RESEARCH ARTICLE

Statistical Design based on Response Surface Methodology to Optimize the Production of a Yellow Pigment by *Streptomyces thinghirensis* AF7

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ABSTRACT

Although its wide utilization in microbial cultures, the one factor-at-a-time method, failed to find the true optimum, this is due to the interaction between optimized parameters which is not taken into account. Therefore, in order to find the true optimum conditions, it is necessary to repeat the one factor-at-a-time method in many sequential experimental runs, which is extremely time-consuming and expensive for many variables. This work is an attempt to enhance bioactive yellow pigment production by *Streptomyces thinghirensis* based on a statistical design. The yellow pigment demonstrated inhibitory effects against *Escherichia coli* and *Staphylococcus aureus* and was characterized by UV-vis spectroscopy which showed lambda maximum of 449. The FTIR and GC-MS analysis showed that the colorings in this type of product are due to the presence of chromo peptides. Furthermore, the GC-MS measurement determined the presence of 4 compounds, as it gave 4 different retention times within this yellow pigment, but with different percentages, except for the compound BHT when the retention time was 17.86 minutes. Starch casein broth (SCB) was selected as an optimized medium for yellow pigment production. The optimization process was first started with one factor at time method, revealing that maltose and casein were the best carbon and nitrogen sources. Response surface methodology based on central composite design was conducted to obtain the optimal combinations of maltose and casein concentrations, pH, and inoculum size for maximum production of yellow pigment. The results showed that casein was the most effective parameter with F-value 393.1 and the model exhibited good fitting with a correlation coefficient of 0.946. Moreover, the actual maximum yellow pigment product 0.80 nm which aggregated with a predicted value 0.835 nm at maltose concentration 8 g/L, casein 5 g/L, KNO3 0.01 g/L, pH 6 and inoculum size 5%.

Keywords: Yellow pigment, *Streptomyces thinghirensis*, Classical optimization, Statistical optimization.

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INTRODUCTION

Streptomyces is gram-positive, filamentous bacteria belonging to the group actinomycetes.[1] They are ubiquitous in soil, conferring the characteristic earthy smell, and they have an important ecological role in the turnover of organic material.[2] Actinomycetes act as a major component of the microbial population in most of the soil. About 90% of the total actinomycetes population consists of *Streptomyces* species.[3] They produce a wide range of secondary metabolites and more than 70% of the naturally derived antibiotics that are currently in clinical use are derived from soil actinomycetes.[4] The genus *Streptomyces,* in particular, accounts for about 80% of the actinomycetes natural products reported to date.[5] In fact, the most interesting property of *Streptomyces* is the ability to produce bioactive secondary metabolites such as antibiotics.[6] Bio-pigments from microorganisms, especially from *Streptomyces* strains, are attractive due to their broad range of activities (i.e. antibiotic, antifungal, anticancer) that make them an excellent target for multifunctional applications.[7]

Optimization of the fermentation process has long been used in enhancing the yield of many bioprocesses. The classical method of 'one factor at a time' approach permits the determination of specific requirements for growth and product formation by systematically adding or deleting components from the medium, with minimal complicated medium interactions.[8] Response surface methodology (RSM), has been extensively applied in the optimization of medium composition, conditions of enzymatic hydrolysis, fermentation, and food manufacturing processes.[9] Response surface methodology is a combination of statistical and mathematical techniques for model constructions, assessing the effect of several independent variables to reach the optimum value of variables to obtain desirable products.[10,11] The traditional 'one factor at a time' (OFAT) is not only effort and time-consuming

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but also often misses in representing the interaction effect between different factors.[12] However, OFAT could be used as a preliminary experiment to determine the effect of medium components on metabolites production,[13] that provide an introductory knowledge for the optimization using a central composite design (CCD) and response surface analysis. Hence, the current research intended to optimize culture conditions of identified Iraqi *Streptomyces thinghirensis* in shake flask to achieve high product of yellow pigment via OFAT and RSM.

MATERIAL AND METHODS

Source of *S. thinghirensis* AF7

S. thinghirensis AF7 was chosen from among 85 *Streptomyces* isolates all were collected from the rhizosphere of plants in the university of Baghdad. The isolation method was as follows: one gram of soil sample was suspended in 9 mL of sterile water and serial dilutions were made up to 10^{-4}. An aliquot (0.1 mL) of suspension from the last dilution test tube was spread on starch casein agar (SCA) with a pH 7 supplemented with tetracycline and nystatin (50 µg/mL) to inhibit unwanted fungal and G- bacterial growth and then, plates were incubated for 7 days at 30°C. *S. thinghirensis* AF7 produced a bioactive yellow pigment which has significant activity against *Staphylococcus aureus* and *E. coli.*

S. thinghirensis AF7 was already identified based on the molecular identification method as follows: the genomic DNA of the selected pigment-producing *Streptomyces* was extracted and purified according to the protocol of ABIOpure. The 16S rRNA gene was amplified by using the universal prokaryotes primers 27F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-TACGGTTACCTTGTTACGACTT-3), the amplification reaction was performed using master mix (Promega, USA), 10 ng/µL of genomic DNA as a template with 10 µM of each primer and the final volume was 25 µL. The amplification program was initiated by [initial denaturation: 95ºC for 1-minute, (denaturation:95ºC for 30 seconds, annealing: 60ºC for 30 seconds, extension: 72ºC for 1-minute) 30 cycles, final extension: 72ºC for 7 minutes]. The amplified product was examined by 0.8% agarose gel electrophoresis stained by ethidium bromide. Macrogen Corporation – Korea, sequenced the purified products. Furthermore, using BLAST software, the determined sequences were compared with the sequences deposited in NCBI GenBank as 16S rDNA gene of different *Streptomyces* species.

UV–vis Spectrophotometer Analysis

UV–vis absorption spectra of the bioactive yellow pigment was determined with a UV–vis spectrophotometer at 300–600 nm to determine the lambda maximum of the selected pigment.

FTIR

FTIR spectrum of yellow pigment was detected using Shimadzu IR-470 plus in the 400 to 4000 cm^{-1} range for the solid pigment without KBr as ATR was used. The data was plotted as intensity versus wave number (Augustine *et al.* 2005).

GC-MS

Yellow pigment was analyzed by gas chromatography-mass spectrometry (GC-MS) using a THERMO GC - TRACE ULTRA VER: 5.0, Thermo MS DSQ II with a TR 5- MS Capillary Standard Non-polar Column (30 m, film 0.25 µm, ID 0.25 mm). The temperature was 80 to 250°C at 8°C/min. The carrier gas was Helium with a 1.0 mL/min flow rate. The chemical constituent was identified using NIST08.LIB library spectra provided by the software on a GC/MS system.

Inoculum Preparation

The spore stock for *S. thinghirensis* AF7 was spread on soya bean agar slant and incubated for 6 days at 30°C to allow sporulation. Distilled water (5 mL) was added to each slant and the surface gently scraped by wire loop to release the spores. Suspensions were harvested by centrifugation and washed twice with distilled water. Inoculum were adjusted to a final concentration of 10^9 spores/mL.

Selection of Medium for Best Pigment Production

The best fermentation medium used for maximum pigment yield was identified by growing *S. thinghirensis* AF7 on three different media, yeast extract malt extract (YEME), starch casein broth (SCB) and mannitol soya bean (MS). The inoculation was carried out using a spore suspension of the isolate slanted on mannitol soya bean agar medium, inoculated in the tested media and kept in an incubator shaker at 150 rpm, 30ºC for 5 days. The culture supernatant containing the crud yellow pigment was separated from mycelium by centrifugation at 10000 rpm for 10 minutes, the absorbance of pigment was estimated using UV–vis spectrophotometer at 449 nm.

Optimization of Batch Fermentation Parameters

Classical optimization (one factor at a time)

Yellow pigment production was primarily optimized by the classical method' one factor at a time' with carbon and nitrogen sources parameters. 5% of inoculum was inoculated into 150 mL Erlenmeyer flask containing 10 g/l starch, 0.30g/l casein, 2 g/l KNO_3, 2 g/l NaCl, 0.05 g/l $MgSO_4$, 2 g/l K_2HPO_4, 0.02 g/l $CaCO_3$ and 0.01 g/l FE_2SO_4 at pH 7 in 50 mL of distilled water. The flasks were incubated on a shaker with 150 rpm at 30°C for 5 days. Various carbon and nitrogen sources were separately added to the production media in two steps, first the carbon sources (glucose, glycerol, mannitol, sucrose, mannitol, methylcellulose, starch, maltose) and second the nitrogen sources (urea, peptone, yeast extract, ammonium chloride, ammonium sulfate, casein, malt extract).

Statistical optimization of medium composition

After the identification of components affecting the production by one factor at a time approach, five variables (casein, KNO_3 and maltose concentrations, inoculum size, and pH) were selected for the statistical optimization using response surface methodology. In this study, the experimental design involved 50 runs and the independent variables were studied at five different levels as shown in Table 1. The data were generated by using the design expert software.

Table 1: Experimental range and levels of the independent factor variables

Factor	*Alpha*	*-1*	*0*	*1*	*Alpha*
Maltose g/l	3.8	8	11	14	18
Casein g/l	1.5	5	7.5	10	13.4
KNO3 g/l	-0.6	0	0.5	1	1.6
pH	4.6	6	7	8	9.3
Inoculum size %	1.6	3	4	5	6.3

*Where: +α: upper axial point; -α: lower axial point; +1: upper factorial point; -1: lower factorial point; 0: canter point.

RESULTS AND DISCUSSION

Pigments from natural sources, such as microorganisms, acquired special attention due to most synthetic dyes' carcinogenic and toxic effects. Some natural pigments have shown notable antimicrobial activity besides providing bright colors; therefore, they could be utilized as functional dyes in many applications such as making colored antimicrobial cloths. In this work, the production of an antimicrobial yellow pigment was studied and optimized based on statistical methods. This pigment was produced from *S. thinghirensis* AF7 which was selected from among 84 local isolates of *Streptomyces* via primary and secondary screening programs. In brief, in primary screening, isolates were selected on the basis of its pigment diffusion ability on starch casein agar (SCA). Out of 84, seven isolates produced different colors varied accordingly from yellow, blue, light blue, black and red (Table 2). Secondary screening was performed via evaluating the antimicrobial potential of pigments produced from the seven *Streptomyces* by well diffusion method against four indicator strains *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter sp* and *Escherichia coli*. The yellow pigment from *Streptomyces* AF7 showed good antimicrobial activity against *S. aureus* and *E. coli*, but did not show activity against *Acinetobacter sp* and *P. aeruginosa.* PCR amplification of 16S rRNA was performed using set of universal primers 27 F and 1492 R resulting in 1500bp to identify the isolate *Streptomyces* AF7. PCR product which was detected on 1% agarose gel electrophoresis, was compared to 100bp ladder marker. As a result of 16S rRNA gene sequencing, the BLAST revealed that yellow pigment-producing isolate was found to be 99% similar to *S. thinghirensis*.

Yellow Pigment Analysis

UV–vis spectrophotometer analysis

The UV–vis spectrum was found by UV–vis spectrophotometer. The spectra showed that the lambda max was at 449 (Figure 1).

FTIR spectrum

The infrared spectrum of the yellow pigment showed a band at position 3440 cm^{-1}, which was attributed to the stretching vibration of the N-H and H-N-H groups. This band appeared as broadband due to the overlap of stretching vibrations of N-H and H-N-H groups. Furthermore, the spectrum showed an intense medium band at 3020 cm^{-1}, which was attributed

Table 2: Screening and isolation of pigment producing *Streptomyces* from soil

Isolate	*Source*	*Diffusible pigment*	*Bioactivity*
Streptomyces AF 1	Abu Nuwas sediment	Blue	Negative
Streptomyces AF 2	Abu Nuwas sediment	Red	Negative
Streptomyces AF 3	rhizosphere of plants	Black	Negative
Streptomyces AF 4	rhizosphere of plants	Light blue	Negative
Streptomyces AF 5	rhizosphere of plants	Red	Negative
Streptomyces AF 6	Abu Nuwas sediment	Black	Negative
Streptomyces AF 7	Abu Nuwas sediment	yellow	Positive

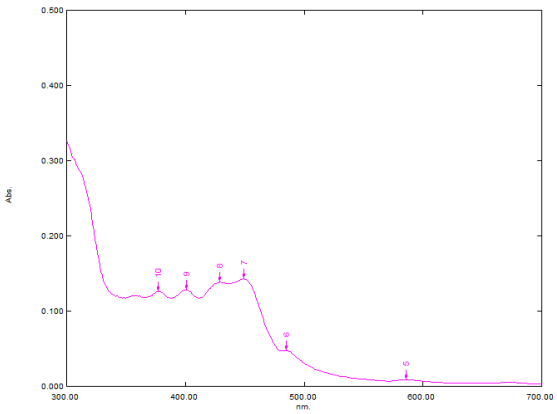

Figure 1: UV–vis absorption spectrum of yellow pigment

to the stretching vibration of aromatic =C-H, while the two weak intensity bands at 2974 and 2839 cm^{-1} were assigned to the stretching vibration of the aliphatic -C-H. Moreover, the spectrum showed the following characteristic bands; 1644, (1604/1519/1477), 1423, 1334, 1211 and (744/667) cm^{-1} which attributed to the vibration of ν (C=O amide), ν(C=C), ν(C=C)/ bending CH_2, ν(C-O)/CH rocking, ν(C-N) and the ν(CH) out of plane for the substituted aromatic rings, respectively (Figure 2).

The colorings in this type of product are due to the presence of chromo peptides, by observing the bands in the infrared spectrum, this yellow pigment can be attributed to the presence of actinomycins, but since there are more than 40 types of actinomycin, depending on the change in the peptide, the existing actinomycin cannot be determined. Furthermore, the GC-MS measurement determined the presence of 4 compounds, as it gave 4 different retention times within this yellow pigment, but with different percentages, except for the compound BHT when the retention time was 17.86 minutes. However, FTIR spectrum of the yellow pigment also shows this molecule's characteristic bands too. while the other compounds were in small percentages, which are likely not to be found in the FTIR spectrum (Figure 2).

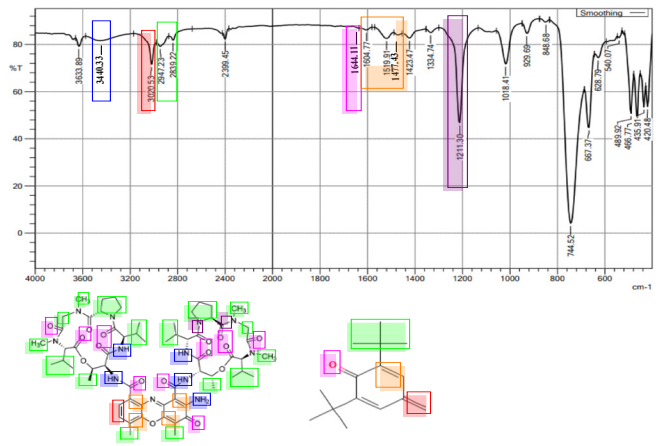

Figure 2: FTIR spectrum of the yellow pigment

GC-MS

The GC-MS measurement of the yellow pigment confirmed the presence of four compounds in the sample, which were BHT-quinone-methide with a retention time of 17.86 minutes with abundance equal to 4800000, as well as tris-(2,4-di-t-butylphenyl) phosphite, a retention time of 46.906 minutes with abundance equal to 1400000 and alpha-mono palmitin with a retention time of 34.406 minutes with abundance equal to 600000, and the lowest abundance was for the compound myristoyl chloride, with a retention time of 36.985 accurate and with abundance equal to 450000. From these percentages, it can be concluded that the percentage of BHT-quinone-methide is 66.20% (Table 2 and Figure 3). Mass fragments of BHT-quinone-methide were illustrated in Figure 4.

Selection of Optimal Fermentation Medium

Three different broth media: yeast extract malt extract (YEME), starch casein broth (SCB) and mannitol soya bean (MS) were tested in order to select the medium that support the highest production of yellow pigment. Based on the results shown in Figure 5, a prominent pigment production was observed in SCB media using a spectrophotometer at 449 nm, in contrast to MS broth, pigmentation was not too low. Therefore SCB medium was selected in the subsequent submerged fermentation process.

Classical Optimization

Effect of carbon sources

In order to select a suitable carbon source for highest yellow pigment production, *S. thinghirensis* was cultivated in the SCB containing various carbon sources. The results revealed that the isolate produced variable levels of pigment based on carbon source used (Figure 6). The best carbon source was maltose, which exhibited high ability to stimulate pigment production in which the optical density was 0.7.V (Karuppiah *et al.,* 2013) concluded that maltose (4.06 g), was required for the maximum production of pigment by the *Streptomyces* sp. PM4. Optimization of medium with maltose increased the pigment yield by 4.6 fold.[14]

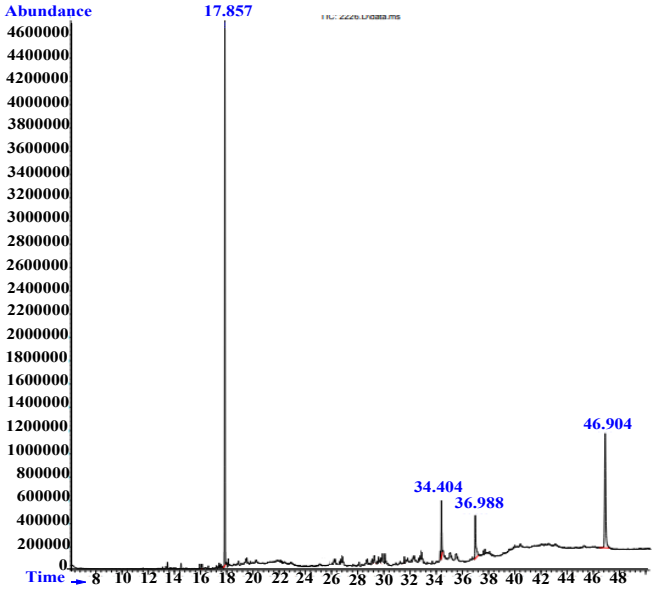

Figure 3: GC graph of the yellow pigment

Table 2: Retention time of the compounds in the yellow pigment

No	*RT (min)*	*Area%*	*Name*	*Quality*	*CAS Number*
1	17.86	39.65	BHT-quinone-methide	99	002607-52-5
2	34.406	5.03	alpha.-Monopalmitin	74	000542-44-9
3	36.985	6.68	Myristoyl chloride	49	000112-64-1
4	46.906	23.09	Tris-(2,4-di-t-butylphenyl) phosphite	83	085454-97-3

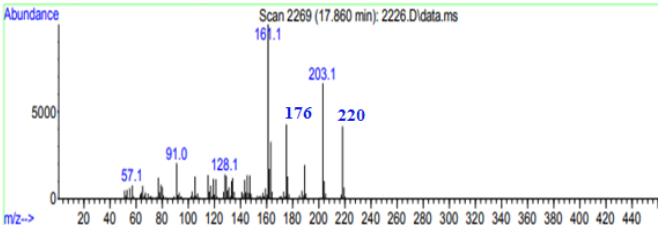

Figure 4: Mass spectrum of BHT-quinone-methide

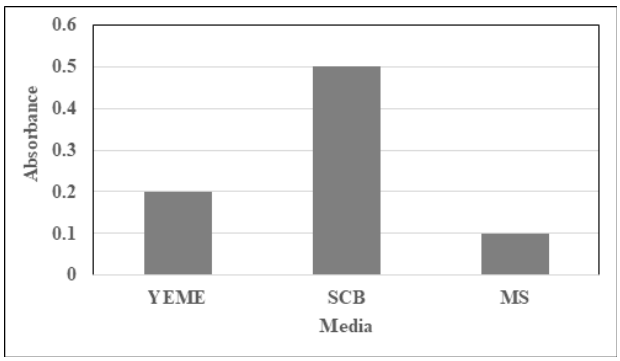

Figure 5: Growth of *S. thinghirensis* on different broth media

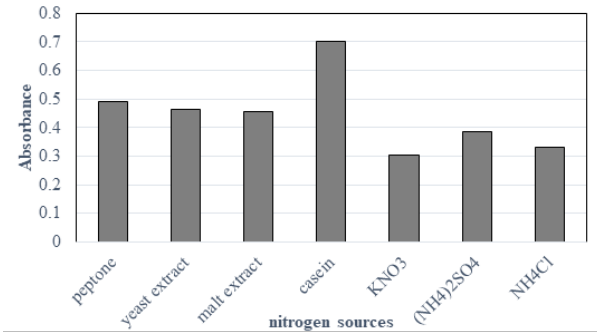

Figure 6: The effect carbon sources on yellow pigment production by *S. thinghirensis*

Effect of nitrogen sources

In the present study, casein was the best nitrogen source that yielded better pigmentation (Figure 7). A previous study conducted by (M. Srinivasan *et al*. 2017) casein, yeast extract, peptone and asparagine were used on the optimization of pigment production from *Streptomyces bellus*, as a result the finest nitrogen sources were found to be casein and peptone.[15]

Statistical Optimization of Medium Composition

Optimization of culture conditions by the classical method involves changing one independent factor at a time while keeping others at a fixed level. Obviously, this method works if no interaction between parameters used is found and hence this method failed to find the true optimum. In contrast, the optimization process based on statistical method helps to minimize the number of experiments and assists in constructing an approximation model that used to study the interaction between numbers of fermentation variables. In this study, 50 experiments with actual and predicted values of response (yellow pigment), were carried out to obtain the optimal combinations of maltose and casein concentration, PH and inoculum size for maximum yellow pigment production.

According to response values and data analysis, and from fit summary analysis, quadratic model is the most suggested model for yellow pigment production with the lake of fit test with *p-value* 0.1653. Analysis of variance, ANOVA, for quadratic model was shown in Table 4, which was performed to check adequacy and significance of model. Model fitness was evaluated using determination coefficient (R2), which is in this case for yellow pigment model. The R2 value was

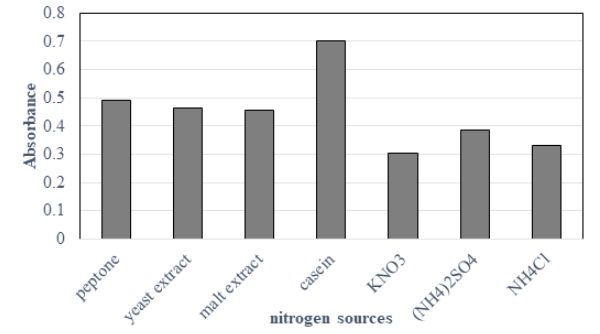

Figure 7: The effect nitrogen sources on yellow pigment production by *S. thinghirensis*

0.9463 indicating that the model did not explain 5.37% of total variation. Adequate precision for yellow pigment was 22.11; this value used for measuring signal to noise which was believed to be desirable greater than 4. The adjusted and predicted determination coefficient for yellow pigment was 0.909 and 0.820, respectively, which are accepted values as the difference between them was less than 0.2. Based on the ANOVA table for yellow production, all interactions and square terms showed insignificant effect for yellow pigment production except AB, B^2 and C^2, which are significant. Since most of the *p-value*, data showed 0.0001 in the ANOVA table. Therefore, the highest significant factors can be determined through F-value.

Moreover, B-casein showed the most significant factor affecting on yellow pigment production with F- value of 393.1 followed by A-maltose with F- value 46.5.

The experimental data were further analyzed by fitting to a second order polynomial model, which was statistically validated by performing analysis of variance equation obtained full actual model on pigment production.

Results of central composite design were more analyzed by contour plots for yellow pigment production against any two independent variables while keeping the other at zero levels were presented in Figures 8-10. The contour plot in Figure 8 shows that the highest productivity of yellow pigment (the red spot) occurred with higher inoculum size and low concentration of casein (1.5 g/l). In addition, Figure 11 shows that the lines were changed with the concentration of maltose from green to blue, while with KNO_3 was constant, suggesting that the effect of potassium nitrate concentration on the response was insignificant. Consequently, it can be said that casein's presence in the production medium provided an adequate amount of nitrogen source instead of KNO_3. Furthermore, from chart 8, it can be seen that the interaction between maltose and pH with a concentration of (3.80–10.90 g/l) and (4.6–5.78), respectively, promote pigment yield.

Furthermore, Figure 8 shows that yellow pigment production increased when inoculum size concentration was higher (6.30–5.13%) and decreased when maltose concentration was beyond the range of 18 g/l (blue spot). Carbon sources are mainly used for cell growth and product formation. However, the production of secondary metabolites usually occurs when carbon source in the media is reduced; that is, the presence of carbon sources would repress the formation of the secondary metabolites.[16]

Moreover, plot 9 showed that the highest pigment yield presented in the red spot, was observed at (1.9 g/l) casein, whereas at (13.4 g/l) level, no effect on pigment production was seen, though KNO_3 has no effect on pigment yield. Figure 9 demonstrated that, at low concentrations of casein, high production was appeared, whereas higher concentrations of casein decreased pigment production. On the other hand, the production was almost constant with different pH values (4.60–9). Maximum yellow pigment production presented in the red plot was observed at very low concentrations of

Table 4: Analysis of variance (ANOVA) for the quadratic modal of yellow pigment production obtained from the experimental results

	Sum of		*Mean*	*F*	*p-value*	
Source	*Squares*	*df*	*Square*	*Value*	*Prob > F*	
Model	4.765741051	20	0.238287	25.56683	< 0.0001	significant
A-Maltose	0.433850003	1	0.43385	46.54962	< 0.0001	
B-casine	3.664621946	1	3.664622	393.1929	< 0.0001	
C-KNO3	0.00064161	1	0.000642	0.068841	0.7949	
D-pH	0.358382737	1	0.358383	38.45241	< 0.0001	
E-inoculum size	0.078129082	1	0.078129	8.382802	0.0071	
AB	0.050562	1	0.050562	5.425012	0.0270	
AC	0.000882	1	0.000882	0.094634	0.7606	
AD	0.0018	1	0.0018	0.19313	0.6636	
AE	0.006498	1	0.006498	0.697198	0.4106	
BC	0.000162	1	0.000162	0.017382	0.8960	
BD	0.010368	1	0.010368	1.112427	0.3003	
BE	0.002592	1	0.002592	0.278107	0.6020	
CD	0.004608	1	0.004608	0.494412	0.4876	
CE	0.0072	1	0.0072	0.772519	0.3867	
DE	1.8E-05	1	1.8E-05	0.001931	0.9652	
A^2	0.040391003	1	0.040391	4.333723	0.0463	
B^2	0.085960464	1	0.08596	9.223064	0.0050	
C^2	0.001312193	1	0.001312	0.140791	0.7102	
D^2	0.00104141	1	0.001041	0.111737	0.7406	
E^2	0.000270852	1	0.000271	0.029061	0.8658	
Residual	0.270284729	29	0.00932			
Lack of Fit	0.234105854	22	0.010641	2.058887	0.1653	not significant
Pure Error	0.036178875	7	0.005168			
Cor Total	5.03602578	49				

Yellow pigment = 0.368031536-0.100082263*A-0.290871991*B-0.003848778*C-0.090962179*D0.042471116* E0.03975 *A*B0.00525*A*C0.0075*A*D-0.01425*A*E-0.00225*B*C0.018 *B*D-0.009*B*E0.012*C*D0.015*C*E 0.00075*D*E-0.026960402*A^20.0 39330858*B^20.004859403* C^20.004329073*D^20.002207752*E^2

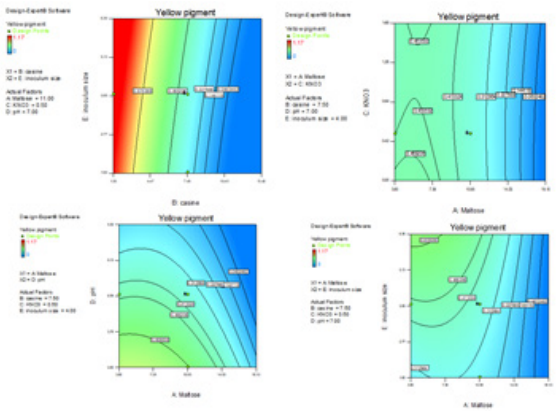

Figure 8: Effect of factors interactions on yellow pigment production by *S. thinghirensis*. A: casein and inoculum size, B: maltose and KNO3, C: maltose with pH, D: maltose with inoculum size.

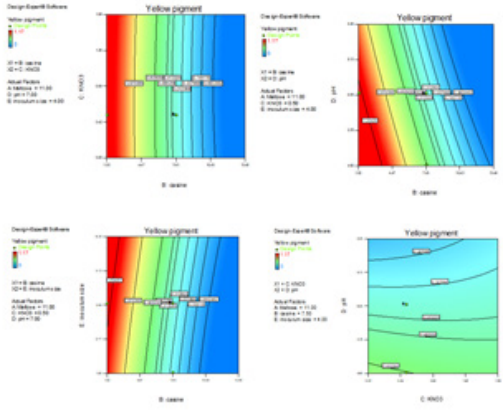

Figure 9: Effect of factors interactions on yellow pigment production by *S. thinghirensis*. A: KNO3 with casein, B: pH with casein, C: inoculum size and casein, D: pH and KNO3.

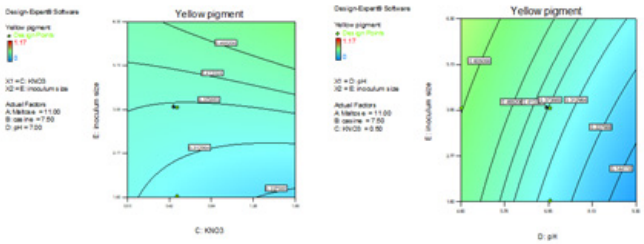

Figure 10: Effect of factors interactions on yellow pigment production by *S. thinghirensis*. A: inoculum size and KNO3, B: of pH and inoculum size

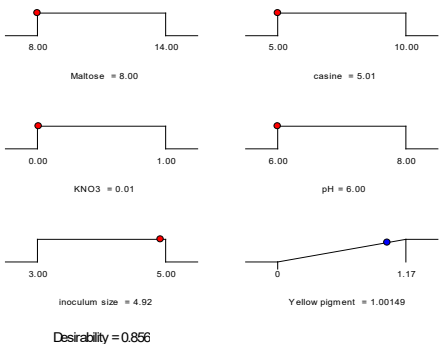

Figure 11: ramp charts of suggested optimal concentrations of maltose, casein, pH, inoculum size and kno3 for maximum yellow pigment production

casein (1.5 g/l), while dramatic decrease in the productivity occurred near the concentrations of (13.4 g/l) nevertheless, different concentrations of inoculum size enhanced high pigment production. The contour plot in Figure 9 also shows that the color is constant with different concentrations of KNO_3, meaning that it is an ineffective factor, and we notice a change in color occurred with different pH levels giving high production at (4.8–5.7).

Finally, the plot in Figure 10 showed that an increase in the inoculum size induced an increase in the pigment yield with the factor KNO_3 having no effect on production. The contour plot in Figure 10 also shows that inoculum size does not affect pigment production, while production increases with decreasing pH value

Validation of Optimum Conditions

The model was validated by conducting experiments in shake flasks under predicted conditions. As can be seen from the ramp chart presented in Figure 9, the suggested optimal concentrations of maltose, casein, KNO_3, pH and inoculum size were 8, 5, 0.01, 6 and 5%, respectively. In order to verify the optimization results and determine accuracy of the model, an experiment was conducted with the suggested conditions. The results showed that response of yellow pigment yield of *S. thinghirensis* was 1.28233 which is nearly approximate to the predicted value (0.856).

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