1	1.600E-005	2.766E-004	0.9871	
A^2	1.57	1	1.57	27.16	0.0012	
B^2	0.08	1	0.08	1.53	0.2554	
C^2	0.55	1	0.55	9.54	0.0175	
Residual	0.40	7	0.058			
Lack of Fit	0.03	3	0.012	0.1246	0.9407	
Pure Error	0.37	4	0.093			Not significant
Cor Total	4.70	16				

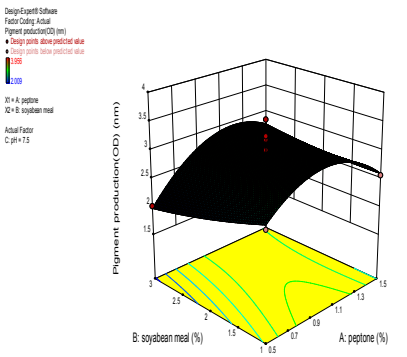

Figure 4 Response surface plot of soyabean meal and peptone

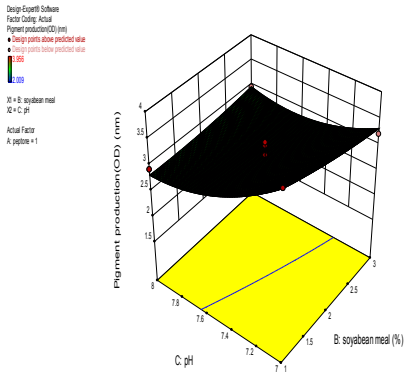

Figure 5 Response surface plot of pH and soyabean meal

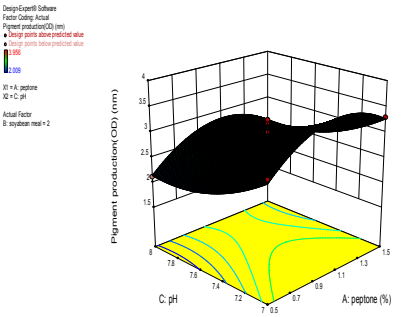

Figure 6 Response surface plot of pH and peptone

The figure 4 demonstrates correlation between soya bean meal and peptone. Figure 5 demonstrates correlation between pH and soya bean meal. Figure 6 demonstrates correlation between pH and peptone.

DISCUSSION

From the present study, it is clear that nitrogen sources and pH are very important for the selected bacterium to produce pigments. At neutral pH, many bacterium produce maximum pigments. Many reports tell us that nitrogen source plays an important role in pigment production. F. Stanly Pradeep *et.al.,* (2013) in his study has reported the same results as ours. For the maximum production of microbial pigment by *Fusarium moniliforme*, 1% peptone in basal medium is needed but the optimum pH was 5.5. Peptone was also found as best nitrogen source in a study carried out by M.P. Prasad, (2015) on optimization of pigment by *Serratia marcescence* and *Brevibacterium maris*. 2% of peptone and 1.5% of peptone in a basal medium was found as best for maximum pigment production by *Brevibacterium maris* and *Serratia marcescence* respectively. The optimum pH observed for the organisms *Brevibacterium maris* and *Serratia marcescence* were 6, but for *Arthrobacter species* the optimum pH was 7 as like ours findings. In a report by B. V. Latha *et al*., (2004) sodium nitrate was found as the best nitrogen source for production of carotenoids (3.3 mg/L),in contrast to our findings ,peptone gave poor yield of carotenoid pigment. In another report by Amr A El-Banna *et al.* (2011), ammonium sulphate was reported as best nitrogen source. Tallapragada *et.al.,* (2013) in their research study, observed that malt extract serve best nitrogen source for pigment production by *M. purpureus* with concentration of 8 g/L in a basal medium. From the results and observation by various researchers, we can conclude that nitrogen source and pH plays critical role in pigment production process but different microorganisms with distinct pigments might require different nitrogen sources with specific concentration. pH also plays very crucial role in microbial pigment production. Some microbes are able to produce pigments in slight alkaline and acidic conditions. M. R. A. Manimala *et al.,* (2017) optimized medium for carotenoid production by using response surface methodology and reported that the suitable pH for maximum carotenoid production is 5.The present study revealed that for the maximum pigment production by bacterium NR[1], both physical and chemical parameters are important.

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