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Classical and Statistical Optimization of Medium Composition for Promoting Prodigiosin Produced by Local Isolate of Serratia Marcescens

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Abstract

Prodigiosin is a natural red pigment produced by *Serratia marcescens* which exhibits immunosuppressive and anticancer properties in addition to antimicrobial activities. This work presents an attempt to maximize the production of prodigiosin by two different strategies: one factor at time (OFAT) and statistical optimization. The result of OFAT revealed that sucrose and peptone were the best carbon and nitrogen sources for pigment production with concentration of prodigiosin of about 135 mg/ L. This value was increased to 331.6mg/ L with an optimized ratio of C/N (60:40) and reached 356.8 with pH 6 and 2% inoculum size at end of classical optimization. Statistical experimental design based on Response surface methodology was conducted to optimize the composition of trace element. The design revealed that the predicted prodigiosin production of 406 mg/L can be achieved when concentrations of trace element $CaCl_2 \cdot 2H_2O$, $FeSO_4 \cdot 4H_2O$, $MgSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot 4H_2O$ were equal to 9.22, 0.32, 0.67 and 2.48 g/L, respectively. The actual production of prodigiosin in the optimized medium was 375 ± 12 mg/L. Growth kinetics of *S. marcescens* were evaluated in optimized medium which revealed that prodigiosin was 'non-associated growth' secondary metabolite with maximum production of approximately 365.7 mg/L obtained after 54 hours of incubation.

***Keywords**: Classical optimization, prodigiosin, Serratia marcescens , statistical optimization.*

1. Introduction

Prodigiosin is a red pigment produce by Serratia marcescens which is Gram-negative bacteria belong to the family of *Enterobacteriaceae* [1]. It is belong to the family of prodiginines which are a group of tripyrrole red pigments produce as a secondary metabolite by many terrestrial (soil) and marine bacterial strains including species of *Serratia*, mainly *S. marcescens* [2]. In recent years, there has been an increasing interest in prodiginines compounds due to their immune suppressive and anticancer properties in addition to antimicrobial activities [3]. The common way for improving production of antibiotic is to optimize one independent variables while, other independent variables remain constant. However, the experimental results obtained from such strategy are not reflecting the interaction effect between factors. Optimum values of factors are difficult to determine from experiment simply due to large number of experiment required. Therefore statistical optimization technique was developed to reduce the number of experiments that optimize all independent variables in order to maximize the response (dependent variable)as well as build models evaluating effect of factors on response

[4]. The aim of the present study was to find the optimal medium composition for cultivating the local isolate Serratia marcescens in order to improve the production of prodigiosin.

2. Experimental Works

2.1 Microorganisms and Media

Prodigiosin producing isolate of *Serratia* sp. was used throughout this work. It was provided by Fermentation laboratory, Department of Biotechnology, Collage of Science, University of Baghdad. Brain heart infusion was used for maintenance of S. marcescens. A chemically defined liquid medium described by Chen and coworkers (2013) [5] was used for the cultivation of Serratia and production of prodigiosin which contains g/L: (Starch, 10; Peptone, 5; $CaCl_2.2H_2O$, 8.82; $FeSO_4.4H_2O$, 0.33; $MgSO_4.7H_2O$, 0.61; $MnSO_4.4H_2O$, 2). pH was adjusted to 7 prior to autoclaving.

2.2 Preparation of Inocula and Cultivation Methods

Seed culture of *Serratia marcescens* isolate was prepared as follows: a few loop full of bacterial growth from an overnight culture on Brain-heart infusion medium was inoculated into 100 ml Erlenmeyer shake flask contained 20 ml of chemically defined liquid medium. The flask was then incubated for 24 hr in an orbital shaker incubator at 30ºC and 200 rpm which then used as seed culture for inoculating production medium at level of 2% (v/v) under the same incubation conditions for 48h. During the incubation, samples were taken for the analyses of prodigiosin production, biomass and substrate concentration. Each run was conducted either in triplicate or duplicate.

2.3 Classical Optimization

Prodigiosin production was primarily optimized by classical method one factor at time with the parameters carbon and nitrogen sources, C/N ratio, pH and inoculum size%. For this purpose, different carbon sources (glucose glycerol, methanol, sucrose, arabinose, mannitol, mannose, methyl cellulose, starch, maltose) and nitrogen sources (urea, peptone, trypton, yeast extract, ammonium oxalate, ammonium acetate) were investigated. The best concentration ratio of carbon to nitrogen sources that maximize prodigiosin production was studied using different ratios of (C/N) (9/1, 8/2, 7/3, 6/4, 1/1, 4/6, 3/7, 2/8, 1/9). In addition, the effect of pH (5 to 9) and size of seed culture (1 to 5%) were also investigated.

2.4 Statistical Experimental Design

Response surface methodology (RSM) was used to optimize trace elements concentrations (micronutrients) that maximize prodigiosin production based on a statistical method using Box-bhenken with three levels for each factor and design matrix as shown in table (1). The effects of each variable and their interaction as well as the statistical analysis were studied in order to obtain a predict production as explained in the following quadratic equation[6]:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_0 x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k-1} \sum_{i=2}^{k} \beta_{ij} x_i x_j \quad \ldots(1)$$

Where Y is the predicted response, β0 is the intercept term, βi is the linear effect, βii is the squared effect, βij is the interaction effect, and Xi and Xj are input variables that influence the response variable Y.

The experimental design and regression analysis of experimental data was conducted with Design expert7 analysis of variance ANOVA to evaluate the model. The quality of the polynomial model equation was judged statistically by the determination coefficient (R^2), and its statistical significance was determined by Fisher's test [7].

2.5 Growth kinetics Study of Serratia Marcescens

The behavior of *Serratia marcescens* in terms of growth and substrate consumption with time as well as prodigiosin production in the optimized and un-optimized medium was studied. Fifty ml of the chemically defined liquid medium was prepared in 250 ml Erlenmeyer flasks contained. After autoclaving, the flasks were inoculated with the optimal size of *Serratia* inoculum and incubated in an orbital shaker at 30°C and 200rpm for 48hrs. Samples were taken at different time during the incubation period at 0 time and after (3, 6, 12, 15, 18, 33, 48, 51, 54, 63) hours of the incubation for the determination of biomass, substrate concentration and prodigiosin.

2.6 Analytical Methods

Prodigiosin concentration was determined by a colorimetric method [8]. Biomass concentration of *Serratia marcescens* growth was measured as dry weight of cell weight (DCW). Briefly, methanol was used to extract prodigiosin from the cell pellets for at least three hours. Then, centrifuge at 10000 rpm for 10 min was used to remove cell debris and the absorbance at 530 nm of supernatant was determined by spectrophotometer (Aurora instrument Ltd-U.K). The concentration of prodigiosin was detected according to the following equation

Prodigiosin g/ L = ($O.D._{530}$ *323.4 *Dilution factor)/ 7.07 *10^4 ...2

Where: O. D_{530}: Optical density nm; 323.4: molecular weight of prodigiosin; E_{530} =7.07*10^4 M^{-1} cm^{-1} (molar extension coefficient of prodigiosin at 530 nm); Dilution factor = final volume / initial volume

Starch concentration in the fermented broth was determined by iodine test based on the reaction between iodine/potassium iodide and starch (amylose and amylopectin) that resulting a change in the colour [9]. The absorbance is measured at 580 nm. Sucrose concentration was measured in term of glucose concentration after hydrolysis of sample in acid solution [10], the glucose concentration was detected by enzymatic colorimetric method (C cromatest).

3. Results and Discussion

The optimization of cultural parameters that required for elevating prodigiosin production was conducted by classical optimization method such as carbon and nitrogen sources, pH and inoculum size. Some physical parameters that were not investigated in this study have been adjusted according to the literature such as agitation rate in the shaker 200 rpm and temperature 30^0C [5].

3.1 Classical Optimization of Medium Composition

Different carbon sources were investigated to find the best and most suitable carbon source for prodigiosin production. Based on the literature, *S. marcescens* is capable of consuming a wide range of different carbon sources including *monosaccharides* (*e.g.* glucose), *disaccharides (e.g. sucrose and* maltose) as well as polysaccharide (*e.g.* starch) to produce energy and metabolites [11] . According to the results, *S. marcescens* was able to metabolize all types of carbon sources used in this study for growth. As shown in figure (1) prodigiosin production was observed with media supplemented with glycerol, mannitol, mannose, sucrose and maltose and decrease in other media. Maximum production is achieved with sucrose 130.7 mg/L. Prodigiosin as a secondary metabolic product is produced in the stationary phase. In general, simple carbon sources are consumed in the growth phase and therefore, are not supporting secondary metabolite production as it noted in the culture supplemented with glucose.

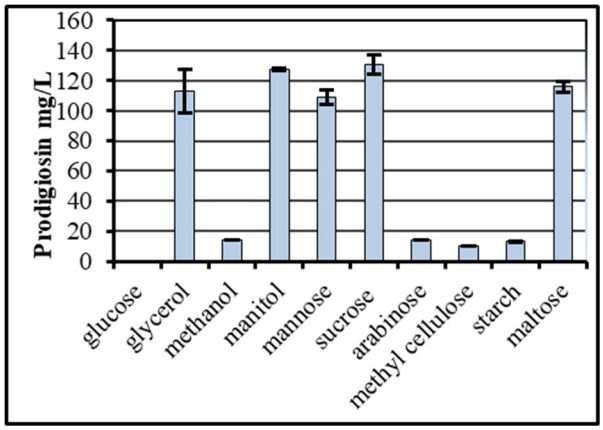


Fig. 1. The effect carbon source on prodigiosin production by S. marcescens in chemically defined liquid medium.

Nitrogen sources cannot be neglected during prodigiosin production and other antibiotics. It has been shown that antibiotic production by many microorganisms is influenced by the type and concentration of nitrogen sources in the culture medium [12]. As shown in figure (2), most of nitrogen sources used in this study except peptone did not support the production of prodigiosin by *S. marcescens.* The maximum prodigiosin production of 139.34 mg/L was achieved in the culture contained peptone as a nitrogen source. Whereas, the minimum pigment production was observed with ammonium acetate 7.47 mg/L. The use of complex nitrogen sources for antibiotic production is favourable because these sources may help to create physiological conditions in trophophase which favour antibiotic production in idiophase [13]. In this context, Wei and coworkers [14] demonstrated that prodigiosin production was increased with peptone because it contains certain amounts of pyrrole amino acid. On the other hand, low prodigiosin production

with ammonium sulfate was observed in the work undertaken by [15].

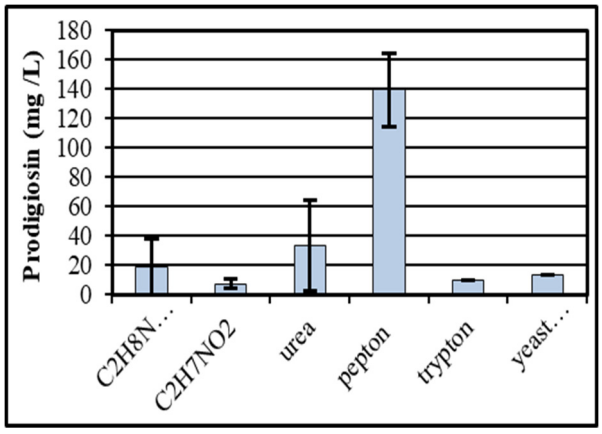


Fig. 2. The effect of nitrogen sources on prodigiosin production by S. marcescens in chemically defined liquid medium.

The requirements of carbon in living organisms are usually larger than nitrogen and therefore the balance between the concentrations of them in the culture medium is an important aspect as it can determine how microorganisms use these sources [16,17]. Thus, in order to enhance the production of prodigiosin, C/N ratios of 1:1,1:9, 2:8, 3:7, 4:6, 6:4 , 7:3 , 8:2 and 9:1in the medium were investigated. It was found that microbial growth and production of prodigiosin was significantly affected at high C/N ratio Figure (3). The optimal C/N concentration ratio of 6/4 was found to be the best ratio that conferred the maximum prodigiosin production. The maximum concentration of prodigiosin obtained in this culture was331.6 mg/L.

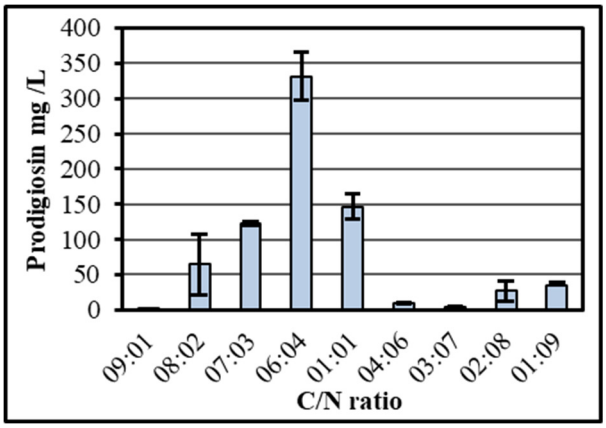


Fig. 3. The effect of carbon to nitrogen ratio on prodigiosin production by S. marcescens in chemically defined liquid medium.

Microbial growth and synthesis of secondary metabolite are highly affected by pH of the medium, therefore it was important to identify the optimum pH that maximize prodigiosin production. As illustrated in Figure (4), maximum production of prodigiosin356.8 mg/L was obtained at pH 6. When the pH of the medium was less or more than 7, prodigiosin production was consequently decreased.

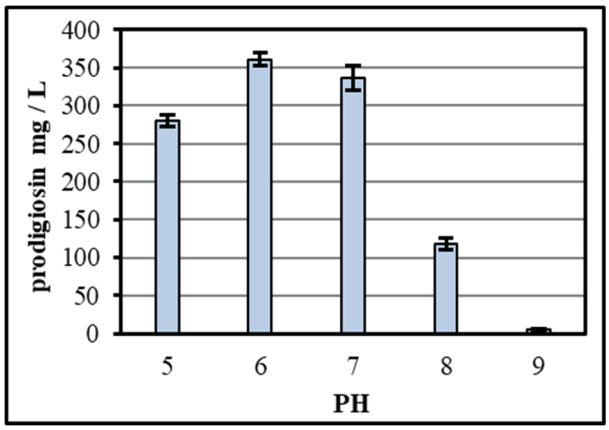


Fig 4. The effect of pH on prodigiosin production by S. marcescens in chemically defined liquid medium.

Inoculum size represents an important factor in the fermentation process that usually affects the production of metabolites. Therefore, this factor was investigated in this study using different inoculum size ranging from 1 to 5% (v/v). As shown in figure (5), maximum prodigiosin production was obtained when inoculum size was used at 2% (v/v) level yield in approximately 354.6 mg/L under the experimental conditions used in this work. The results showed that prodigiosin production was significantly decreased with less or more than that inoculum size. In this context, Mahmoud [18] have found that the best inoculum size for the maximum production of prodigiosin was 2%.

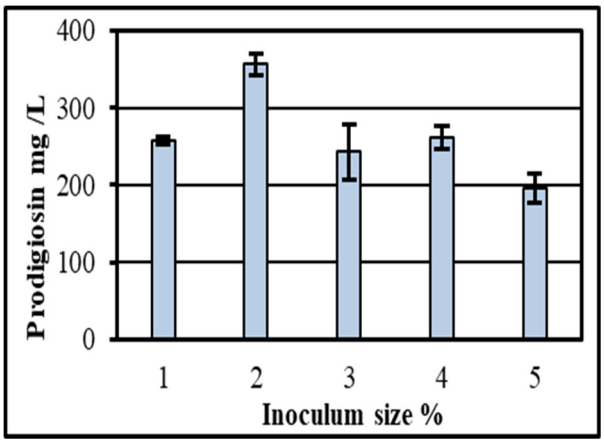


Fig 5. The effect inoculum size on prodigiosin production by S. marcescens in chemically defined liquid medium.

3.2 Statistical Optimization of Medium Composition

In this study, composition of trace element was optimized by statistical experimental design based on Response surface methodology. Four variables representing the concentrations of $CaCl_2 \cdot 2H_2O$, $FeSO_4 \cdot 4H_2O$, $MgSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot 4H_2O$ were selected to determine the optimal composition that maximizes prodigiosin production [19]. The trace compounds were used in three levels for each variable using Box–Behnken design which provided twenty-nine experiments as shown in table (1).

Table 1,
Coded experimental design and results for the response surface of prodigiosin production from *S.* marcescens as a function of trace element concentration.

run	$CaCl_2$ (g/L)	$FeSO_4$ (g/L)	$MgSO_4$ (g/L)	$MnSO_4$ (g/L)	Prodigiosin (mg/L)	
					Actual	Predict
1	9	0.3	0.6	2	391	396.4
2	8	0	0.6	2	15.5	33.78
3	8	0.3	1.2	2	275	261.8
4	10	0.3	0.6	3	450	380.4
5	9	0.3	0.6	2	396	396.4
6	8	0.6	0.6	2	100	110
7	9	0	0.6	1	0.567	26.4
8	9	0.3	0	1	154.4	174.8
9	9	0.3	0	3	259	272.6
10	9	0	0.6	3	0.14	67.76
11	9	0.6	0	2	0.14	22.01
12	10	0.6	0.6	2	65	81.3
13	9	0.3	0.6	2	400	396.4
14	10	0	0.6	2	0.141	24.60
15	8	0.3	0	2	241.6	221.7
16	10	0.3	1.2	2	220.5	255.5
17	9	0.6	1.2	2	110.3	124.9
18	9	0.3	1.2	3	280	294.1
19	9	0	0	2	70	5.57
20	8	0.3	0.6	3	246	230.8
21	8	0.3	0.6	1	313.2	323.1
22	9	0.3	0.6	2	405	396.4
23	10	0.3	0	2	161.6	190.0
24	9	0.6	0.6	1	120	67.7
25	9	0.6	0.6	3	170	159.4
26	9	0.3	1.2	1	238	258.96
27	9	0.3	0.6	2	390	396.4
28	10	0.3	0.6	1	180	145.3
29	9	0	1.2	2	80	8.32

Based on values of response determined in designed experiment, empirical improved model was generated. The insignificant coefficients which are represented by terms (AB, AC, BC and BD) which p-value greater than 0.5 were omitted except $CaCl_2 \cdot 2H_2O$ due to it is hierarchical model [20]:

$$Y = +396.4-9.5(A)+33.26(B)+26.42(C)+33.25(D)+84.30(AD)-70.92(A^2)-263.02(B^2)-93.17(C^2)-53.08(D^2) \quad ...(3)$$

Where Y denoted predicted response of prodigiosin in mg/L and A, B, C, D denoted to CaCl2·2H2O, FeSO4·4H2O, MgSO4·7H2O and MnSO4·4H2O concentration in g/L respectively.

The significance of empirical model was examined by Fisher test and analysis of variance for response surface. Second order model is given in Table (2) in which F-value 32.37 and P<0.0001implies that the model is highly significant. The fitness of model can be determined by determination coefficient (R^2) [21].The R^2value 0.9388 indicated that 93.8% of total experiment are explained by the model. The adequate precision used to measure signal to noise, it is believed to be desirable greater 4.here the value 16.644 revealed that the empirical model is of an adequate signal, and can be used to navigate the design space. The "Pred R Squared" 0.8208 were in reasonable agreement with the" Adj R-Squared" 0.9098. The coefficient estimates of Eq. (3), along with the corresponding P values are given in Table (2). The P value implies the significant of each factor and its important on prodigiosin production. As can be seen in table (2) FeSO4·4H2O* FeSO4·4H2O (B^2) is the most significant with F-value 274.85 and P<0.0001.

Table 2,
Analysis of variance (ANOVA) for the quadratic modal of prodigiosin production obtained from the experimental results

Source	Sum of square	df	Mean square	F-value	P value Prob> F
Model	$5.2*10^5$	9	58607.0	32.3	0.0001*
A	1083.1	1	1083.16	0.6	0.448**
B	13272.8	1	13272.8	7.33	0.0140*
C	8373.56	1	8373.56	4.62	0.0446*
D	13263.6	1	13263.6	7.33	0.0140*
AD	28425.9	1	28425.96	15.7	0.0008*
A^2	32628.7	1	32628.7	18.0	0.0004*
B^2	$4.4*10^5$	1	$4.48*10^5$	274	0.0001*
C^2	56309.3	1	56309.3	31.1	0.0001*
D^2	18277.2	1	18277.2	10.0	0.005*
residual	34400.0	19	1810.53		
Lack of fit	34242.8	15	2282.85	58.0	
Pure error	157.2	4	39.30		0.0007*
Cor total	$5.61*10^5$	28			

R^2=0.9388 R^2adj=0.9098 R^2pred= 0.8208 adeq precision =16.644 *significant**in significant

Response surface methodology provides method of visualizing interaction between independent variables and response by contour plot [22]. The maximum prodigiosin (403.389 mg/L) dominant red region when concentration of $CaCl_2.2H_2O$ was 9.19 g/L and $MnSO_4.4H_2O$ was 2.45 g/L. The minimum prodigiosin production (175.55mg/L) which dominant green region when concentration of $MnSO_4.4H_2O$ and $CaCl_2.2H_2O$ were 1.03 and of 9.9 g/L respectively as explain in figure (6).

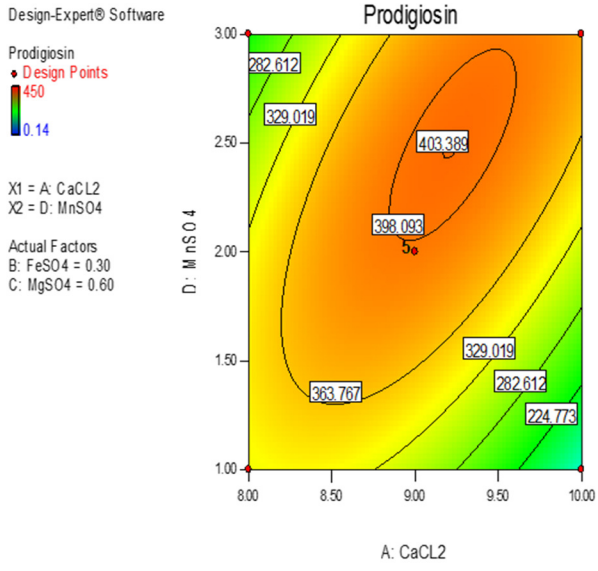
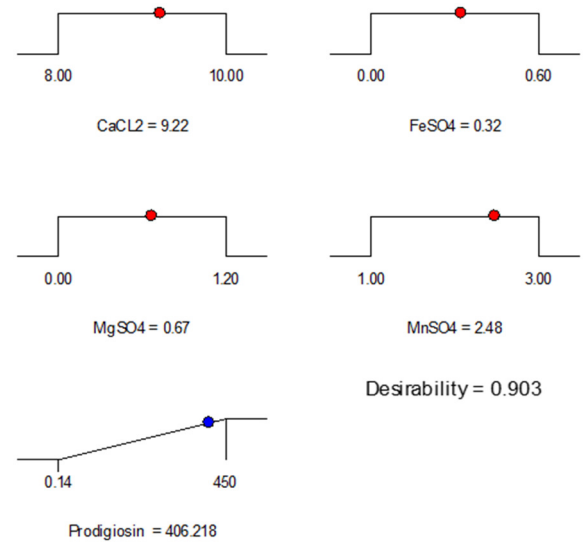


Fig. 6. effect of interaction factors $CaCl_2$ and $MnSO_4$ in medium composition for prodigiosin production.

3.3 Validation of Optimum Condition

Based on the enhanced regression model, optimization plot can be generated using the Design expert 7 software in order to determine the optimum trace element in the medium composition for prodigiosin production. The optimum concentrations of $CaCl_2$ $.2H_2O$, $FeSO_4.4H_2O$, $MgSO_4.7H_2O$ and $MnSO_4.4H_2O$ using statistical media optimization are 9.22, 0.32, 0.67 and 2.48 g/L respectively . The ramps chart for statistical optimization is explained by figure (7) .



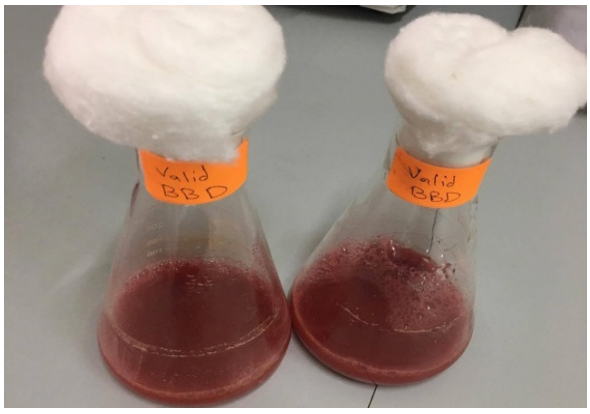


Fig. 7. ramp chart for numerical optimization condition of prodigiosin based on Box –Behnken design matrix

In order to verify the optimization result and determine accuracy of model, an experiment was conducted in duplicate with optimized media contain 9 g/L sucrose, 6 g/L peptone and the optimum concentrations of $CaCl_2$ $.2H_2O$, $FeSO_4.4H_2O$, $MgSO_4.7H_2O$ and $MnSO_4.4H_2O$ as shown in figure (7) with pH value 6 and inoculum size 2%. The results showed that response of prodigiosin yield of S. marcescens was 375 ± 12 mg/L which is nearly approximate to the predicted value .

3.4 Growth kinetic study of S. marcescens in shake flask

S. marcescens produce only low quantities of prodigiosin in un-optimized medium. The onset production of prodigiosin occurred after 6 hrs of inoculation and reached its maximum after 42hr to 10.95 mg/L. The maximum biomass concentration of 22.4 mg/5ml was observed after 33hr in which starch concentration was dropped from 9.5 g/L to 3.9 g/L Figure (8). On the other hand, optimized medium stimulated production of prodigiosin by S. marcescens. As explained in figure (9), production of prodigiosin started after 3hrs after inoculation and reached its maximum of 365.71 mg/L at 54 hr of incubation . Furthermore, maximum biomass obtained was 9.4mg/ml and sucrose concentration dropped from 4.61 g/l to 0.019 g/L during 63 hr.

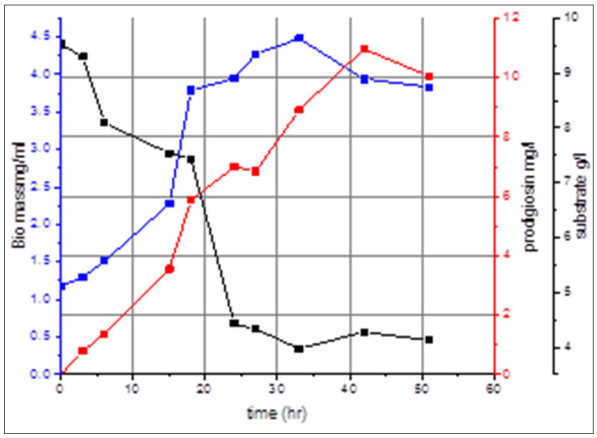


Fig. 8. Time course of cell growth (■), substrate consumption (■) and prodigiosin production (■) by S. marcescens in un-optimized medium in shaker incubator at 200 rpm and 30 °C.

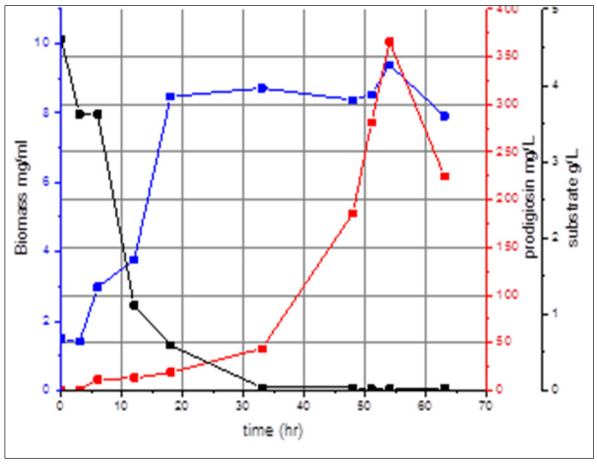


Fig. 9. Time course of cell growth (■), substrate consumption (■) and prodigiosin production (■) by *S. marcescens* in optimized medium in shaker incubator at 200 rpm and 30 °C.

3.5 Kinetic and stoichiometric parameters

The Kinetic and stoichiometric parameters associated to prodigiosin production, growth of *S. marcescens*and substrate consumption in both statistically optimized and un optimized medium in shake flasks are presented. The stoichiometric and kinetics parameters were evaluated according [23].Table 3 presented a comparison between these parameters which showed that the maximum biomass and prodigiosin production was obtained in shake flask experiment using the optimized medium. It was found that, the most notable different in prodigiosin yield was in relation to substrate consumption.

Table 3,
Stoichiometric and kinetic parameters associated with prodigiosin and S. marcescensgrowth calculated from experimental result of shaking flask.

Stoichiometric and kinetic parameters	Shaking flask	
	Optimized medium	Un-optimized medium
X_{max} (mg/ml)	9.4	4.48
P_{max} (mg/L)	365.7	10.95
(%)SΔ	0.9957	0.5826
μ_{max} h^{-1}	0.0733	0.0693
$Y_{X/S}$g/g	1.72	0.594
$Y_{P/S}$ mg/g	79.629	2.0909
$Y_{P/x}$ mg/g	46.29	3.97
P_p (mg/L. h)	6.772	0.2609
q_p(mg_{prod}/g_{cell}.h)	0.720	0.058
μ h^{-1}	0.333	0.1666
t_d h	9.44	9.99034
Ks g/L	0.0196	4.2784

4. Conclusion

Prodigiosin production by *S. marcescens* in the Chen's medium was low (13.1 mg/L) and therefore, medium optimization is necessary to enhance prodigiosin production. Based on results, prodigiosin production significantly increased by 10 fold with sucrose and peptone as carbon and nitrogen sources. Additional increased in prodigiosin production by 3.45 fold was obtained with 60/40 carbon nitrogen ratio and with pH 6 and inoculum size 2%. Further increased was also achieved with RSM to optimize the concentrations of trace elements in the production medium. According to RSM, the maximum predicted prodigiosin production of 406 mg/L can be achieved when concentrations of trace element $CaCl_2 \cdot 2H_2O$, $FeSO_4 \cdot 4H_2O$, $MgSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot 4H_2O$ were 9.22, 0.32, 0.67 and 2.48 g/L respectively. The growth profile of *Serratia marcescens* in shake flake in optimized medium revealed that prodigiosin was 'non-associated growth' secondary metabolite.

Nomenclature

Ks	limiting substrate g/L
Pmax	maximum prodigiosin production g/L
Qp	prodigiosin production to cell per hour (gprodigiosin/gcell.h)
ΔS %	substrate consumption
Td	Biomass doubling time (h)
X	Biomass concentration (g /L)
X max	Maximum biomass concentration (g /L)
YX/S	Yield factor of biomass to substrate
YP/ x	Yield factor of prodigiosin to biomass
μ	growth rate (h-1)
μmax	Maximum specific growth rate (h-1)
DCW	dry cell weight
OD	optical density
BBD	Box-Behnken design
ANOVA	analysis of variance

Acknowledgments

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تحديد الظروف المثلى للوسط المنتج للبرودجيوسين من عزله محليه لبكتريا Serratia marcescens باستخدام الطريقه التقليديه و الاحصائيه

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الخلاصة

يُعد البرودجيوسين واحدا من اهم المواد التي تحمل صفات تؤهلها لتكون ماده اوليه في الصناعة ومن اهم الصفات التي تميز هذا الصبغه الحمراء المايكروبيه المنتجه من قبل بكتريا S.marcescens هي فعاليتها ضد العديد من المايكروبات فضلاً عن الى ان العديد من البحوث التي اشارت الى امكانيه استخدامه كمثبطاً مناعياً و مضاداً للاورام السرطانيه .الدراسة الحاليه صممت لايجاد الظروف المثلى لانتاج الصبغة الحمراء باستخدام الطريقة التقليدية لايجاد الظروف المثلى وقد بينت النتائج ان السكروز يعد افضل مصدر كربون والبيبتون بوصفه أفضل مصدر نايتروجيني 60:40 وافضل نسبة كربون الى نايتروجين ,٦ أفضل رقم هيدروجيني و ٢% افضل حجم لقاح وكانت الانتاجيه في نهاية هذة الخطوة هي ٣٦٥ملغم /لتر . وتضمنت الخطوة الثانية أيجاد افضل التراكيز للاملاح الموجودة في الوسط باستخدام الطريقة الاحصائية لايجاد الظروف المثلى وكانت النتائج لتراكيز الاملاح $CaCl_2{\cdot}2H_2O$, $FeSO_4{\cdot}4H_2O$, $MgSO_4{\cdot}7H_2O$ و $MnSO_4{\cdot}4H_2O$ 9.22, 0.32, 0.67, 2.48 g/L بالتتابع والتي تعطي انتاجية ٤٠٦ ملغم/ لتر بينما القيمة الحقيقية للانتاج12 ± 375 ملغم/ لتر . الخطوة الاخيرة من البحث تضمنت دراسة القوة الحركية لبكتريا S.marcescens في وسط الانتاج المثالي وغير المثالي وأظهرت النتائج ان اعلى انتاجية لصبغة ٣٦٥,٧ ملغم/ لتر بعد ٥٤ ساعة بالاضافة الى ان البروجيوسين يعد ناتجاً غير متعلق بالنمو .