RESEARCH ARTICLE

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Response Surface Methodology (RSM) and electromagnetic optimization of pigment production by *Sporobolomyces* sp S5 and *Rhodotorula* sp A21 in submerged fermentation

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ABSTRACT

This study aimed at enhancing pigment production by *Sporobolomyces* sp S5 and *Rhodotorula* sp A21 in submerged fermentation using statistical method and electromagnetic inducement technique. One-factor-at-a-time (OFAT) experiments were initially employed to screen cultural and physical parameters that significantly affect pigment production in both cultures. The most significant medium components and physical parameters were selected using Plackett-Burman (PB) experiment design while a Central Composite Design (CCD) of the response surface methodology (RSM) was used to determine the optimal levels and the interactive effects among the selected components. Results of OFAT experiments suggested that glucose, yeast extract, calcium sulphate and sodium nitrate, incubation temperature of 30 °C, the incubation period of 72 hours and, pH 6.5 and 7.0 for *Sporobolomyces* sp S5 and *Rhodotorula* sp A21 respectively were best for pigment production. The results of the Plackett-Burman (PB) design showed that yeasts extract incubation temperature significantly affected Pigment production by *Rhodotorula* sp A21 while glucose, incubation time and pH affected pigment yield in *Sporobolomyces* sp S5 cultures RSM optimization revealed maximum pigment yield of 170.34±0.02 $\mu g.mL^{-1}$ (*Sporobolomyces* sp S5) and 242.48±0.02 $\mu g.mL^{-1}$ (*Rhodotorula* sp A21) when the optimal levels and the interactive effects among the selected parameters were investigated. Exposure to electromagnetic field (EMF) further enhanced pigment production with a pigment yield of 202.70±0.02 $\mu g.mL^{-1}$ by *Sporobolomyces* sp S5 at 40 minutes exposure under statistically optimized conditions.

Key words: Biopigments, medium components, central composite design, *Sporobolomyces* sp., electromagnetic field

Introduction

The use of chemically synthesized pigments in food, textile, cosmetic and pharmaceutical industries is drastically being minimized and/or avoided due to problems including but not limited to high cost and toxicity (Seyedin et al., 2015; Tran et al., 2019). These challenges have caused a shift in attention to the pigments of biological origin (Hosseinpour et al., 2017; Eman, 2019). Although the main production sources of biopigments are plants and microorganisms, the problems of seasonal and geographic variability have greatly hampered the use of plants (Manimala & Murugesan, 2017). Microbial synthesis using bacteria, fungi, yeasts and algae are however are advantageous compared to plants, particularly in terms of availability and independence on weather conditions, stability, cost efficiency and easy downstream processing (Joshi et al., 2014). Among the microbial sources, the yeasts are considered to be more suitable for pigment production primarily due to their high yield, ease of cultivation for large scale production in bioreactors, unicellular nature and high growth rate (Frengova & Beshkova, 2009; Manimala & Murugesan, 2017). Despite these advantages, the current rate of pigment production by yeasts has not been able to meet the very high industrial demands for pigments by the various industries, thereby affecting its cost and availability.

Strategies to meet the high industrial demand for pigment use include bioprospecting for high yield-producing strains, optimization of fermentation technologies, and use of strain

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improvement technologies. Optimization is usually done using classical one-factor-at-a-time (OFAT) or statistical techniques (Wang et al., 2017). However, the OFAT is expensive, time-consuming and laborious while statistical experimental techniques including Plackett-Burman Design (PBD) and Response Surface Methodology (RSM) are being widely used to improve product yield and the overall process efficiency (Singh et al., 2017; Tran et al., 2019). Additionally, the use of electromagnetic radiation, by the simple exposure of microorganisms to electromagnetic fields (EMF), to improve fermentation processes has also been reported to have the potential to boost fermentation processes particularly in terms of yield and productivity on microorganisms (Hristov & Perez, 2011). Available literature showed that EMF is capable of eliciting a variety of biological effects such as the alteration of the substructure of cell membranes which may enhance permeability (Bejenaru et al., 2017).

In this study, pigment production by *Sporobolomyces* sp S5 and *Rhodotorula* sp A21 in submerged fermentation was studied and further optimized using statistical techniques. The effect of exposure of the yeasts to electromagnetic field on pigment production by the yeasts was also investigated. To the best of our knowledge, this is one of the few pieces of literature documenting the enhancement of pigment production by yeasts under the influence of electromagnetic fields.

Materials and Methods

Microorganisms and inoculum preparation

Sporobolomyces sp S5 and *Rhodotorula* sp A21 used in this study were obtained from The Culture Collection of the Department of Microbiology, University of Ibadan, Ibadan, Nigeria. They were maintained on Yeast Malt Extract Agar (YMEA) medium, stored at 4 ºC and sub-cultured at regular intervals. The inoculum used was prepared by inoculating 50 mL of sterile Sabouraud Dextrose Broth with a loopful of 24 hours old culture of each of the yeasts and incubated for another 24 hours. An aliquot of 1 mL containing approximately 4.8×10^6 was then taken as the inoculum except otherwise stated.

Pigment production, extraction and estimation

Yeasts were grown in 250 ml Erlenmeyer flask containing 100 ml of fermentation medium composed ($g.L^{-1}$) of glucose (30.0), $(NH4)_2SO_4$ (2.5), K_2HPO_4 (1.0), $MgSO_4.7H_2O$ (0.5) and yeast extract (4.0) and incubated under rotary shaker condition (150) rpm) at 30 °C for 72 hours after which pigment extraction was carried out using the method of Panesar et al. (2014) and the pigment yield quantified using the spectrophotometric technique. Pigment yield was expressed in $\mu g.ml^{-1}$ using the standard curve for β-carotene as described by Sanusi & Adebiyi (2009).

Influence of physiological parameters on pigment production

Single factor experiments

The effect of different carbon sources (glucose, maltose, sucrose, fructose, and glycerol at a concentration of 5 %, w/v), nitrogen sources (Yeast extract, Urea, Peptone, potassium nitrate, and sodium nitrate at 1 %, w/v concentration), metallic salts ($CaSO_4$, $ZnSO_4$, $MgSO_4$, $MnSO_4$, $NaSO_4$ at a concentration of 0.5 %, w/v), incubation temperature (25 - 40 °C), the incubation period (24 - 120 Hours) and pH (6.0 - 8.0) were examined by using one-factor-at-a-time experiments (Singh et al., 2017; Wang et al., 2017).

Response Surface Methodology (RSM) experiments

From the results obtained from the single factor experiments, a Plackett-Burman experiment was further used to screen the significant variables among those parameters that best support pigment production. All the variables were investigated at two intervals specified at negative values (low level, -1) and positive values (high level, +1) in total of twelve runs. The detail of the design is shown in Tables 1a and 1b. Response surface methodology was then used with Central Composite Design (CCD) to optimize the selected fermentation parameters: glucose (A), pH (E) and incubation period (G) for *Sporobolomyces* sp S5 (Tables 2a and 2b) and, yeast extract (B) and temperature (F) for *Rhodotorula* sp A21 (Tables 3a and 3b), for enhanced pigment production in a total of twenty and thirteen runs respectively. The selected components were studied at three different levels: (-), (0), and (+) for low, intermediate, and high levels respectively. Minitab software Version 18.0 (Minitab Inc., USA) was used for the experimental design and analysis of the data obtained.

Effect of electromagnetic inducement on pigment production under optimized conditions

The modified method of Ibraheim & Darwish (2013) was employed in the construction of the electromagnetic field exposure system. The field was generated by a solenoid consisting of 94 turns of electrically insulated 2 mm copper wire wound around a cylinder (10 cm diameter and 5 cm length). The ends of the solenoid were connected to a step-down transformer fed from the mains (220 V, 50 Hz) (Figure 1). The electromagnetic flux density was calculated using Biot-Savart law (Ahmed et al., 2013) as indicated below;

$B = \frac{2\text{-}\mu_o NIR^2}{2(R^2+x^2)^{3}/_{2}}$, where B = Magnetic flux density (T), I = coil current (A), R = coil radius (m), x = coil distance on axis, to pint, (m), N = number of wire loops and μ0 = permeability constant and equals 1.257×10− 6 T.m/A.

Table 1a. *Variables, symbol codes and, actual low and high level of the selected parameters used in Placket-Burman design.*

Code	Variable	High level (+1)	Low level (-1)
A	Glucose (%, w/v)	8	2
B	Yeast extract (%, w/v)	1.5	0.5
C	Sodium nitrate (%, w/v)	1.5	0.5
D	Calcium sulphate (%, w/v)	0.8	0.2
E	pH	8	6
F	Incubation temperature (^{0}C)	35	25
G	Incubation period (Hours)	120	24

Table 1b. *Plackett-Burman experiment design and response value*.

Run	Variables							Pigment yield* (µg.mL^{-1})	
	A	B	C	D	E	F	G	*Rhodotorula* sp A21	*Sporobolomyces* sp S5
1	+1	-1	+1	-1	-1	-1	+1	93.46 ± 15.10	134.12 ± 10.51
2	+1	+1	-1	+1	-1	-1	-1	110.18 ± 1.49	112.10 ± 5.26
3	-1	+1	+1	-1	+1	-1	-1	180.01 ± 2.97	96.23 ± 0.51
4	+1	-1	+1	+1	-1	+1	-1	28.05 ± 1.00	121.13 ± 1.98
5	+1	+1	-1	+1	+1	-1	+1	175.75 ± 2.31	147.51 ± 2.80
6	+1	+1	+1	-1	+1	+1	-1	51.00 ± 6.57	132.23 ± 11.33
7	-1	+1	+1	+1	-1	+1	+1	138.38 ± 9.85	121.89 ± 0.02
8	-1	-1	+1	+1	+1	-1	+1	89.69 ± 2.64	131.80 ± 1.00
9	-1	-1	-1	+1	+1	+1	-1	68.71 ± 2.31	108.07 ± 1.00
10	+1	-1	-1	-1	+1	+1	+1	39.53 ± 3.13	138.87 ± 7.22
11	-1	+1	-1	-1	-1	+1	+1	70.02 ± 0.67	101.38 ± 4.11
12	-1	-1	-1	-1	-1	-1	-1	58.05 ± 0.02	95.87 ± 8.87

*Mean values ± Standard deviation

Table 2a. *Variable levels used in Central Composite design for optimization of pigment production by Rhodotorula sp A21.*

Code	Variable	High level (+1)	Central level (0)	Low level (-1)
B	Yeast extract (%, w/v)	1.5	1.0	0.5
F	Incubation temperature (^{0}C)	35	30	25

Table 2b. *Central Composite design and response value for Rhodotorula sp A21*.

Run	B (Yeast extract (%, w/v))	F (Temperature (^{0}C))	Pigment yield* (µg/mL)
1	-1	-1	174.12 ± 12.48
2	+1	-1	169.20 ± 0.34
3	-1	+1	182.64 ± 9.03
4	+1	+1	240.84 ± 0.18
5	-1	0	169.85 ± 1.33
6	+1	0	171.49 ± 0.15
7	0	-1	156.57 ± 3.99
8	0	+1	242.48 ± 0.02
9	0	0	88.51 ± 0.02
10	0	0	88.61 ± 11.00
11	0	0	87.12 ± 0.67
12	0	0	86.25 ± 0.02
13	0	0	88.71 ± 1.33

*Mean values ± Standard deviation

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Table 3a. *Variable levels used in Central Composite design for optimization of pigment production by Sporobolomyces sp S5.*

Code	Variable	High level (+1)	Central level (0)	Low level (-1)
A	Glucose (%, w/v)	8	5	2
E	pH	8	7	6
G	Incubation period (Hours)	120	72	24

Table 3b. *Central Composite design and response value for Sporobolomyces sp S5.*

Run	A (Glucose (%, w/v))	E (Incubation Period (Hrs))	G (pH)	Pigment yield* (µg.mL^{-1})
1	-1	-1	-1	11.16 ± 0.51
2	+1	-1	-1	39.69 ± 0.15
3	-1	-1	+1	39.85 ± 0.67
4	+1	-1	+1	69.69 ± 0.67
5	-1	+1	-1	27.23 ± 0.15
6	+1	+1	-1	79.53 ± 0.02
7	-1	+1	+1	148.21 ± 1.82
8	+1	+1	+1	105.75 ± 0.18
9	0	0	0	161.15 ± 1.16
10	0	0	0	168.21 ± 5.10
11	0	0	0	170.34 ± 0.02
12	0	0	0	168.54 ± 4.77
13	-1	0	0	62.15 ± 1.33
14	+1	0	0	83.46 ± 4.61
15	0	0	-1	36.57 ± 0.02
16	0	0	+1	64.28 ± 1.49
17	0	-1	0	67.72 ± 3.13
18	0	+1	0	126.25 ± 0.34
19	0	0	0	169.03 ± 0.34
20	0	0	0	162.15 ± 0.82

*Mean values ± Standard deviation

The modified method of Bejenaru et al. (2017) was used to evaluate the influence of electromagnetic field treatment on pigment production by the yeasts. Briefly, yeast cells were grown under the same conditions as indicated in each of the runs from RSM results. Cultures of the cells in the logarithmic growth phase (48 hours) were exposed to electromagnetic flux intensity of 5 mT for the varying time of 10, 20, 30, 40, 50, and 60 minutes. The tubes containing the culture cells were placed at the Centre of the coil. The temperature during the exposure period was monitored using a hand-held mercury-in-glass thermometer and was found to be 35 ± 7 ^{0}C; it was controlled using a mini fan (Plate 1).

Results and Discussion

Screening of medium components by OFAT experiments

Figure 1 shows the effects of different nitrogen sources, carbon sources, metallic salts, incubation period, incubation temperature, and pH on pigment production. As shown in Fig 1(a), of the five-carbon sources investigated, glucose was found to support the highest pigment production with a titre of 163.62±11.4 µg.mL^{-1} and 145.75±8.1 µg.mL^{-1} by *Sporobolomyces* sp S5 and *Rhodotorula* sp A21 respectively. A similar result was also observed by Panesar et al. (2014), Tran et al., (2019), and Budsabun et al., (2020). Glucose supporting the highest pigment production may be attributed to its easy and efficient assimilation by these organisms. All the nitrogen sources investigated in this study were observed to support pigment production. However, yeast extract best-supported pigmentation with a production titre of 205.15±3.6 µg.mL^{-1} and 168.82±2.2 µg.mL^{-1} by *Rhodotorula* sp A21 and *Sporobolomyces* sp S5 respectively (Figure 1b).

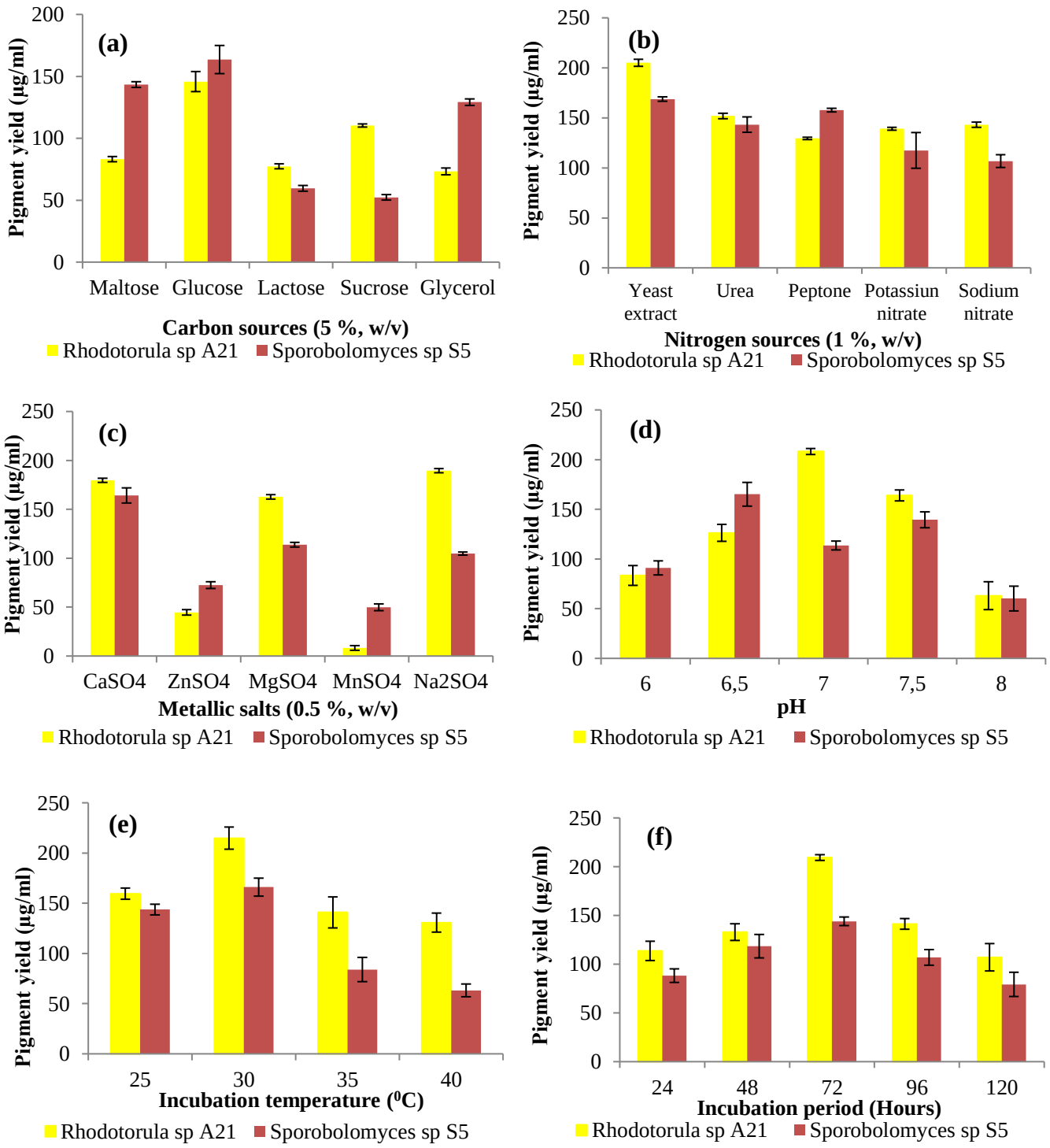

Figure 1. *Single factor experiments showing the effect of different carbon sources (a), nitrogen sources (b), metallic salts (c), pH (d), incubation temperature (e) and incubation period (f) on pigment production by Rhodotorula sp A21 and Sporobolomyces sp S..*

Metallic salts have been reported to play active roles in the pigmentation process by yeasts (Komemushi et al., 1994). In this study, $CaSO_4$ and $MgSO_4$ best-supported pigment production while $MnSO_4$ gave the lowest pigment yield by the two isolates (Figure 1c). Similar results were reported by Buzzini et al. (2005) and Mata-Gomez et al. (2014) in which Mn^{2+} (added as $MnSO_4$) had an inhibitory influence on pigment production by *R. graminis*. Fermentation conditions such as pH, incubation period and incubation temperature were also evaluated and as shown in Figure 1d,

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Sporobolomyces sp S5 and *Rhodotorula* sp A21 could produce pigments over the pH range investigated with a peak yield of 165.18±1.0 µg.mL^{-1} and 208.21±2.01 µg.mL^{-1} obtained at pH 6.5 and pH 7.0 by *Sporobolomyces* sp S5 and *Rhodotorula* sp A21 respectively. Similar results have been reported in the literature that acidic (usually below pH 5.0) and alkaline (above pH 8.0) does not support pigment production (Jissa, 2008). An increase in pigment production was observed with respect to an increase in the incubation period up to 72 hours, with a decrease with further increase in incubation time Figure 1e. This may be attributed to a decrease in the nutrient level in the fermentation medium. Similarly, the effect of different incubation temperatures is shown in Figure 1f. It was observed that 30 ºC best-supported pigment production in both *Sporobolomyces* sp S5 and *Rhodotorula* sp A21. This corresponds with the reports of Latha et al. (2005) and Manimala & Murugesan (2017) that 30 °C was the best incubation temperature for *Rhodotorula graminis* and *Sporobolomyces* sp respectively.

Screening of significant medium components by PB design

Recently, several researchers working on the production of microbial metabolites have employed Plackett Burman and Response Surface Methodology as statistical tools to screen, select and optimize influencing physiological parameters and reported increased yield in various bioprocesses (Cui et al., 2005; Manimala & Murugesan, 2017; Wang et al., 2017; Tran et al., 2019). In this study, the Plackett-Burman experiment design (Table 1) was used to screen and select parameters that have a significant influence on pigment production. From the results obtained and analyzed, and as seen in Figure 2 (the Pareto graph generated), yeast extract (B) and incubation temperature (F) had an apparent influence on pigmentation by *Rhodotorula* sp A21, while glucose (A), the incubation period (G) and pH (E) were found to show significant influence on pigment production by *Sprorobolomyces* sp S5 (Figure 2). These parameters were selected and further optimized using RSM.

Optimization by Response Surface Methodology

The Central Composite Design (CCD) was employed to study the interactions among the significant variables (parameters) based on the results of the Plackett-Burman design. Furthermore, their optimal levels were also investigated. Table 2 shows the results of the central composite experiment design and response value for *Rhodotorula* sp A21. The highest pigment yield observed was 242.48±0.02 µg.mL^{-1} at run 8. The second-order polynomial equation is shown below

$$Y\ (ug.ml^{-1}) = 2707 - 484B - 163.8F + 156.3B^2 + 2.717F^2 + 6.31BF,$$

where Y is the predicted pigment yield, B is yeast extract and F is temperature.

Similarly, the CCD and response value for pigment production by *Sporobolomyces* sp S5 is shown in Table 3, and the second-order polynomial model for pigment yield is expressed in terms of the following equation;

$$Y(ug.ml^{-1}) = -984 + 65.2A + 3.05G + 213E - 4.22A^2 - 0.02620G^2 - 13.8E^2 - 0.0811AG - 2.02AE + 0.231EG,$$

where Y is the predicted pigment yield, A is glucose, E is pH and G is the incubation period.

The result of the regression analysis for pigment production by *Rhodotorula* sp (Table 4a) showed that the model had a low *P*-value of 0.012, which implies that the model fitted the experimental data significantly. The fitness of the model was examined by the determination coefficient (R^2 = 0.8314), which suggests that the sample variance of more than 83 % was attributed to the variables. Also, the result of the regression analysis for pigment production by *Sporobolomyces* sp (shown in Table 4b) indicates that the model had a *P*-value of 0.003.

The fitness of the model as revealed by the determination coefficient (R2 = 89.45 %) indicates that more than 89 % of the total variance could be explained by the model. The linear terms of the independent variables including incubation and pH exerted a significant effect on the pigment yield. Notably, A2, G2, E, and G P-values were higher than the other effects

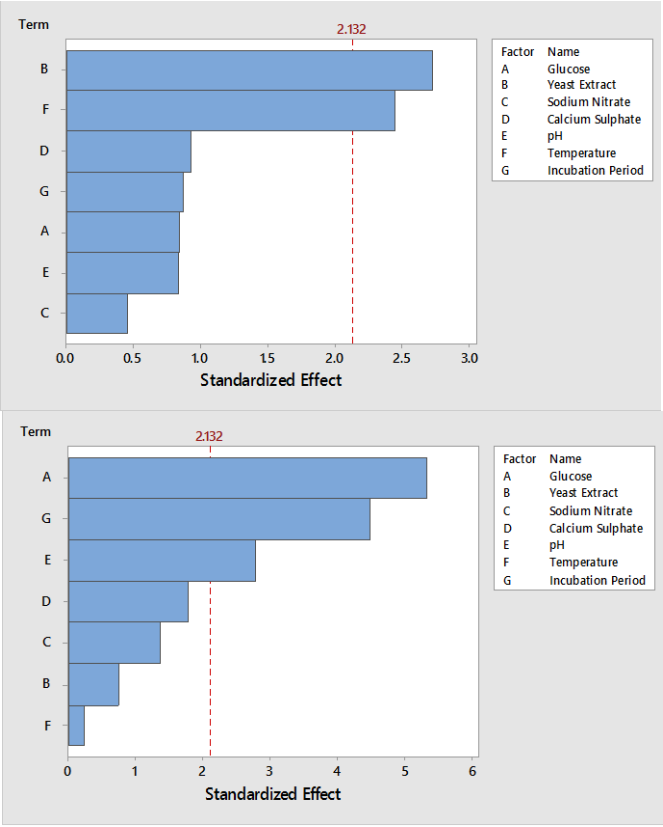

Figure 2. *Pareto graph of the variables on pigment production based on the observation of Plackett-Burman design (a) Rhodotorula sp A21 (b) Sporobolomyces sp S5.*

Table 4a. *Regression analysis of the Central Composite Design for pigment production by Rhodotorula sp A21.*

Source	Coefficient estimate	*F*-Value	*P*-Value	Confidence level (%)
Model	100.3	6.90	0.012	
B: Yeast extract	9.2	0.53	0.488	51.2
F: Temperature	27.7	4.89	0.063	93.7
B*B	39.1	4.48	0.072	92.8
F*F	67.9	13.55	0.008	99.2
B*F	15.8	1.06	0.338	66.2

Table 4b. *Regression analysis of the Central Composite Design for pigment production by Sporobolomyces sp S5.*

Source	Coefficient estimate	*F*-Value	*P*-Value	Confidence level (%)
Model	149.20	7.63	0.003	
A: Glucose	8.95	1.15	0.312	68.8
E: pH	25.89	9.61	0.013	98.7
G: Incubation period	23.36	7.83	0.021	97.9
A*A	-38.0	5.56	0.043	95.7
E*E	-13.8	0.74	0.413	58.7
G*G	-60.4	14.4	0.005	99.5
A*E	-6.07	0.42	0.532	47.8
A*G	-11.68	1.57	0.242	75.8
E*G	11.07	1.41	0.266	73.4

R^2 = 89.45%; R^2_{Adj} = 77.73%

at a 90 % confidence level. The graphical representation, three dimensional (3D) plots, of the regression equations II and III shown in Figure 4 illustrated the individual and interactive effects of the various variables on pigment production. Figures 3a and 3b showed the response surface plots and the corresponding contour plots generated by the predicted models on pigment yields by *Sporobolomyces* sp S5 and *Rhodotorula* sp A21 respectively. The shape of the corresponding surface plots indicated that the mutual interactions among the independent variables were the orientation of principal axes of contour plots (Cui et al., 2005; Manimala & Murugesan, 2017).

Optimization by electromagnetic inducement technique

The influence of 5 mT electromagnetic flux density was evaluated on pigment production at different exposure times, using each of the runs that gave the highest yield in the response surface methodology experiments and the result shown in Figure 4. As shown, there is a decrease in the pigment yield of *Rhodotorula* sp A21 at 10 to 60 minutes duration of exposure as compared with the control. However, electromagnetic treatment resulted in increased yield in *Sporobolomyces* sp S5 at 40-50 minutes of exposure. The disparity in response to EMF treatments by these two organisms could be due to differences in their electromagnetic properties in their cell membranes (Bejenaru et al., 2017).

Conclusion

In this present study, the statistical approaches employed gave relatively increased yields as compared to those obtained from the OFAT experiments and allowed a rapid screening and selection of the influencing physiological parameters on pigment production. There was also an indication that EMF inducement could be used to improve pigment yield as about a 19 % increase was observed in pigment production by *Sporobolomyces* sp S5 at 40 minutes of exposure. There is, therefore, the need for more extensive studies to validate the influence and mechanisms of action of EMF on pigmented yeasts, and further establish the use of electromagnetic fields in biotechnological processes.

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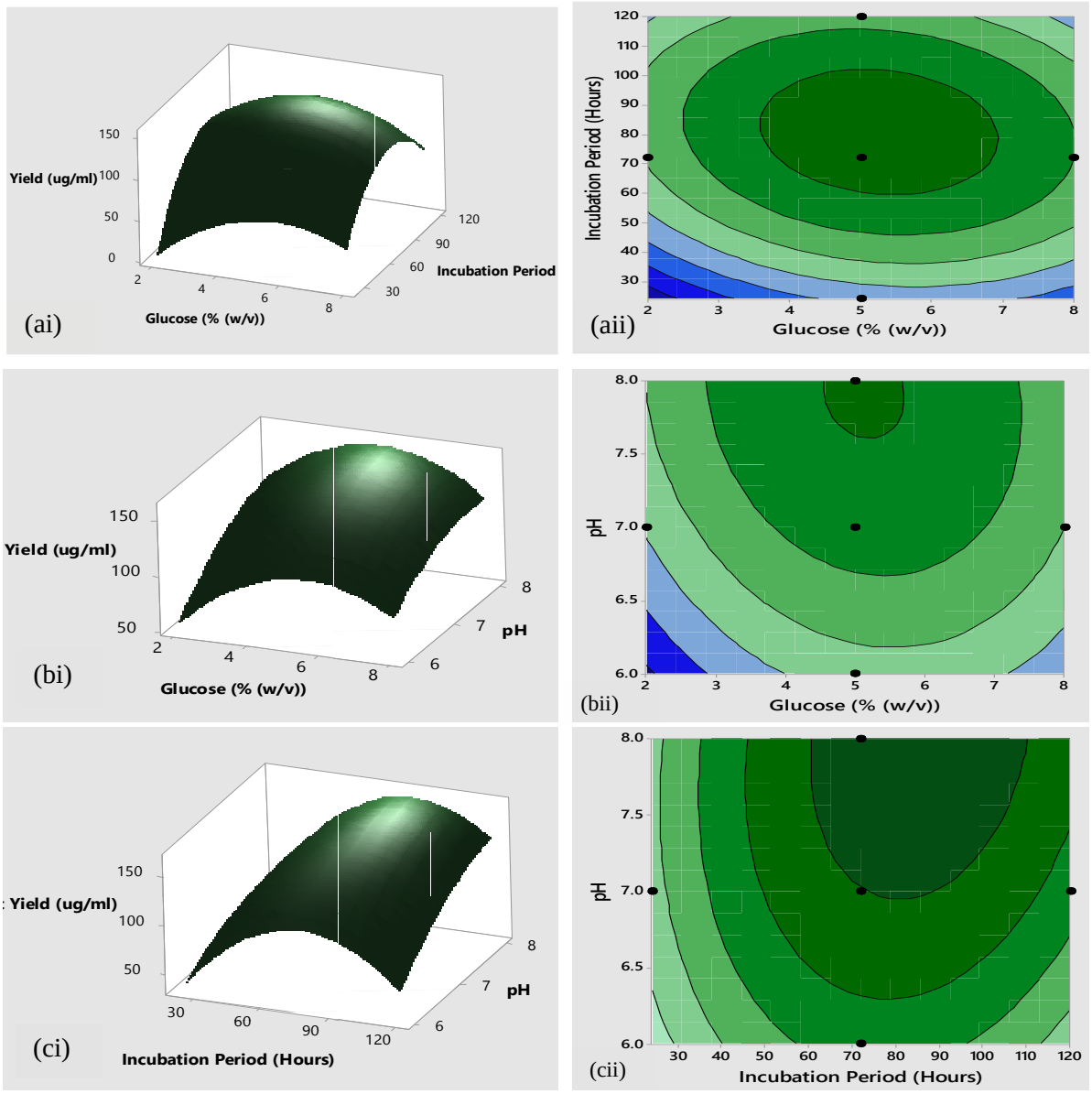

Figure 3a. *Response surface plots of the effects of three most significant parameters on pigment production in Sporobolomyces sp S5. (ai) and (aii): Interaction between A (glucose) and G (Incubation period); (bi) and (bii): interaction between A (glucose) and E (pH); (ci) and (cii): interaction between E (pH) and G (incubation period).*

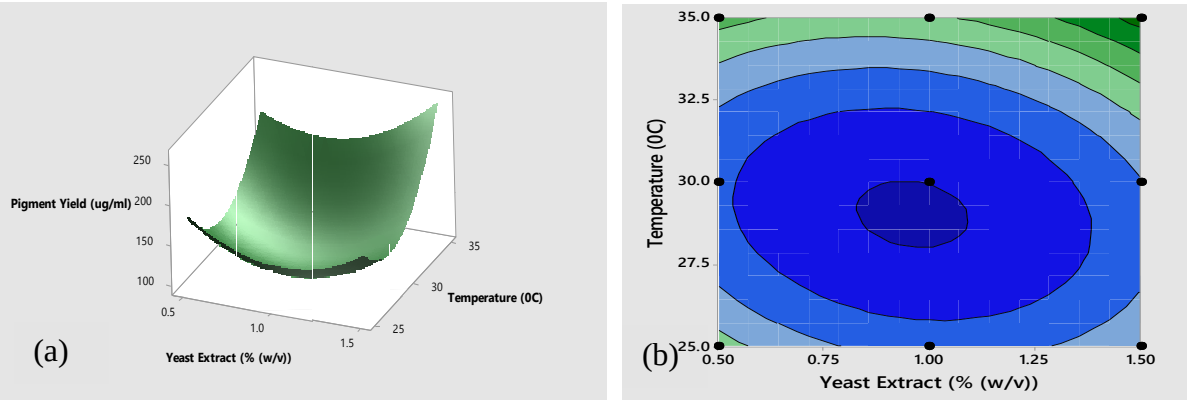

Figure 3b. *Response surface plots (a) and (b) of the effects of yeast extract (B) and temperature (F) on pigment production by Rhodotorula sp A21.*

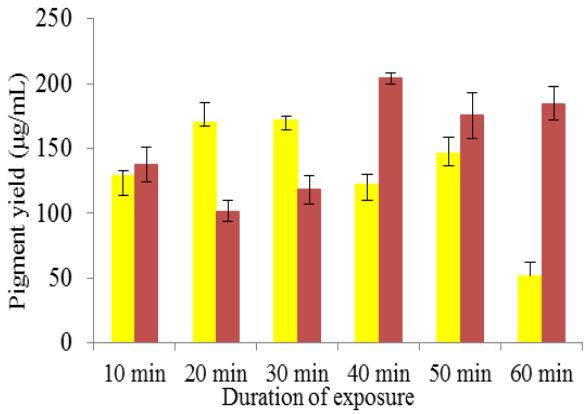

Figure 4. *Influence of electromagnetic inducement on pigment production by Sporobolomyces sp S5 (pink bar) and Rhodotorula sp A21 (yellow bar) under optimized conditions.*

Note: Optimum yields obtained from statistical optimization for Sporobolomyces sp S5 and Rhodotorula sp A21 are 170.34 and 242.48 µg.mL^{-1} respectively.

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