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STATISTICAL OPTIMIZATION OF MEDIUM COMPOSITION FOR MELANIN PRODUCTION BY LOCAL ISOLATE OF *STREPTOMYCES ATROVIRENS* TA4

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ABSTRACT

Melanin pigment has broad applications in medicine, cosmetics, and agriculture. In this study, *Streptomyces atrovirens* TA4, a soil isolate was used for the first time to produce bioactive melanin pigment. This work mainly focuses on media optimization and using statistics to obtain optimum concentrations of the medium composition for maximum pigment production. Starch casein broth was the best melanin pigment-producing medium (127.6 mg/L) among 8 tested media. Central composite design analysis using (Design-Expert-13), a statistics-based method, revealed casein as the most significant factor affecting melanin production with an F-value of 1878.8 and the model exhibited good fitting, with a correlation coefficient of 0.88. An experimental model was generated according to the values of response resolved in the designed experiment and the R^2 value was 0.8174. The suggested optimal concentrations of (starch, casein, KNO3 and Nacl) were (20 g\L, 3 g\L, 0.5 g\L and 1 g\L), respectively. The model's accuracy was determined and results showed that the melanin pigment yield was 123 mg\L which was Approximately matches the predicted value (109 mg\L).

Keywords: central composite design ,pigment , starch casein agar , validation , Response surface methodology.

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INTRODUCTION

Melanin pigment has received massive attention in the last decades, it is a natural brown-black pigment found not only in melanin-producing animal cells, but also in bacteria, fungi, and plants(Cabrera-Valladares *et al*,2006, Rudrappa *et al*,2013). Melanin pigment can be produced by a wide species of bacteria, like *Bacillus*, *Pseudomonas*, *Aeromonas* , *Azotobacter*, *Mycobacterium*, and many species of the *Streptomyces* genus. They offer significant benefits like environmentally friendly and cost-effective over colors derived from synthetic or inorganic sources. These microbial pigments are not poisonous, do not cause cancer, and break down naturally, making them valuable for many industrial applications. Microorganisms can provide a variety of colored byproducts, including bacteriochlorophylls, carotenoids, flavins, indigoids, and melanin (Nuanjohn *et al*,2023). Among the microbial genera exploited for this purpose, *Streptomyces* spp. is the most extensively studied actinomycetes species, they are gram-positive filamentous and can produce a remarkable variety of helpful compounds, including antibiotics, enzymes, antitumor agents, colorful pigments, and antioxidants. These compounds can be used for their antioxidant, anti-inflammatory, and antimicrobial properties (Al zaidy *et al*,2023, Gurme *et al*,2014). Melanin pigments are classified according to their chemical compositions: pyomelanin, pheomelanin, eumelanin, and allomelanin (Colombo *et al*,2019). Pyomelanin is significant and wildly produced by *Streptomyces avermitilis* and other microorganisms like *Ralstonia pickettii*, *Pseudomonas aeruginosa*. Eumelanin, Originating from the oxidative

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polymerization of tyrosine or phenylalanine, the black-brown pigment arises through the subsequent transformation of L-DOPA into melanin. (Ly *et al*,2020, Rana &Umar ,2017). Previous studies examined melanin properties like cleaning out free radicals and chelate metal ions, however, applications of melanin were used in industries, agriculture, and food production (Jang *et al*,2018, Mohammed &Luti,2021). Biomedical aspects, such as radiation protection, magnetic resonance imaging contrast agents, anti-tumor, immunomodulatory, antimicrobial, photothermal properties, and other activities have gradually attracted attention in recent years (Ebrahimi & Zarinpanjeh,2015). Classical optimization of cultural and fermentation conditions by using physical and nutritional parameters is a powerful method that improves the development and designation of any successful cultivation process and fermentation due to their beneficial effects in economics and applications (Ali &Haq, 2010). High cost, time-consuming, and laborious processes are the disadvantages of classical optimization, so, some researchers have developed statistical approaches like Response Surface Methodology (RSM), that help to reduce the number of experiments (Almaliki,2018, El-Zawawy *et al*,2024). The present study aims to use statistical optimization for improving a medium composition by using RSM and central composit design (CCD) for the highest melanin production by *Streptomyces atroverins.*

MATERIALS AND METHODS

Microorganisms: A melanin-producing isolate of *Streptomyces atrovirens* TA4 was used throughout this work. It was isolated from soil and subjected to different morphological and genetic identification to ensure its belonging to this species (data not shown).

Preparation of *Streptomyces* inoculum

Spore inoculum of *Streptomyces sp*. was prepared by cultivating *Streptomyces atrovirens* on Starch Casein (SC) agar for 7 days at 30°C. The culture from the original SC slant was used for inoculation. Five milliliters of sterile distilled water was then added to the grown culture. The spores were gently released by scraping the culture with a sterile loop, and the resulting suspension was collected in a sterile tube. To collect the spores, this spore suspension was centrifuged at 4000 rpm for 10 minutes. Next, the Spores were washed and re-suspended in 1 ml of sterile distilled water. The suspension was then counted using a hemocytometer to determine the number of spores per milliliter. The concentration of spores in this inoculum was adjusted to approximately 1×10^9 spores/mL by adding more sterile distilled water if necessary. (Choi, 2021). The manufacturing company provided virtual technical support, advice, and mathematical equations of the hemocytometer. UV-visible spectroscopy measurement (Shimadzu/ Japan) over 400 nm was used to measure melanin pigment (Nuanjohn *et al*,2023).

Cultivation conditions

A starch casein broth medium was inoculated with a spore suspension of *Streptomyces atrovirens* TA4 for pigment production. After inoculation with the spore suspension, the medium was incubated at 30 °C and pH 7.0 (1×10^9) spores/mL in a rotary shaker at 150 rpm until pigment production was observed. Following a 7-day incubation period, the culture was centrifuged at 13,000 rpm for 15 minutes. This centrifugation step separated the biomass, which was discarded. The supernatant containing the pigment (extra celluler) was then collected and used for the quantitative analysis of melanin, as detailed in the following section.

Quantification of melanin pigment

Melanin was extracted from the culture supernatant by precipitation via adding 6M HCl gradually to obtain pH 2. Then, precipitated melanin was collected and redissolved in a defined volume of DMSO. The absorbance was measured by spectrophotometer (Beckman DU70) at 400nm and melanin concentration was estimated from a standard curve of melanin (Sigma Chemical Co.), which was in a range of 0.1 to 0.5 g/L prepared in DMSO (9,17) .Melanin was quantified by applying a conversion constant

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of 0.1 grams per liter (g/L) per unit of absorbance (OD) at 400 nanometers (nm).

Selection of fermentation medium for pigment production: To select an appropriate medium for maximum melanin production, eight liquid media including peptone yeast extract broth, starch casein broth, peptone yeast iron broth, oatmeal broth, mannitol soya bean broth, dextrose yeast malt broth, glycerol yeast extract broth, and glycerol asparagine broth were screened. Spore suspension of *S. atrovirens* TA4, isolate slanted on mannitol soya bean agar medium(to get a heavy growth culture) , was used for inoculation. All flasks were then incubated in the shaker incubator at 150 rpm and 30ºC for 7 days.After incubation, the biomass in each medium was discarded by centrifugation at 13000 rpm for 15 minutes and the produced melanin was quantified as described above (Ahn et al, 2021).

Medium optimization

Central composite design (CCD) was employed within the framework of response surface methodology (RSM) to optimize media composition for maximizing melanin production. Four variables (starch, casein, KNO_3, and NaCl concentration) were used to determine the optimum concentrations for each factor and their interactions that significantly affect pigment production through a statistical model using (Design-Expert-13) software (Nair *et al*,2023). To evaluate the model, the experimental design and analysis of experimental data were conducted. This included analysis of variance (ANOVA) to assess the model's fit, followed by statistical judgment of the polynomial model equation's quality based on the coefficient of determination (R^2) and its significance using Fisher's test (Anna *et al*,2015).

Response surface methodology based on central composite design: Response surface methodology based on central composite design (CCD) was utilized using design expert software with four variables representing the composition of the selected medium to optimize the pigment production (response). The four variables (Starch, Casein, KNO3, NaCl) and their range (low and high level) uploaded to the RSM program were shows in Table (1).

Table 1. Levels and Ranges of the Independent Variables

Factor	Alpha -α	-1	0	+1	Alpha +α
Starch g/l	-2.5	5	12.5	20	27.5
Casine g/l	-0.75	0.5	1.75	3	4.25
KNO3 g/l	-0.75	0.5	1.75	3	4.25
NaCl g/l	0	1	2	3	4

***Note. +1 upper factorial point. -1 lower factorial point. 0 center point. +α upper axial point. -α lower axial point.**

A central composite design was employed with six replicates at the center point, two replicates for each factorial point, and two replicates for each axial point. Each factor was investigated at four levels. The (Design-Expert Software-13) was used to generate and randomize the data. A total of 30 experiments were conducted, with each experiment representing the interaction between the independent variables within a single flask. Table(2). Flasks were prepared and inoculated in all runs as described earlier in the previous section. The inoculated flasks were incubated at 30°C and 150 rpm for seven days in a rotary shaker incubator. Pigment production was measured at the end of incubation, and obtained data were then analyzed by using the (Design-Expert-13) software.

RESULTS AND DISCUSSION

Bacteria is an important source of pigment such as melanin that is exploited in various commercial applications like cosmetics, pharmaceuticals, and agriculture. In recent years, there has been an increasing interest in the natural pigment produced particularly from microorganisms, compared with those produced by chemical methods due to environmental considerations. *Streptomyces atrovirens* TA4 was used in this study which was obtained from the soil and showed a significant production of brown to black pigment of melanin in early investigations conducted in our laboratory. To the best of our

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knowledge, this is the first observation of the melanin production from *Streptomyces atrovirens* in the literature. This work focused on selecting an optimum medium and designing its composition to support the maximum pigment production using statistical optimization methods.Eight fermentation media were tested to select the best fermentation medium that can be used and optimized to obtain maximum melanin production by *Streptomyces atrovirens*. Based on results presented in Figure(1), starch casein broth (SCB), showed the highest melanin production with 127.6 mg/L, followed by mannitol soybean broth which was 41.7 mg/L. The minimum melanin production was noticed in peptone yeast iron broth and peptone yeast extract broth with 10 and 9mg/L production levels respectively. However, no pigment production observed in dextrose yeast malt broth, glycerol yeast extract, and glycerol asparagine broth. According to the results, starch casein broth was more suitable for melanin production due to the nature of the carbon and nitrogen sources presented in this medium, which induced melanin production compared with other media used in this experiment. Therefore, this medium was used in this work and was subjected to an optimization strategy using response surface methodology. Several researches have documented that starch casein broth was the best medium for growth and production of pigments from *Streptomyces* spp. In this context, (Njoku &Otisi,2023) found that starch casein agar was the best medium for the growth of *Streptomyces*. In addition, Al-Tekreeti and Luti (Al-Tekreeti &Luti,2023) reported that starch casein broth was the better medium to support bacterial growth and production of bioactive yellow pigment by *Streptomyces thinghirensis* AF7 among different media. As mentioned earlier, the main target for this work was to design an optimum medium that supports the maximum production of melanin from *Streptomyces atrovirens* TA4. The optimization of the medium composition plays a vital role in the cost-effectiveness of microbial *secondary metabolites* production (Sholkamy *et al*,2020). The classical method of optimization of culture conditions involves changing one independent factor at a time while keeping others at a fixed level. This method failed to find the true optimum as the interaction between parameters is not considered. while the optimization by statistical method helps us to construct an approximation model that is used to study the interaction between numbers of fermentation variables and decrease the number of experiments (Al-Tekreeti *et al*,2023, Mahmood et al,2020). In this study, response surface methodology was employed as the experimental design to optimize melanin production. Data analysis was performed using Design Expert 13 software based on a central composite design (CCD) incorporating four parameters: starch, casein, KNO3 concentration, and NaCl. The experimental design consisted of 30 runs, and The independent variables, as shown in Table(2), were studied at four distinct levels. Production was estimated by measuring pigment concentration, which was then analyzed using (Design-Expert-13) software. Table (2) indicates the actual and predicted values of the response variable (melanin pigment).According to the values of response resolved in the designed experiment, an improved model was generated. The insignificant coefficients represented by terms (A and D) with p-values greater than 0.5 were excluded. The B, C, AB, AC, AD, BC, BD, and CD are significant model terms. Y= 36.5406667- 1.69791667* A+8.84791667* B-5.46708333* C-3.70791667* D+5.735625 * A * B -13.704375 * A * C-6.015625* A * D-9.751875* B * C-8.428125* B * D+9.706875* `Where, (Y) denoted the predicted melanin response in mg/L and A, B, C, and D denoted starch, casein, KNO3, and NaCl concentration in g/L,respectively. The significance of the experimental model was detected and the variance for the response surface was analyzed using the Fisher test.

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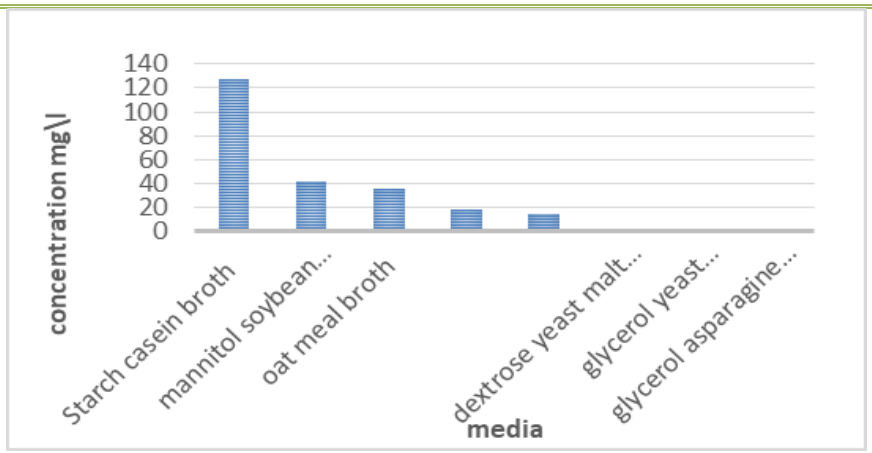

Figure 1. The influence of various broth media on melanin pigment production by *Streptomyces atrovirens*, incubated at 30 C° for 7 days and measured at a wavelength of 400 nanometers

Table 2. The central composite design matrix along with actual and predicted response achieved by (Design Expert 13) Statistical Software

std	Run	point type	Starch	Casein	KNO3	NaCl	Melanin pigment mg/l	
							actual	predict
2	1	Fact	20	0.5	0.5	1	39.98	44,08
16	2	Fact	20	3	3	3	18.4	15.4
28	3	Center	15	1.75	1.75	2	35	36.5
29	4	Center	15	1.75	1.75	2	37	36.5
19	5	Axial	15	-0.75	1.75	2	20	18
21	6	Axial	15	1.75	-0.75	2	43.8	47.4
10	7	Fact	20	0.5	0.5	3	25.16	22.07
7	8	Fact	10	3	3	1	35.5	32.8
12	9	Fact	20	3	0.5	3	50	53.89
24	10	Axial	15	1.75	1.75	4	30	29
9	11	Fact	10	0.5	0.5	3	16.4	14.77
22	12	Axial	15	1.75	4.25	2	27.1	25.6
8	13	Fact	20	3	3	1	28.5	32
27	14	Center	15	1.75	1.75	2	38	36.5
23	15	Axial	15	1.75	1.75	0	40	43
25	16	Center	15	1.75	1.75	2	33	36.5
26	17	Center	15	1.75	1.75	2	13.464	36.5
13	18	Fact	10	0.5	3	3	62.5	70
1	19	Fact	10	0.5	0.5	1	15.5	12.7
6	20	Fact	20	0.5	3	1	8.7	5.8
11	21	Fact	10	3	0.5	3	26.5	23.6
20	22	Axial	15	4.25	1.75	2	50	54.24
30	23	Center	15	1.75	1.75	2	60.126	36.5
15	24	Fact	10	3	3	3	41.91	40
5	25	Fact	10	0.5	3	1	30.95	29
4	26	Fact	20	3	0.5	1	123	109
14	27	Fact	20	0.5	3	3	24	22.6
17	28	Axial	5	1.75	1.75	2	34	33.14
3	29	Fact	10	3	0.5	1	51.73	55
18	30	Axial	25	1.75	1.75	2	36	39.94

The second-order model is presented in Table (3), where the F-value is 1878.8 and $p<0.0001$, indicating a highly significant model. R^2 (determination coefficient) was used to determine the model's fitness, with an R^2 value of 0.8174. This indicates that 81.7% of the total experiment is explained by the model. Adequate precision, a measure of signal to noise, is desirable with a value greater than 4. The value of 19.0894 reveals that the empirical

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model has an adequate signal, and can be used to navigate the design space. The Pred R Squared (0.7445) was in reasonable agreement with the Adj R-Squared (0.8174). Table(3) shows the coefficient estimates of the equation, along with their corresponding P values. The P value indicates the significance of each factor and its influence on melanin production. Casein is identified in Table (3) as the most significant factor, with an F-value of 23.30898 and a (P value) 0.0001.

Table 3. An analysis of variance (ANOVA) was conducted to assess the quadratic model of melanin production by *Streptomyces atrovirens* TA4, based on the experimental results

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	11271.3617	10	1127.13617	13.983193	< 0.0001	significant
A-starch	69.1901042	1	69.1901042	0.8583689	0.3658	
B-casine	1878.8551	1	1878.8551	23.30898	0.0001	
C-KNO3	717.336004	1	717.336004	8.8992336	0.0076	
D-NaCl	329.967504	1	329.967504	4.0935599	0.0573	
AB	526.358306	1	526.358306	6.5299741	0.0193	
AC	3004.95831	1	3004.95831	37.279358	< 0.0001	
AD	579.003906	1	579.003906	7.1830927	0.0148	
BC	1521.58506	1	1521.58506	18.876706	0.0003	
BD	1136.53266	1	1136.53266	14.099766	0.0013	
CD	1507.57476	1	1507.57476	18.702895	0.0004	
Residual	1531.52335	19	80.6064923			
Lack of Fit	426.646199	14	30.4747285	0.13791	0.9985	not significant
Pure Error	1104.87716	5	220.975431			
Cor Total	12802.8851	29				

Figure (2) shows the predicted values plotted against the actual response values. Notably, the model generates predictions for all the data points. Furthermore, the residual behavior follows a normal distribution, which holds greater significance in validating the statistical model. The values calculated using the predictive quadratic model can be noted that. The experimental values showed satisfactory correlation with the model's predictions, indicating good agreement between them. Therefore, the developed model is well-suited for forecasting melanin pigment production (mg/l media) for the proposed composition.

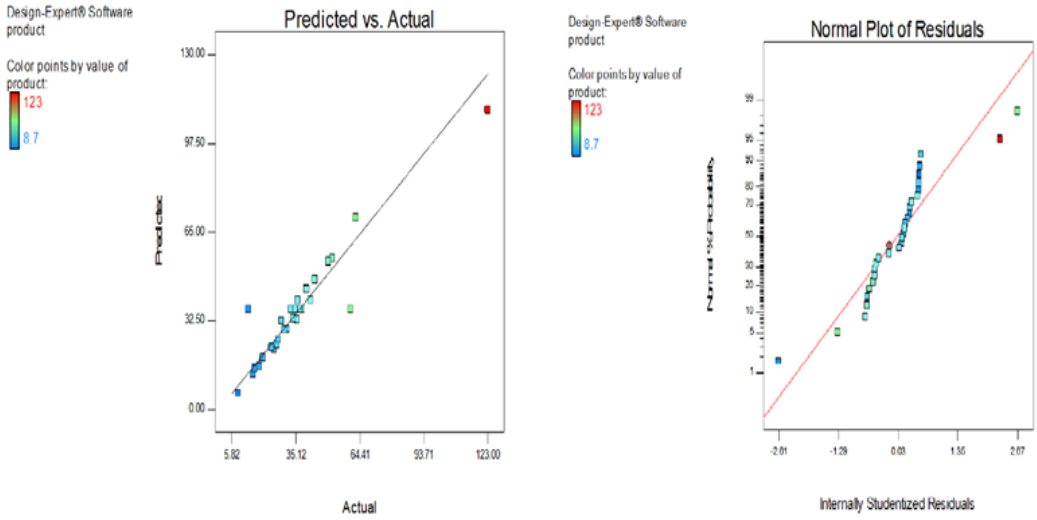

Figure 2. Normal probability plot of standardized residuals of the quadratic model, along with actual versus predicted values for melanin pigment production by *S. atrovirens* based on CCD

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Contour plots were generated to analyze the results of the central composite design and assess melanin production in relation to two independent variables, one at a time, while the other variable was held at zero concentration. These are presented in Figure (4). Figure (4A) depicts the contour plot, where the highest melanin pigment productivity is observed at higher concentrations of starch and casein (20 g/l and 3 g/l), respectively. Whereas, Figure (4B) shows that the lines were changed with the concentration of starch and KNO3 from blue to green, suggesting that the effect of both concentrations on the response was significant. Certainly, both casein and KNO3 provided an adequate amount of nitrogen. source for the microbial cells. This result is further supported by the study of Bundale and his colleagues (Al zaidy *et al*,2023), who found that casein and KNO_3 significantly enhanced the production of bioactive metabolites in the tested isolates. Figure (4C), shows the interaction between starch and Nacl with a concentration of (12.5-20 g/l) and (3.25-4 g/l), respectively, promoting pigment yield. Moreover, Figure (4D) shows that the highest melanin production of (59.379 mg\l) can be obtained with casein concentration (2.25–3 g/l). However, melanin production was decreased when NaCl concentration was beyond (1.5 g/l) suggesting that the effect of casein concentration on the response was significant. Ebrahimi and Nassim mentioned that biomass and secondary metabolite production can be enhanced by casein hydrolysate in the culture medium (D'Ischia *et al*,2013). In their study (Saud &Alaubydi, 2019) reported While sodium chloride (NaCl) is not essential for all microorganisms, it can be an important element for their growth by influencing both the water activity and the osmotic pressure of their surrounding environments. (Saud &Alaubydi, 2019). Furthermore, plot (4E) showed that the highest pigment yield presented in the green spot was observed at (57.69 mg\l) with casein concentration (2.25–3 g/l) while melanin production decreased when KNO_3 concentration was beyond (1.5 g/l). these results suggest a further significant effect of casein concentration on the response, while KNO_3 showed no effect on pigment production. This may be due to the presence of casein in the production medium which provides an adequate amount of nitrogen source instead of KNO3. This is certainly the reason for the increase observed in melanin production. The same result was obtained by Al-Tekreeti and Luti (Mohammed &Luti. 2021). Rana and Umar presented in their study that among nitrogen sources used (potassium nitrate. ammonium sulfate, sodium nitrate, casein, and urea); casein gave a higher amount of tannin in fermentation media (Polapally *et al*,2022). In addition, Figure(4F), showed that the highest pigment yield presented in the green spot was observed at (50.36 mg\l) with KNO_3 concentration (1.3 g\l), however, pigment production was decreased with the increase of NaCl concentration. The pigment production was decreased with the increase of KNO_3 and NaCl concentration.

Validation of Optimum Conditions

To validate the model for the optimum medium predicted by statistical optimization, an optimization plot in the form of a ramp chart was generated using Design Expert 13 software. This plot identified the optimum concentrations of (starch, casein, KNO3, and NaCl) that maximized melanin production based on the enhanced regression model. To verify the optimization results and determine the accuracy of the model, an experiment by shake flask experiments was conducted with the suggested conditions. As can be seen from the ramp chart presented in Figure(4), the suggested optimal concentrations of (starch, casein, KNO3 and Nacl) were (20 g\L, 3 g\L, 0.5 g\L, and 1 g\L), respectively. The results showed that the response of melanin pigment yield of *S. atroviran* was 123 mg\l which is nearly approximate to the predicted value (109 mg\l). (Mohammed *et al*,2018) reported that response surface methodology is a highly beneficial tool for finding the optimum conditions to increase microbial production (Mohammed *et al*,2018).

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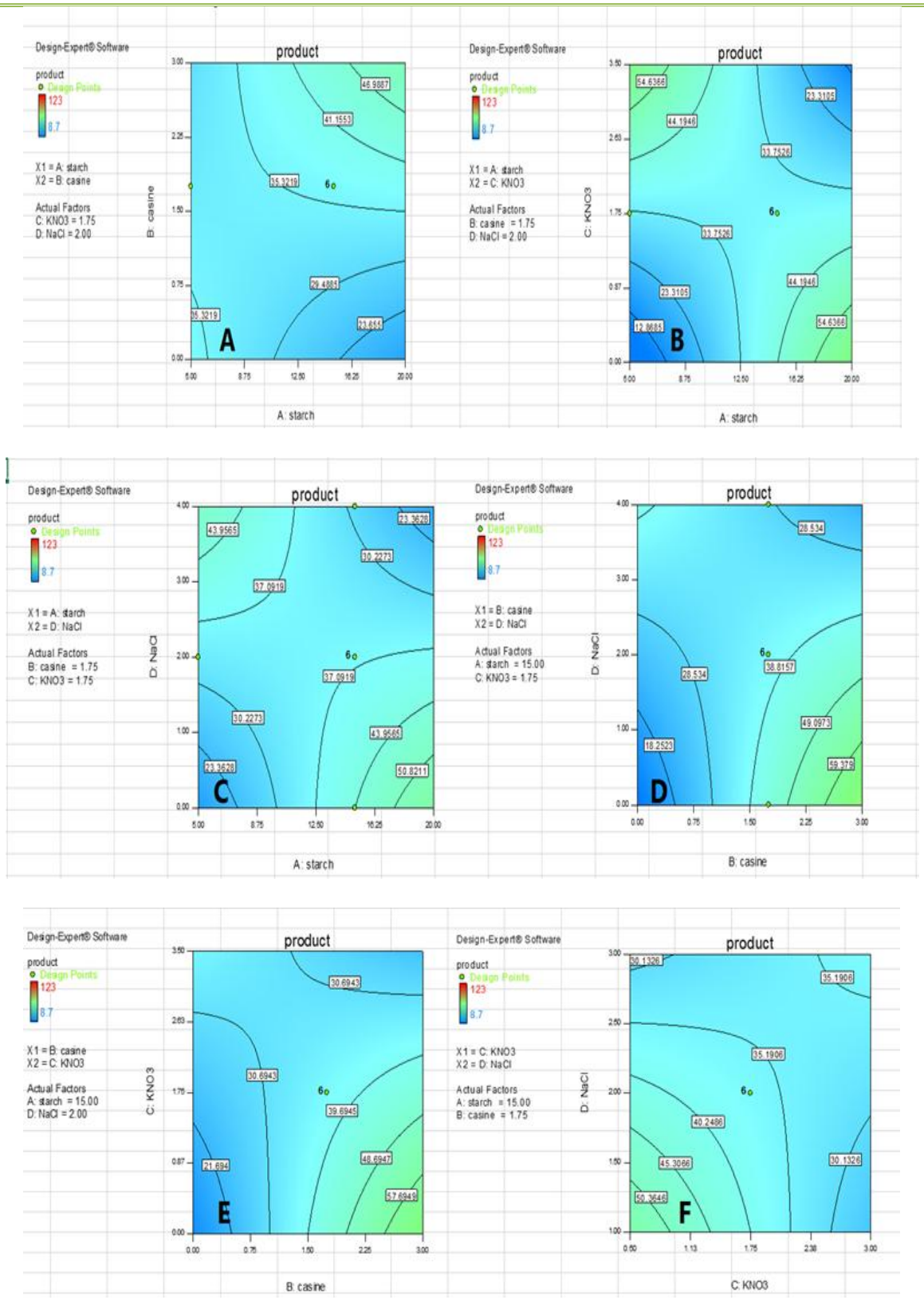

Figure 4. Effect of interaction factors on melanin pigment production by *S. atrovirans*: A. Starch and casein B.Starch and KNO_3 (potassium nitrate),C.Starch and NaCl (sodium chloride), D.Casein and NaCl, E.Casein and KNO_3, F.KNO_3 and NaCl

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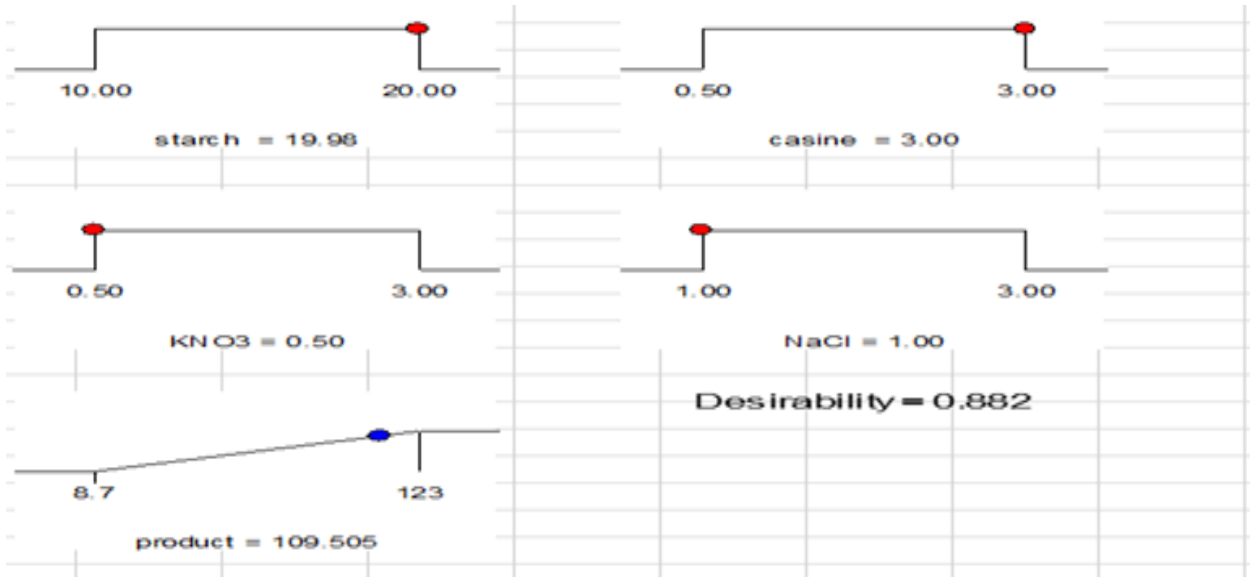

Figure 5. Ramp charts depicting the suggested optimal concentrations of starch, casein, KNO3, and NaCl for maximizing melanin pigment production

CONCLUSION

The current study is focused on determining the optimum composition of the culture medium, using a highly beneficial statistical method (Response surface methodology) to increase the melanin production from *Streptomyces atrovirens* TA4. The results of this research support the effective approach of using statistical strategy to construct a mathematical model (correlation) to predict the output variable (response) depending on the combinations of parameter levels. This approach allows studying the parameters that affect the response and illustrating the relative magnitude and interactions between them which can not be investigated using the classical method of one factor at a time. Therefore, the current findings add to a growing body of literature on using statistical optimization as an effective strategy to reach the true optimum conditions that support maximum production

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare that they have not received a fund.

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التحسين الإحصائي لمكونات الوسط المنتج للميلانين من العزلة المحلية TA4

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المستخلص

صبغة الميلانين لها تطبيقات واسعة في الطب ومستحضرات التجميل والزراعة. تم في هذا العمل لأول مرة استخدام بكتريا المعزولة من التربة في إنتاج صبغة الميلانين النشطة بايولوجيا. يركز هذا العمل بشكل أساسي *Streptomyces atrovirens*TA4 على تحسين الوسط واستخدام الإحصائيات للحصول على التراكيزالمثلى لمكونات الوسط لتحقيق أقصى إنتاج للأصباغ. كان مرق كازين النشا أفضل وسيلة لإنتاج صبغة الميلانين (127.6 ملغم / لتر). منهجية سطح الاستجابة هي الإحصائيات المعتمدة على التصميم أظهر أن الكازين كان العامل الأكثر فعالية في التأثير على إنتاج الميلانين بمعامل (Design Expert 13) المركب المركزي. تم إنشاء نموذج تجريبي وفقا لقيم الاستجابة التي تم واظهرالنموذج ملاءمة جيدة مع معامل ارتباط قدره 0.88 1878,8 تباين) هي KNO3و Nacl 0,8174كانت التراكيز المثالية المقترحة (للنشا والكازين و R^2 حلها في التجربة المصممة وكانت قيمة20 جمالتر و3 جمالتر و0.5 جمالتر و1 جمالتر) على التوالي. كانت إنتاجية صبغة الميلانين 123 ملغم/لتر وهي مقاربة (للقيمةالمتوقعة(109ملغم/لتر).

الكلمات المفتاحية: تصميم المركب المركزي، الصبغة، أجار نشا الكازين، التحقق، منهجية سطح الاستجابة.