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Classical and Statistical Optimization by Response Surface Methodology For Enhancing Biomass and Bacteriocin Production by *Lactobacillus Plantarum*

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Abstract

Response surface methodology (RSM) based on central composite design was successfully applied to redesign MRS media for maximizing both biomass and bacteriocin production from *Lactobacillus plantarum* NH40. First, glucose and yeast extract were chosen as the best carbon and nitrogen sources based on classical optimization results of one factor at time which also revealed the possibility of eliminating peptone and meat extract from the original composition of medium without affecting the growth and bacteriocin production. Statistical experimental design based on a regression model generated using the Design expert 7 software showed that the optimum concentrations of glucose, yeast extract, tween80, NH_4Cr, CH_3COONa and K_2PO_4 were 40, 19.9, 1, 3.06, 7, 1.25 g/L respectively for maximum production of biomass (15.87 mg/mL) and bacteriocin (634.74 U/mL). In addition, from the analysis of variance, yeast extract with F-value 77.2 and glucose with 185.4 were the most effective factors on biomass and bacteriocin production. Formulation of empirical model explained that the interaction among factors showed that the determination coefficient R^2 of biomass and bacteriocin production were 0.8777 and 0.8539 respectively. Furthermore, the accuracy of model of the optimized MRS medium suggested by design expert 7 for both biomass and bacteriocin was verified and results showed that concentrations of biomass and bacteriocin were 15 mg/mL and 640AU/mL respectively, which were approximately closed to predicted values.

Keywords: optimization; RSM; biomass; Bacteriocin; *Lactobacillus plantarum*

التحسين بالطريقه الكلاسيكيه والإحصائية باستخدام منهجية استجابة السطح لتعزيز انتاج الكتلة الحيوية و البكتريوسين بواسطة *Lactobacillus plantarum*

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الخلاصة

في هذه الدراسة ، تم تطبيق منهجية استجابة سطح (RSM) على أساس التصميم المركب المركزي بنجاح لاعادة تصميم وسط MRS لغرض زيادة إنتاج كل من الكتلة الحيوية والبكتريوسين من بكتريا *Lactobacillus plantarum* NH40. أولًا ، تم اختيار الكلوكوز وخلاصة الخميرة كأفضل مصادر كاربوني ونايتروجيني اعتمادا إلى نتائج تحديد الظروف المثلى بالطريقه الكلاسيكيه باستخدام طريقة العامل واحد والذي كشف أيضًا عن إمكانية الاستغناء عن الببتون ومستخلص اللحوم من التركيبة الأصلية للوسط دون التأثير على نمو وإنتاج البكتريوسين. أظهر التصميم التجريبي الإحصائي المستند إلى نموذج انحدار تم إنشاؤه

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باستخدام برنامج Design expert 7 ، أن التراكيز المثلى للكلوكوز وخلاصة الخميرة والتوين 80 وكرومت الصوديوم و خلات الصوديوم وفوسفات البوتاسيوم كانت 40 ، 19.9 ، 1 ، 3.06 ، 7 ، 1.25 غم/لتر على التوالي حيث بلغ انتاج للكتلة الحيوية 15.87 ملغم/ مل والبكتريوسين 634.74 وحده/مل . بالإضافة إلى ذلك ، ومن خلال تحليل البيانات ، وجد ان مستخلص الخميرة مع قيمة F−77 والكلوكوز مع 185.4 كانت أكثر العوامل تاثيرا في إنتاج الكتلة الحيوية والبكتريوسين. بين النموذج التجريبي أن معامل التحديد R2 للتفاعل بين العوامل للكتلة الحيوية وإنتاج البكتريوسين كانت 0.8777 و 0.8539 على التوالي. تم التحقق من دقة النموذج الأمثل لوسط MRS الذي اقترحه برنامج Design expert 7 لانتاج كل من الكتلة الحيوية والبكتريوسين وقد أظهرت النتائج أن تركيز الكتلة الحيوية والبكتريوسين كانت 15 ملغم /مل و 640 وحدة / مل على التوالي ، والتي كانت مقاربه إلى القيم المتوقعة.

Introduction

Production of both primary and secondary metabolites by microorganisms are strongly affected by the composition of medium such as carbon and nitrogen source and therefor, in order to elevate a particular product, optimization of these factors is strongly required. In industrial biotechnology, the use of a usable, adequate, and low economical cost medium is important as the deployment of a producer microorganism. Unless the medium is satisfying the nutritional needs, the full industrial potential of the microorganism cannot be reached [1]. In fact, not only may the production of the target product be reduced in the incorrect medium, but undesired products such as toxic materials may be produced. Thus, it is imperative that the correct medium is used and formulated to promote the synthesis of the target product which is either cell biomass or a specific metabolite for both laboratory investigations and industrial bioprocessing applications.

Medium optimization is considered to be one of the most critically investigated stage that should be achieved before any large-scale production. Before 1970s, classical methods were used for the optimization of culture media which were expensive and time consuming as it already includes performing many experiments with compromised accuracy [2]. In addition, as this approach based mainly on determine the optimum level of one factor while holding others constant, the interaction between variables is not considered. However, modern mathematical and statistical techniques provided an effective and economical tool for design and optimization of media which became more efficient and robust in obtaining useful results [3].

One of the best methods for process of statistical optimization is response surface methodology (RSM) which is not only determine optimum conditions, but also provide the data necessary to design a process [4,5]. RSM is a collection of mathematical and statistical techniques useful for modeling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response [3].

Lactobacillus spp is one of the most important industrial microorganisms that used widely in bioprocess applications. They have been used for many centuries as starter culture responsible for the fermentation process of a variety of food products such as dairy products, meat and vegetables as well as production of valuable compounds in particular lactic acid [5]. However, one of the most important features that recognize this genus is the production of wide range of bacteriocins with significant antimicrobial activity against wide spectrum of pathogens that make these bacteria to be used as probiotic for human health [6]. In this work, the classical method of one factor at a time and a statistical method based on response surface methodology were used to investigate the minimal nutritional requirements of *Lactobacillus plantarum* NH40 via redesigning the MRS medium in order to maximize biomass and bacteriocin production.

Materials and methods

Microorganism and culture medium

Bacteriocin producing isolate of *Lactobacillus plantarum* NH40 was obtained through screening program of 120 isolates all were collected from ready and homemade yogurt. This isolate was chosen based on its ability to produce an effective and wide spectrum bacteriocin (data not shown). *Lactobacillus plantarum* NH40 was maintained in MRS medium after cultivation at 37^{o}C for 24 hrs under anaerobic condition.

Cultivation conditions

Lactobacillus plantarum NH40 was cultivated for classical and statistical optimization in MRS broth at 37°C for 24 h with 150 rpm in a rotary shaker. In all experiments, inoculum was kept at 2% level contained approximately 1×10^8 cells/mL. After the incubation, samples were taken for the analyses of bacteriocin

Classical optimization

MRS medium was primarily optimized by classical method of one factor at time (OFAT) with two parameters carbon and nitrogen sources. For this purpose, ten different carbon sources (glucose, fructose, arabinose, lactose, xylose, mannose, maltose, dextrose, starch, cellulose) were investigated at 20 g/L to elevate biomass and bacteriocin production by *Lactobacillus plantarum* NH40. MRS medium is already contained three organic components as nitrogen source (peptone, yeast extract, meat extract) which subjected in this study to the optimization procedure in order to choose one source that support both growth and bacteriocin production. The procedure was based mainly on the removal experiment optimization approach as follow: first, each nitrogen source was investigated separately at 24 g/L concentration level, then half amount for each two nitrogen sources and finally equally amount for the three different nitrogen sources.

Statistical experimental design

Further optimization by response surface methodology RSM based on central composite design CCD was achieved to redesign the MRS medium that support both biomass and bacteriocin production. Six parameters (glucose, yeast extract, tween80, NH_4Cr, CH_3COONa and K_2PO_4) were investigated with upper and lower limit pre-coded according to previous studies [7] as shown in Table-1. The design was generated with five replications of center point and data were generate and randomize by central composite design as explained in Table-2. The effect of each variable and their interactions as well as the statistical analysis were studied in order to obtain a predict production of bacteriocin and biomass as explained in the following quadratic equation.

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

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.*

Data analysis

Design expert 7 was used for designing an experimental matrix and regression data analysis. The Model was statistically analyzed to evaluate the analysis of variance (ANOVA). Determination coefficient R^2 was used to express the quality of the fit of polynomial model equation. F-test was used to identify the statistical significant and significance of regression coefficient was determined by t-test [8].

Analytical methods

Determination of growth

The growth of *Lactobacillus plantarum* NH40 isolate was determined as the dry weight of cell material. A known volume of the growth culture was filtered through 0.2µm filter paper which then dried at 60°C and weighted. The difference in weight represented the biomass.

Determination of bacteriocin activity

Bacteriocin activity was detected by agar well diffusion method described by [9], *Lactobacillus plantarum* NH40 was first grown in MRS broth at 37°C for 24 hours in anaerobic conditions. Then, cell free supernatant was obtained by centrifugation at 10000 rpm for 20 min and 4°C. The supernatant was neutralized to pH 6.5 by 1M NaOH to eliminate the effect of lactic acid and 5 mg /100ml of catalase was added to reduce the effect of H_2O_2. Next, the cell free supernatant was filtered through 0.22 millipore unit (cellulose acetate filter). Twofold dilution series of free cells culture supernatant were prepared in PBS pH 7. 100 µl of an overnight growth culture of *Klebsella penumonia* containing approximately 1×10^8 cells /ml was mixed with 25 mL of a sterile Muller Hinton agar kept at 45-50°C in a water bath. The mixtures were kept at the same temperature until poured into sterile plastic Petri dishes and allowed to solidify. Circular wells of 7mm in diameter were cut by using a sterile cork borer and then low melting temperature MHA was used to seal the bottom of the wells. 100µL from each twofold dilution was added to the wells which then incubated aerobically overnight at 37°C. The

highest dilution creating a detectable inhibition zone (DF) was detected which reflected the strength of bacteriocin activity. The arbitrary unites (AU) of bacteriocin activity was calculated using the following equation.

$$\mathrm{AU/ml} = \frac{1}{DF}\frac{1000}{volums\ spotted\ in\ \mu l}$$

Results and discussion

In this work, the minimal nutritional requirements for the cultivation of *Lactobacillus plantarum* NH40 were investigated in order to maximize the production of both biomass and bacteriocin. For this purpose, classical optimization strategy of one factor at time was used to select the best carbon and nitrogen sources. Different carbon sources were investigated to find the ideal and most suitable carbon source for biomass and bacteriocin production. As can be seen in Figure-1, *lactobacillus plantarum* was able to consume all types of carbon sources used in this study with maximum biomass obtained in culture contained glucose. However, bacteriocin production was only observed in media supplemented with glucose, fructose and mannose with 80 Au/ml. Therefore, glucose was selected as the best carbon source with 11.5 mg/mL and 80 Au/ml for biomass and bacteriocin activity respectively. MRS medium contains peptone, yeast extract and meat extract which were subjected to an optimization strategy in order to investigate their effect on biomass and bacteriocin production. Figure-2 shows that most nitrogen sources used supported biomass and bacteriocin production, however, maximum production was observed in culture contained yeast extract as sole nitrogen source with 13.2 mg/mL and 120 AU/mL for biomass and bacteriocin production respectively. These results provide the possibility to eliminate peptone and meat extract from the original composition of MRS medium without affecting the growth and bacteriocin production.

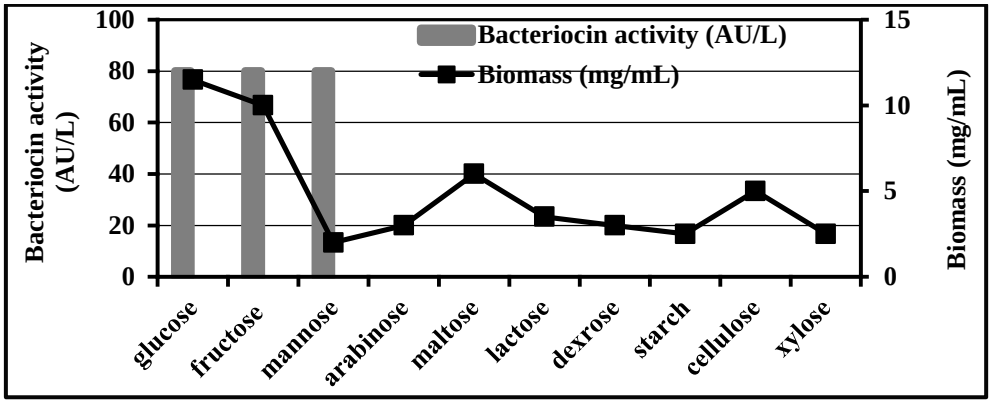

Figure 1-The effect of carbon source on biomass and bacteriocin production by *Lactobacillus plantarum* NH40 in MRS medium

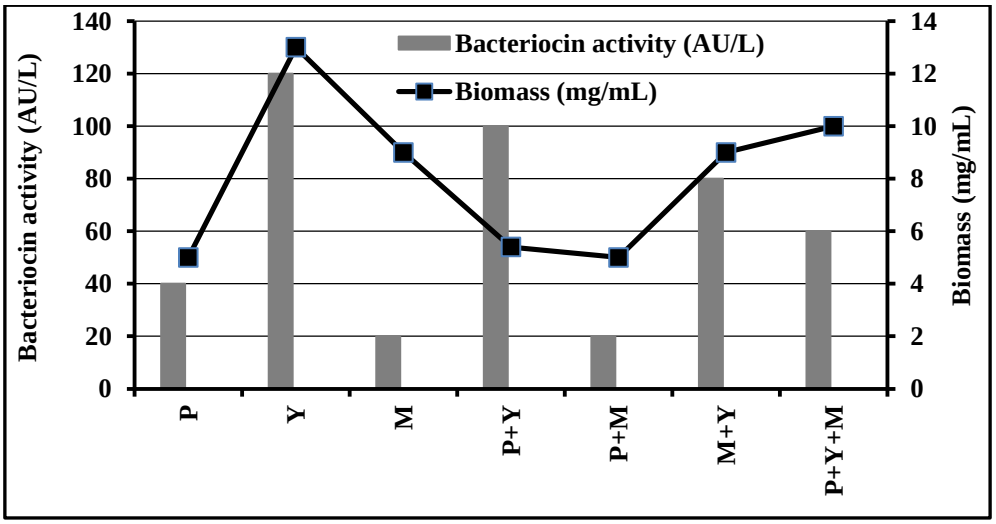

Figure 2-The effect nitrogen sources (Peptone (P), Yeast extract (Y), Meat extract (M)) on biomass and bacteriocin production by *Lactobacillus plantarum* NH40 in MRS medium.

Statistical optimization

A response surface methodology approach (RSM**)** based on central composite design (CCD) was used to investigate the possible combination of factors represent the composition of MRS medium in order to maximize two responses, biomass and bacteriocin production. As can be noticed from Table - 1, six parameters (glucose, yeast extract, tween80, NH_4Cr, CH_3COONa and K_2PO_4) were investigated with upper and lower limit. The values of alpha in lower level with negative values for selected variables was assumed to be zero [10]. The value of alpha was chosen based on the rotatable hypothesis to ensure a constant variance at points that were equidistant from the center point, and therefore provides equal precision of response estimation in any direction of the design [7]. The values of center point are used to detect curvature in the response (they contribute to the estimation of coefficient of quadratic terms). Therefore, in order to obtain good estimation of experimental error (pure error), the center point was replicated six times. In addition, the axial point is used to estimate the coefficient of quadratic terms, whereas factorial points are used mainly to estimate the coefficients of linear terms and two-way interactions [11]. On the other hand, a considerable number of previous studies have mentioned for the important role of tween 80 in bacteriocin production and therefore, it was investigated as one of the variables uploaded to the software with lower limit of zero [12]. The data were generated by using the design expert 7 Software with total numbers of experiments of 82 runs each one represented the interaction between the independent variables in one flask (Table-2).

Table 1-The upper and lower limit of each factor in MRS medium

	Factor	Unit	-alpha	-1	0	+1	+alpha
A	glucose	g/ L	0.86	20	30	40	58.28
B	yeast extract	g/ L	0.86	10	15	20	29.1
C	tween 80	g/ L	-0.91	0	0.5	1	1.91
D	NH_4Cr	g/ L	-1.74	1	2.5	4	6.74
E	CH_3COONa	g/ L	-0.66	3	5	7	10.66
F	K_2PO_4	g/ L	-1.74	1	2.5	4	6.74

Table 2-he central composite design matrix in uncoded units along with actual (Act) and predicted (Pred) response for six parameters (A: glucose, B: yeast extract, C: tween 80, D: NH_4Cr, E: CH_3COONa, F: K_2PO_4)

			MRS composition						Biomass (mg/mL)		Bacteriocin (Au/L)	
std	run	point	A	B	C	D	E	F	Act	Pred	Act	Pred
78	1	Center	30	15	0.5	2.5	5	2.5	15	14.47	320	301.5
29	2	Fact	20	10	1	4	7	1	11.5	10.6	160	173.6
67	3	Axial	30	0.9	0.5	2.5	5	2.5	2	2.01	80	28.4
59	4	Fact	20	20	0	4	7	4	14	12.5	80	117.4
44	5	Fact	40	20	0	4	3	4	14	13.35	640	546.9
56	6	Fact	40	20	1	1	7	4	15	13.04	640	569.3
82	7	Center	30	15	0.5	2.5	5	2.5	14	14.47	320	301.5
54	8	Fact	40	10	1	1	7	4	11	11.9	320	327.9
23	9	Fact	20	20	1	1	7	1	16	15.08	320	310.8
63	10	Fact	20	20	1	4	7	4	15	13.9	80	149.1
53	11	Fact	20	10	1	1	7	4	11.8	11.2	80	101.6
12	12	Fact	40	20	0	4	3	1	14	13.58	320	390
6	13	Fact	40	10	1	1	3	1	10.9	11.1	80	87
50	14	Fact	40	10	0	1	7	4	11	11.2	320	368.1
79	15	Center	30	15	0.5	2.5	5	2.5	16	14.47	320	301.5
81	16	Center	30	15	0.5	2.5	5	2.5	13.1	14.47	320	301.5
25	17	Fact	20	10	0	4	7	1	10	10.07	10	33

60	18	Fact	40	20	0	4	7	4	12.2	13.29	640	660.5
31	19	Fact	20	20	1	4	7	1	15	14.7	320	270.6
33	20	Fact	20	10	0	1	3	4	10	9.57	20	118
77	21	Center	30	15	0.5	2.5	5	2.5	13.9	14.4	320	301.5
35	22	Fact	20	20	0	1	3	4	12	11.38	40	66.5
37	23	Fact	20	10	1	1	3	4	12	10.98	20	70.5
51	24	Fact	20	20	0	1	7	4	11	11.6	80	132.6
49	25	Fact	20	10	0	1	7	4	11	9.6	80	50.01
22	26	Fact	40	10	1	1	7	1	15	13.2	320	371.3
66	27	Axial	58.3	15	0.5	2.5	5	2.5	19	18.8	640	725.3
40	28	Fact	40	20	1	1	3	4	12	12.28	160	226.9
42	29	Fact	40	10	0	4	3	4	13	13.01	320	416
15	30	Fact	20	20	1	4	3	1	15	13.5	160	158.8
13	31	Fact	20	10	1	4	3	1	8.7	9.5	160	196
75	32	Axial	30	15	0.5	2.5	5	1.7	11	10.5	160	198
4	33	Fact	40	20	0	1	3	1	11	10.89	160	213
9	34	Fact	20	10	0	4	3	1	9.9	9.1	80	155.1
48	35	Fact	40	20	1	4	3	4	13.5	13.6	320	387.3
39	36	Fact	20	20	1	1	3	4	13	13.8	160	19.4
52	37	Fact	40	20	0	1	7	4	10.9	11.3	640	608.9
70	38	Axial	30	15	1.91	2.5	5	2.5	14.7	15.02	320	239.3
61	39	Fact	20	10	1	4	7	4	10	10.3	20	41.4
46	40	Fact	40	10	1	4	3	4	12.9	12.07	320	255.9
8	41	Fact	40	20	1	1	3	1	12.8	12.7	160	184
17	42	Fact	20	10	0	1	7	1	9	9.6	40	77.1
47	43	Fact	20	20	1	4	3	4	14	14.1	80	112.9
24	44	Fact	40	20	1	1	7	1	13.9	15	640	602.1
21	45	Fact	20	10	1	1	7	1	11	11.45	320	238.2
72	46	Axial	30	15	0.5	6.7	5	2.5	11.7	11.8	320	254.6
71	47	Axial	30	15	0.5	1.7	5	2.5	9	9.24	80	89.9
3	48	Fact	20	20	0	1	3	1	11	10.44	80	7.8
74	49	Axial	30	15	0.5	2.5	11	2.5	13	13.3	320	328.1
34	50	Fact	40	10	0	1	3	4	10.3	10.7	320	259.4
41	51	Fact	20	10	0	4	3	4	10	10.65	160	208
80	52	Center	30	15	0.5	2.5	5	2.5	14.09	14.47	320	301.5
65	53	Axial	1.72	15	0.5	2.5	5	2.5	15	15.7	160	19.1
76	54	Axial	30	15	0.5	2.5	5	6.7	9	9.8	320	226.5
30	55	Fact	40	10	1	4	7	1	13	13.5	320	373.6
68	56	Axial	30	29	0.5	2.5	5	2.5	7	7.3	320	316.1
38	57	Fact	40	10	1	1	3	4	12	11.23	160	119.9
16	58	Fact	40	20	1	4	3	1	13.2	14.1	320	340
58	59	Fact	40	10	0	4	7	4	14	12.6	320	395.4
1	60	Fact	20	10	0	1	3	1	9	8.06	80	69
32	61	Fact	40	20	1	4	7	1	15	15.7	640	628.7
69	62	Axial	30	15	-0.9	2.5	5	2.5	12	12.05	320	291.2
43	63	Fact	20	20	0	4	3	4	12.5	13	160	180

7	64	Fact	20	20	1	1	3	1	12.7	13.1	20	69.6
62	65	Fact	40	10	1	4	7	4	11	12.05	320	334.6
28	66	Fact	40	20	0	4	7	1	15	15.02	640	579.5
45	67	Fact	20	10	1	4	3	4	11	10.7	40	139.6
18	68	Fact	40	10	0	1	7	1	12	12.3	320	302
10	69	Fact	40	10	0	4	3	1	11.9	12.6	320	270.1
20	70	Fact	40	20	0	1	7	1	13.8	12.98	640	532.2
14	71	Fact	40	10	1	4	3	1	13	12.06	320	219
27	72	Fact	20	20	0	4	7	1	12	13.1	40	129.5
73	73	Axial	30	15	0.5	2.5	-1	2.5	11	10.99	80	16.4
5	74	Fact	20	10	1	1	3	1	9.3	9.72	80	131
26	75	Fact	40	10	0	4	7	1	15	13.8	320	325
2	76	Fact	40	10	0	1	3	1	9.5	10.42	160	117.8
57	77	Fact	20	10	0	4	7	4	10	10.06	160	10.4
55	78	Fact	20	20	1	1	7	4	13.4	14.2	80	185
64	79	Fact	40	20	1	4	7	4	14	13.7	640	600.3
19	80	Fact	20	20	0	1	7	1	12	12.1	80	149.1
36	81	Fact	40	20	0	1	3	4	10.7	10.67	320	365.8
11	82	Fact	20	20	0	4	3	1	11.5	12.1	160	117.1

Based on response values and data analysis of the two responses (biomass and bacteriocin), the quadratic model is the suitable suggested model according to the fit summary analysis which showed that the lake of fit test P-value was 0.6726 and 0.972 for biomass and bacteriocin respectively. Analysis of variance for two responses revealed that many factors showed insignificant effect on responses with P-value >0.1 (data not shown) therefor the model must improve by eliminating insignificant factors except main terms due to hierarchical model [13].

Analysis of variance, ANOVA, for two quadratic improved models are shown in Tables-(3, 4) which performed to check adequacy and significance of the model. Model fitness was evaluated using determination coefficient (R^2) for biomass and bacteriocin model which were 0.8777 and 0.8539 respectively indicating that 12.2% and 14.6% of total variation were not explained by the model. Adequate precision for biomass and bacteriocin were 36.19 and 17.4; these values used for measuring signal to noise which believed to be desirable greater than 4. The adjusted and predicted determination coefficient for biomass (0.8428 and 0.7988) and for bacteriocin (0.8091 and 0.7411) are accepted values as the difference between adjust and predict is less than 0.2.

Table 3-The ANOVA analysis of quadratic reduced model for biomass production based on CCD

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	386.7409	18	21.4856	25.12812	< 0.0001
A-Glucose	23.66804	1	23.66804	27.68055	< 0.0001
B-yeast extract	66.09104	1	66.09104	77.29565	< 0.0001
C-tween80	22.08861	1	22.08861	25.83335	< 0.0001
D-NH_4Cr	16.59546	1	16.59546	19.40894	< 0.0001
E-CH_3COONa	14.32858	1	14.32858	16.75775	0.0001
F-K_2PO_4	1.264254	1	1.264254	1.478587	0.2285*
AB	14.44	1	14.44	16.88806	0.0001
AC	3.330625	1	3.330625	3.895276	0.0528
AD	4.515625	1	4.515625	5.281172	0.0249
AF	5.405625	1	5.405625	6.322057	0.0145

BC	4.730625	1	4.730625	5.532622	0.0218
CD	6.76	1	6.76	7.906043	0.0066
EF	8.555625	1	8.555625	10.00609	0.0024
A^2	13.78248	1	13.78248	16.11906	0.0002
B^2	132.426	1	132.426	154.8765	< 0.0001
D^2	22.29737	1	22.29737	26.07751	< 0.0001
E^2	6.912914	1	6.912914	8.084881	0.0060
F^2	26.68683	1	26.68683	31.21112	< 0.0001
Residual	53.86766	63	0.855042		
Lack of Fit	48.76757	58	0.84082	0.82432	0.6855*
Pure Error	5.100083	5	1.020017		
Cor Total	440.6085	81			

R-sq= 0.877 adj R-sq =0.8428 pred R-sq=0.7988 adeq precession= 36.19 *insignificant

Table 4-The ANOVA analysis of quadratic reduced model for bacteriocin production based on CCD

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	2431796	19	127989.3	19.0729	< 0.0001
A-Glucose	1244418	1	1244418	185.4425	< 0.0001
B-yeast extract	205925.5	1	205925.5	30.68693	< 0.0001
C-tween80	5	1	5	0.000745	0.9783*
D-NH4Cr	68376.13	1	68376.13	10.18938	0.0022
E-CH3COONa	244074.9	1	244074.9	36.37193	< 0.0001
F-K2PO4	1926.177	1	1926.177	0.287038	0.5940*
AB	100806.3	1	100806.3	15.0221	0.0003
AC	33306.25	1	33306.25	4.963283	0.0295
AE	124256.3	1	124256.3	18.51661	< 0.0001
AF	35156.25	1	35156.25	5.238969	0.0255
BE	71556.25	1	71556.25	10.66328	0.0018
CE	39006.25	1	39006.25	5.812694	0.0189
CF	47306.25	1	47306.25	7.049556	0.0101
DE	66306.25	1	66306.25	9.880928	0.0026
EF	23256.25	1	23256.25	3.465636	0.0674
B^2	51833.6	1	51833.6	7.72422	0.0072
D^2	51833.6	1	51833.6	7.72422	0.0072
E^2	51833.6	1	51833.6	7.72422	0.0072
F^2	30953.6	1	30953.6	4.612692	0.0357
Residual	416052.8	62	6710.529		
Lack of Fit	330719.4	57	5802.095	0.339967	0.9802*
Pure Error	85333.33	5	17066.67		
Cor Total	2847849	81			

R-sq= 0.8539 adj R-sq =0.8091 pred R-sq=0.7441adeq precession= 17.43
*insignificant

Based on ANOVA table for biomass and bacteriocin production, all terms show significant effect for the two responses except for K_2PO_4 which is insignificant for biomass and tween 80 and K_2PO_4 for bacteriocin. Since most of P value data shows 0.0001 in the ANOVA table, therefore the highest significant factors can be determined through F- value. Yeast extract shows the most significant factor affecting on biomass production with F-value 77.29 followed by Glucose with F-value 27.68.

Whereas, glucose is the most significant factor affecting on bacteriocin production with F-value equal to 185.44 followed by CH_3COONa with F-value of 36.37.

A regression equation is generated from response surfce methodology which is imperical relationship between tested variables and response. After analysis of variance and estimation of regression coefficient, the experimental design was fitted in second order polynomeal equation 2 and 3 in the coded factors.

Biomass =+14.18+0.54 * A+0.91 * B+0.53 * C+0.46* D +0.42* E -0.13 * F -0.47 *A * B -0.23* A * C+0.27* A * D -0.29* A * F +0.27* B * C -0.32* C * D - 0.37* E * F +0.37 * A^2 -1.13* B^2 -0.47* D^2 -0.26 * E^2 -0.51* F^2……..(2)

Bacteriocin =+335.87+124.72* A+50.74* B+0.25 * C+29.24 * D+55.24* E+4.91* F+39.69 *A* B-22.81* A*C+44.06 * A* E+23.44* A * F+33.44* B * E+24.69* C * E -27.19* C * F-32.19 * D * E-19.06* E * F-22.00* B^2-22.00* D^2 -22.00 * E^2-17.00* F^2.....(3)

Where A: Glucose, B: yeast extract, C: tween 80, D: NH_4Cr, E: CH_3COONa and F: K_2PO_4.

In addition to correlation, regression analyses can be used to assess the best fit of a line using the equation $y = b0 + b1x$. The ideal line of best fit will have as small as possible the sum of squares of distances from x to the line of fit. The diagnostic of normal residual demonstrated in Figure-3 indicate that residual behavior has followed normal distribution and was quadratic, which is an important assumption for checking statistical modeling. On the other hand, the predicted output values versus actual experimental values for biomass and bacteriocin production are presented in Figure-4. From this figure, it can be noted that the values calculated using the predictive quadratic model were in good agreement with the experimental values with a satisfactory correlation between these values. Therefore, the developed model is suitable for predicting biomass and bacteriocin concentration under suggested medium composition.

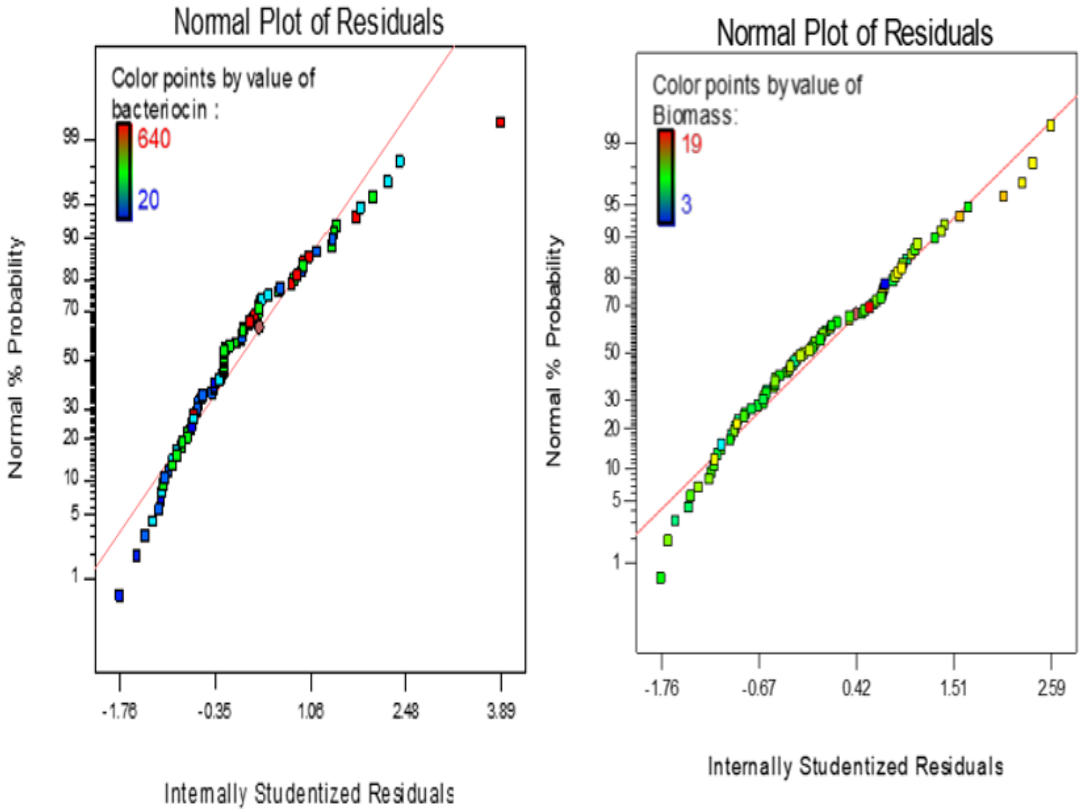

Figure 3-Normal probability plot of standardized residuals of quadratic model based on CCD for biomass and bacteriocin production

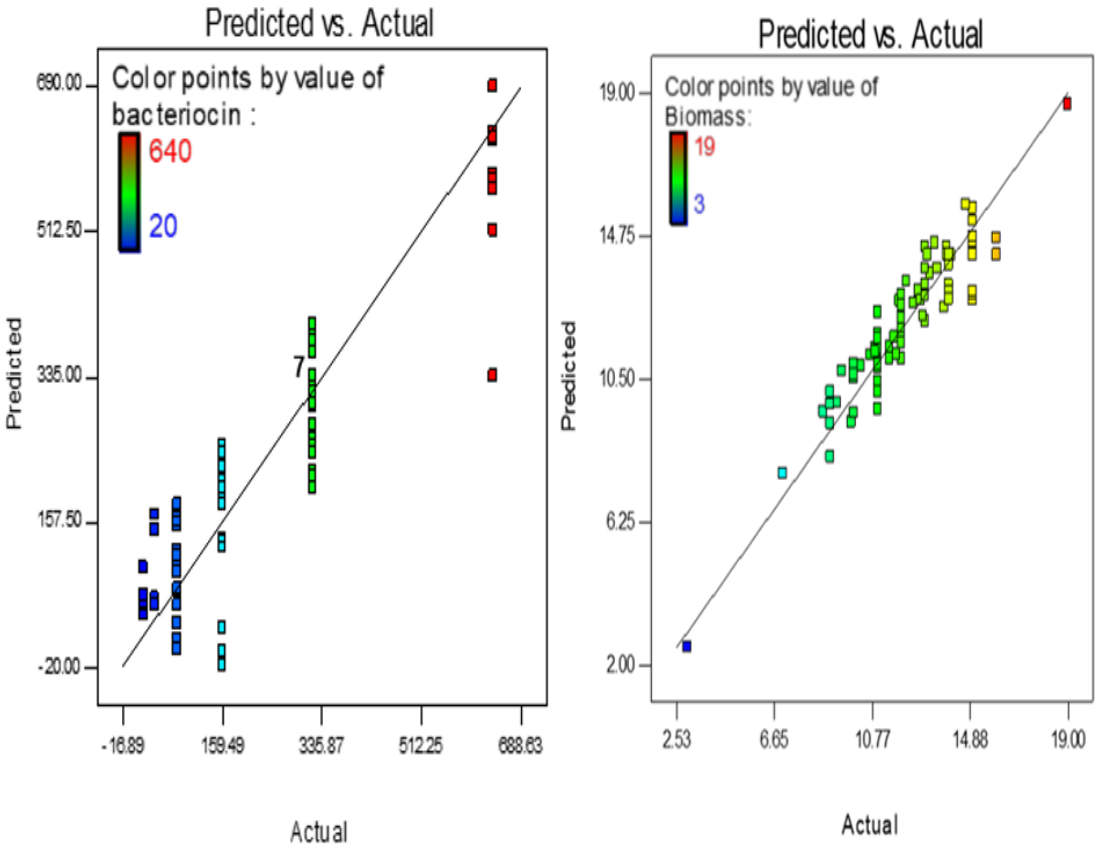

Figure 4-Actual versus predicted values for biomass and bacteriocin production

Contour plots were generated using design expert 7 software which can help to visualize and understand the nature of interaction between different factors and their effect on the responses in order to conclude the optimum conditions. These plots were obtained via calculations based on the model; the values were taken by one factor while the second was varies from -α to +α with constrain of given response. Contour plots presented in Figures-(5, 6) illustrate the effect of interaction between different MRS medium components with various experimental range on the production of both biomass and bacteriocin. For example, the interaction between glucose and yeast extract presented in the contour plot in Figure-5 shows that maximum biomass production of 15.4 g/L can be obtained when the concentration of glucose and yeast extract are 42.9 g/L 18 g/L respectively. Whereas, maximum production of bacteriocin (640.46 Au/mL) can be attained when glucose and yeast extract concentrations are 45.3 g/L and 22.2 g/L respectively. In addition, contour plot shows that 80 Au/mL is the minimum bacteriocin production present in the blue region.

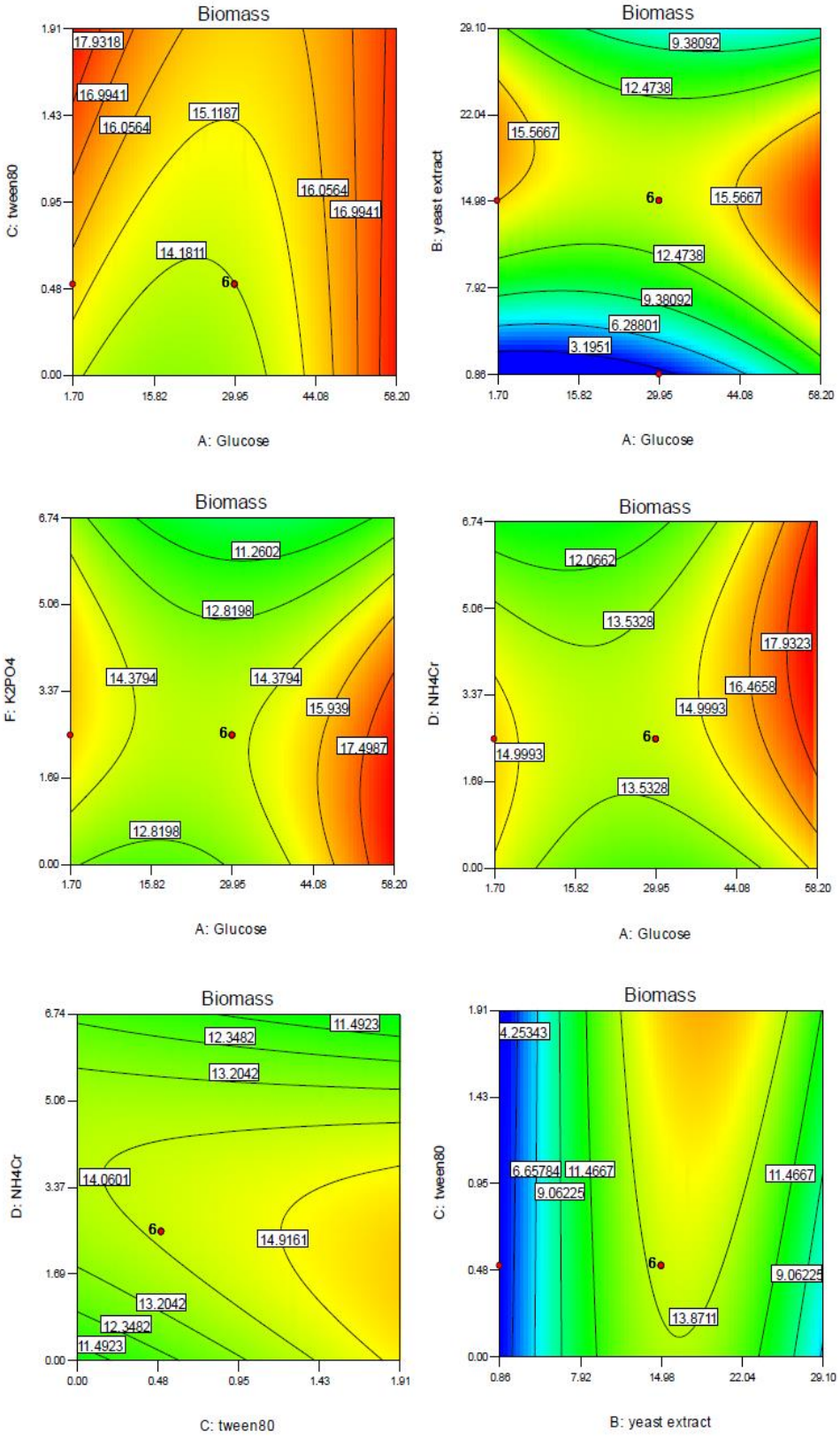

Figure 5-D contour plot response surface curve represents the effect of interaction among different factors represent the composition of MRS medium on biomass production (Design points 193).

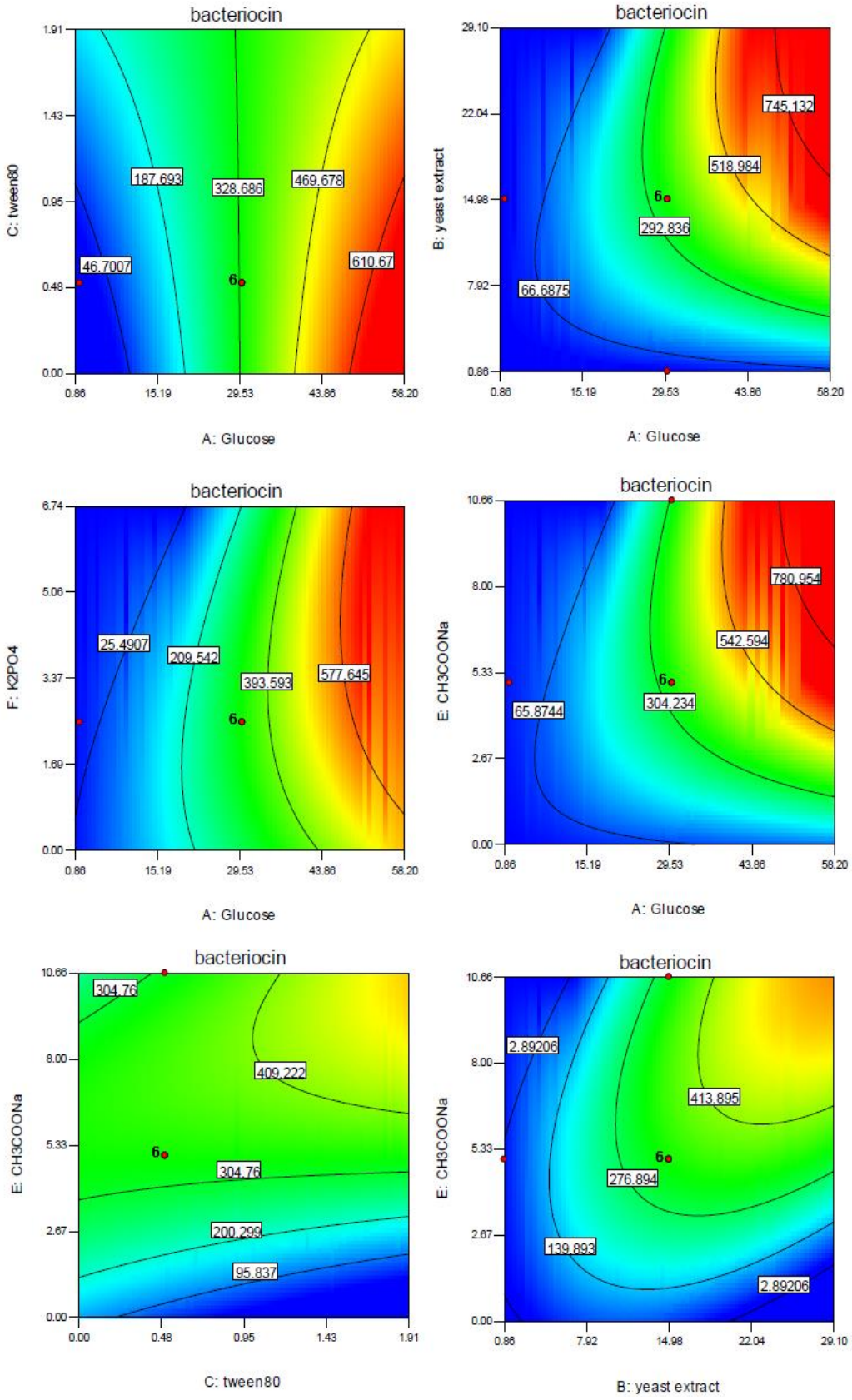

Figure 6-2D contour plot response surface curve represents the effect of interaction among different factors represent the composition of MRS medium on bacteriocin production (Design points 640 20).

Validation of optimum MRS medium

Based on the enhanced regression model, optimization plot can be generated using the Design expert 7 software in order to determine the optimum MRS medium composition (glucose, yeast extract, tween80, NH_4Cr, CH_3COONa and K_2PO) for both biomass and bacteriocin production. Ramp chart presented in Figure-7 shows that the optimum concentrations of glucose, yeast extract, tween80, NH_4Cr, CH_3COONa and K_2PO_4 were 40, 19.9, 1, 3.06, 7, 1.25 g/L respectively for maximum production of biomass (15.87) and bacteriocin (640). Furthermore, in order to verify the optimization results and determine the accuracy of model, an experiment was conducted in duplicate with optimized MRS medium suggested by design expert 7 for both biomass and bacteriocin. Our results revealed that concentrations of biomass and bacteriocin were 15 mg/mL and 640Au/mL respectively, which are approximately closed to the predicted values.

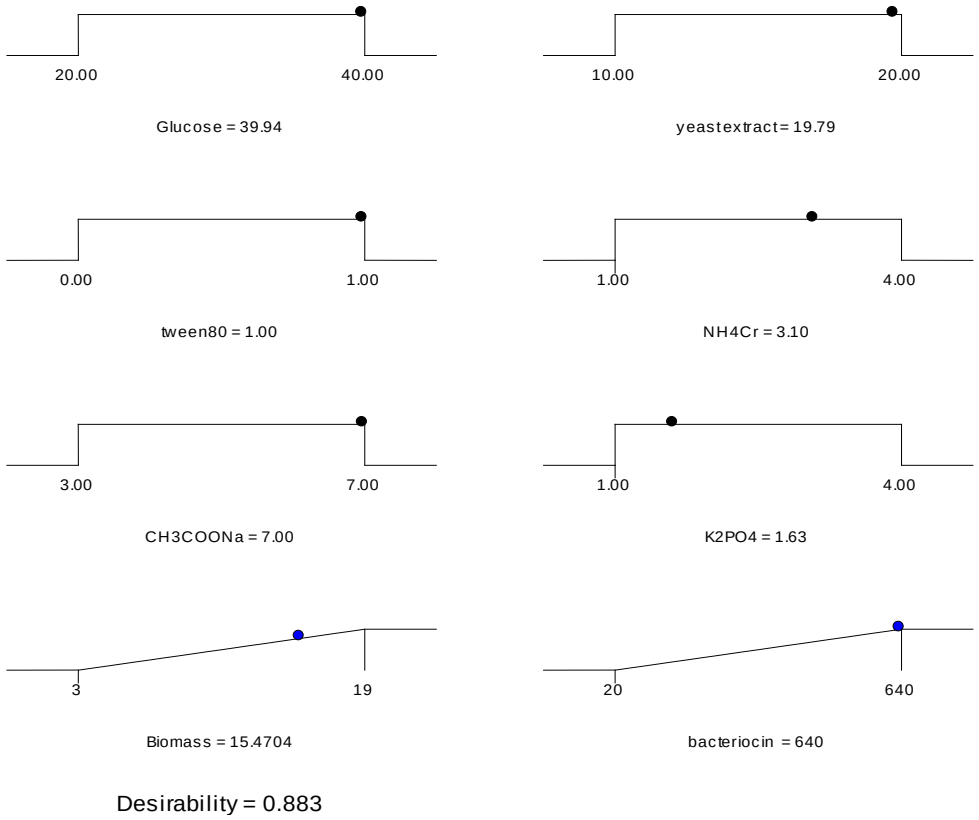

Figure 7-Ramp chart for statistically optimization of MRS medium for both biomass and bacteriocin production by *Lactobacillus plantarum* NH40.

The physiological behavior of a microorganism and production of a particular metabolite varies during growth and it is mainly depending on the composition of media in terms of types of nutrients and their concentration. Different classical and statistical strategies are applied for media optimization and all are focused on detecting the minimal nutritional requirements for growth that make a strain reach to the maximum production of metabolite(s).

Nitrogen source is an important and essential component for biomass and bacteriocin production. Most industrial microorganism strains can utilize organic and inorganic sources of nitrogen. In many instances, microbial growth might be faster in media contain organic nitrogen sources however, few microorganisms have specific requirement for amino acid. Though, amino acids are commonly added as complex organic nitrogen sources which are non-homogenous cheaper and readily available. MRS medium contain peptone, yeast extract and meat extract however, our results revealed that peptone and meat extract can be eliminated from the medium without affecting the growth and production of bacteriocin. On the contrary, maximum growth and bacteriocin production was observed when yeast extract was used as sole nitrogen source in the culture. Previous studies have reported that yeast extract is already containing growth factors and relatively more proportion of free amino acids as

well as short peptides of 2 or 3 amino acids which might be the reason for the increase observed in the production of biomass and bacteriocin [14, 15]. Moreover, what is interesting in this data is that: peptone and meat extract can be eliminated from the original composition of MRS medium without affecting the growth and bacteriocin production. At the end of classical optimization glucose and yeast extract were chosen as optimum carbon and nitrogen sources that maximize biomass and bacteriocin production for further optimization by RSM.

Modern mathematical and statistical techniques for the optimization is useful for designing a production medium with minimal nutritional requirements of microorganism that support growth and maximum production of metabolite(s). This study sets out to modify MRS medium based on detecting the minimal nutritional requirements of *Lactobacillus plantarum* that give maximuim production of both biomass and bacteriocin. In our work, along with modifying the basic concentration of nutrients in MRS medium, we confirmed that peptone and meat extract can be eliminated from the medium without affecting the growth and bacteriocin production. *Lactobacillus* spp. were used for many centuries as a starter culture in several fermentation process and in addition to their widely used in industry, they utilized as probiotics due to their beneficial effects in the health of the host and its role in the treatment of diseases. Therefore, designing a production medium which can support both growth and a particular product such as bacteriocin is necessary and important for the cultivation of this vital genus [16].

Finally, Table-5 summarized the composition of both optimized and original unoptimized MRS medium as well as production of biomass and bacteriocin. Based on results, it can be said that the optimization strategy of MRS medium was more successful for bacteriocin as the production was significantly risen from 80 to 640 AU/ml with an increase of 8-fold. In addition, another increase of approximately 3.5 mg/mL in biomass production was also obtained in the optimized composition.

Table 5-he composition of both optimized and unoptimized MRS medium as well as production of biomass and bacteriocin

		MRS medium composition	
		Unoptimized	Optimized
MRS Composition (g/L)	Glucose	20	40
	Yeast extract	5	19.96
	Pepton	10	0
	Meat extract	10	0
	Tween 80	0	1
	NH4Cr	2	3.15
	CH3COONa	5	7
	K2PO4	2	1.65
	Magnesium sulphate	0.1	0.1
	Manganese sulphate	0.05	0.05
Production	Biomass (mg/mL)	11.5	15
	Bacteriocin (AU/mL)	80	640

References

1. Mahrous, H., Mohamed, A., El-Mongy, M., El-Batal, A. and Hamza, H. **2013**. Study bacteriocin production and optimization using new isolates of *Lactobacillus* spp. isolated from some dairy products under different culture conditions. *Food Nutri Sci*, **4:**342–356.
2. Singh, V., Shafiul, H., Ram, N., Akansha, S., Mukesh, P. and Tripathi, C. **2017.** Strategies for Fermentation Medium Optimization: An In-Depth Review. *Frontiers in Microbiology*, 7**:** 2087-2095.
3. Kumari, V., Vijay, K., Ravin, C., Mohammad, A. and Tek, C.B**. 2016.** Optimization of medium parameters by response surface methodology (RSM) for enhanced production of cutinase from *Aspergillus* sp. *RL2Ct. 3 Biotech*., **6:** 149-158.
4. Ahmad, R., Kaid, N., Al-Shorgani, N., Abdul Hamid, A., Mohta,r W., Yusoff, W. and Daud, F. **2013.** Optimization of medium components using response surface methodology (RSM) for mycelium biomass and exopolysaccharide production by *Lentinus squarrosulus. Adv. Biosc. Biotechnol*, **4**: 1079-1085.
5. Kumari, V., Kumar, V., Chauhan, R., Asif, M. and Chand Bhalla, T. **2016**. Optimization of medium parameters by response surface methodology (RSM) for enhanced production of cutinase from *Aspergillus* sp. *RL2Ct. 3 Biotech*, **6**: 149-154.
6. Vuyst, L. and Leroy, F. **2007**. Bacteriocins from Lactic Acid Bacteria: Production, Purification, and Food Applications. *J Mol Microbiol Biotechnol*, **13**: 194–199.
7. Kanmani, P., Satish kumar, R., Yuvaraj, N., Paari , K. A., Pattukumar, V. and Arul, V. **2011**. Optimization of media components for enhanced production of *Streptococcus phocae* PI80 And Its Bacteriocin Using Response Surface Methodology**.** *Brazilian Journal of Microbiology*, **42**: 716-720.
8. Box, E.P., Hunter, J.S. and Hunter, G.W. **2005**. *Statistics for experiments*. New Jersey: John Willey and Sons, Inc.
9. Montgomery, DC. **1997**. *Design and analysis of experiments*. Wiley; Science, 704.
10. Abd, F.N. and Luti, K.J.K. **2017**. Detection, purification and characterization of a bacteriocin produced by *Bacillus subtilis* NK16 exhibits a significant antimicrobial activity against clinical *Staphylococcus* spp isolates. *J Baghdad for Science*, **14**(3): 524-538.
11. Preetha, S.P., Kanniappan, M., Selvakumar, E., Nagaraj, M. and Varalakshmi, P. **2006**. Lupeol ameliorates aflatoxin B1-induced peroxidative hepatic damage in rats. *Comp Biochem Physiol*, **143**: 333.
12. Montgomery, D.C. **2001**. Design and Analysis of Experiments. New York: John Wiley and Sons.
13. Todorov, S.D., Vaz-Velho, M. and Franco, D. **2013**. Partial characterization of bacteriocins produced by three strains of *Lactobacillus sakei*, isolated from *salpicao*, a fermented meat product from North-West of Portugal. *Food Control*, **30**: 111–121.
14. Dutka, M., Mario, D. and Løvas, T. **2015**. Application of a central composite design for the study of NO emission performance of a low NO_x burner, *Energies*, **8**(5): 3606-3627.
15. Aasen, L.M., Møretrø, T., Katla, T. and Storrø, L. **2000**. Influence of complex nutrients, temperature and pH on bacteriocin production by Lactobacillus sakei CCUG 42687, *Appl. Microbiol. Biotechnol*, **53**(2): 159-66.
16. Benthin, S., Schulze, U., Nielsen, J. and Villadsen, J. **1994**. Growth energetics of *Lactococcus cremoris* FD1 during energy-, carbon-, and nitrogen-limitation in steady state and transient cultures. *Chem. Eng. Sci*, **49**: 589-609.

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