MICROBIOLOGY
INDONESIA
ISSN 1978-3477, eISSN 2087-8575
Vol 9, No 3, September 2015, p 120-128

Available online at
http://jurnal.permi.or.id/index.php/mionline
DOI: 10.5454/mi.9.3.4

Optimization of Surfactin Production by *Bacillus amyloliquefaciens* MD4-12 Using Response Surface Methodology

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Surfactin is a lipopeptide biosurfactant that show potential biomedical application due to its activities such as antiviral, antibacterial, antifungi, anticancer, and antimycoplasma. *Bacillus amyloliquefaciens* MD4-12, isolated from oil-contaminated soil, produced promising yield of surfactin in McKeen medium. The production of surfactin was influenced by many fermentation process parameters such as carbon, nitrogen, minerals and also environmental conditions such as pH and agitation. Therefore, to obtain high yield of surfactin by *B. amyloliquefaciens* MD4-12, optimization of process production was conducted in shake flask fermentation using response surface methodology. McKeen medium composition was used as basal medium. Screening of the best carbon and nitrogen source were selected in preliminary experiments followed by selection of the influencing significant parameters on surfactin production using Plackett-Burman design. Selected parameters were optimized by central composite design and for the data analysis was used response surface methodology. The result showed that the optimum medium composition contained (g L^{-1}) 45.0 glucose, 6.33 urea, 1.0 monosodium glutamate, 1.85 $MgSO_4.7H_2O$, 0.4 KCl, 0.5 K_2HPO_4, and 0.5 mL trace elements. The surfactin yield at optimal condition was 1.25 g L^{-1}, increased 2.4 times compared to condition prior to optimization.

Key words: lipopeptide, optimization, response surface methodology, surfactin

Surfaktin adalah senyawa biosurfaktan lipopeptida yang sangat potensial untuk aplikasi di bidang biomedis karena mempunyai berbagai aktifitas biologi seperti antivirus, antibakteri, antijamur, antikanker, dan antimikoplasma. *Bacillus amyloliquefaciens* MD4-12, yang diisolasi dari tanah yang tercemar minyak menunjukkan kemampuan yang tinggi dalam menghasilkan surfaktin menggunakan medium dasar McKeen. Produksi surfaktin secara fermentasi dipengaruhi oleh berbagai faktor seperti sumber karbon, sumber nitrogen, mineral serta kondisi lingkungan seperti pH dan agitasi. Dengan menggunakan medium McKeen sebagai medium dasar, maka pada penelitian ini dilakukan optimasi produksi surfaktin menggunakan metode permukaan respon. Pada percobaan awal dilakukan pemilihan sumber karbon dan nitrogen terbaik, dan selanjutnya diikuti dengan pemilihan faktor-faktor yang berpengaruh secara signifikan untuk produksi surfaktin menggunakan rancangan Plackett-Burman. Faktor-faktor yang terpilih selanjutnya dioptimasi dengan menggunakan *central composite design* dan analisis data dilakukan dengan menggunakan metode permukaan respon. Hasil penelitian menunjukkan bahwa komposisi medium yang optimal terdiriatas (g L^{-1}) glukosa 45,0; urea 6,33; monosodium glutamat1,0; $MgSO_4.7H_2O$ 1,85; KCl 0,4,; K_2HPO_4 0,5 dan mikroelemen 0.5 mL. Pada kondisi optimum perolehan surfaktin adalah 1,25 g L^{-1}, meningkat 2,4 kali dibandingkan dengan kondisi sebelum optimasi.

Kata kunci: lipopeptida, metode permukaan respon, optimasi, surfaktin

Biosurfactants are microbial metabolites that work as surface active agents produced by some microorganisms, including bacteria, yeast and fungi. Interests in biosurfactants are growing due to their broad possible applications such as in the pharmaceutical, cosmetic, agricultural and food industries, environmental protection and crude oil drilling (Banat *et al*. 2010; Meena *et al*. 2015). Their important characteristics such as biological activities, emulsifying, foaming, soaping and dispersing acquire them for multiple applications. Based on their chemical structures, biosurfactants are divided into five major classes, i.e. lipopeptides, glycolipids, phospholipids, neutral lipids, and polymeric compounds. One of the most important families of lipopeptide class is surfactin. Surfactin is a cyclic molecules consisting of a fatty acid of variable length (hydrophobic moiety) linked to a short

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peptide chain (hydrophilic moiety) of seven amino acids. It has ability to reduce surface tension of water from 72 mN m^{-1} to 27 mN m^{-1} (Cooper *et al*. 1981). Several biological activities have been reported for biomedical application, such as antiviral, antibacterial, antifungi, antitumor, antiinflammatory, antiadhesive, antimycoplasma and thrombolytic activity (Huang *et al*. 2006; Sabate *et al*.2013; Liu *et al*. 2012; Kim *et al*. 2007; Zhang *et al*. 2015; Zeraik *et al*. 2010, Lim *et al*. 2005; Meena *et al*. 2015).

A large variety of *Bacillus* has been reported to produce surfactin, nevertheless microbial exploration is still needed to obtain high productivity strain. *Bacillus amyloliquefaciens* MD4-12 isolated from oil-contaminated soil showed promising ability to produce surfactin. According to previous studies, the surfactin production was influenced by several nutritional factors including the nature and concentration of carbon and nitrogen substrate, the concentration of metal ion such as Mg, Fe, Zn, and Mn in the medium and environmental conditions such as temperature, pH, agitation and aeration (Sen 1997; Yi *et al*. 2013; Sen *et al*. 1997; Al-Ajlani *et al.* 2007; Abdel-Mawgoud *et al.* 2008; Amani *et al.* 2013; Singh *et al.* 2014a). To achieve high yield of surfactin production by *B. amyloliquefaciens* MD4-12, optimization of nutrient supply and environmental condition is very important. Statistical technique, including combination of Plackett-Burman design (PBD), central composite design (CCD) and response surface methodology (RSM) are the most widely method used for optimization of biological processes. The Plackett-Burman experimental design is a two-level factorial design, used to determine the critical physicochemical parameters. Interaction effects among the variables are not considered (Mnif *et al*. 2012). To screen *N* variables using PBD, N+1 experiments are needed. The significant variables obtained by PBD can be further optimized using CCD and (RSM). RSM has been used extensively in biological process optimization due to its efficiency and accuracy for building of a model, evaluating the effects of factors and predicting optimum conditions. The objective of the present study was to optimize of surfactin production by *B. amyloliquefaciens* MD4-12 using response surface methodology.

MATERIALS AND METHODS

Microorganisms. A surfactin-producing microorganism has been isolated from oil-contaminated soil taken from Palembang, Indonesia. Furthermore, it was identified as *B. amyloliquefaciens* MD4-12 by morphological, biochemical, and 16S rRNA sequence analysis.

Inoculum and Culture Conditions. The strain was stored lyophilized and working stock cultures were maintained on nutrient agar slant and stored at 4 °C. For surfactin production, two-stage inoculum were prepared as following: one isolated colony from fresh culture grown onto nutrient agar medium and incubated for 24 h, was dispensed in 5 mL of nutrient broth medium and incubated overnight at 37 °C. The culture (4 mL) was used to inoculate 100 mL nutrient broth medium in 500 mL Erlenmeyer flasks and incubated in a rotatory shaker at 200 rpm and 37 °C (±0.5) for 16 h. At this condition, absorbance around 3 measured spectrophotometrically at 600 nm was reached. For fermentation, 2 mL of culture was inoculated to 250 mL Erlenmeyer flask containing 50 mL of production medium. Modified McKeen medium composition was used as the production medium consist of (g L^{-1}) 20.0 glucose, 2.0 monosodium glutamate, 3.0 yeast extract, 1.0 $MgSO_4.7H_2O$, 1.0 KCl with addition of 1 mL trace element solution (in 100 mL deionized water : 0.64 g $MnSO_4.4H_2O$, 0.16 g $CuSO_4.5H_2O$, and 0.015 g $FeSO_4.7H_2O$). pH was adjusted at 7.0. Fermentation was conducted for 28 h.

Experimental Design and Data Analysis. Preliminary experiments were conducted to screen carbon and nitrogen source. The effect of different carbon sources was evaluated by substitution of glucose in McKeen medium composition with sucrose, fructose, maltose, dextrose, mannitol, sorbitol, galactose, xylose, and starch. The best carbon source generated the highest surfactin production was used for further study. Furthermore, the effect of the different nitrogen source addition was studied similarly by substitution of yeast extract with ammonium nitrate, ammonium sulphate, sodium nitrate, soy flour, peptone, casein hydrolysate, and urea.

The next step was to screen the significant factors affecting surfactin production by *B. amyloliquefaciens* MD4-12 using the PBD, as noted in Table 1. All components in the production medium (seven factors) and some environmental conditions (two-factor) were used to determine the factors that influence significantly. This experiment required 12 runs as presented in Table 2. The factors with confidence levels above 95% were considered as significant effect on surfactin production.

The selected significant influence factor obtained

from PBD experiments was optimized with CCD and RSM as presented in Table 4. Thirty experiments consisted of sixteen factorial points, eight star points and six center points were conducted to optimize four significant factors. For each factor was studied at three level concentration (low, middle and high levels) coded with (-1, 0, +1). All experiments were conducted triplicate and the final result was expressed as the average value.

Statistical Analysis. Design Expert (Version 7.0, Stat-Ease Inc., USA) software was used to generate the experimental designs and data analysis. The quality of polynomial model equation was evaluated statistically using analysis of variance (ANOVA) to determine the coefficient of determination, R^2 and adjusted R^2. The statistical significance of the model and the regression coefficient were determined using the F-test.

Surfactin Analysis. The gravimetric method as described by Liu *et al*. (2012) was used for determination of surfactin concentration with minor modification. The broth culture was centrifuged at 10 000 rpm for 10 min to obtain the cell free supernatant (CFS). An aliquot of the CFS was acidified to pH 2.0 using 6 N HCl, left overnight at 4 °C, and then centrifuged at 10 000 rpm for 20 min. The resulting supernatant was discarded and the remaining pellet was collected, and then extracted three times with methanol. The dry pellet was obtained by removal of methanol using a rotary vacuum evaporator. The net weight of pellet was determined and calculated as crude surfactin. Structural characterization of the crude surfactin by mass spectrophotometry revealed that it contained surfactin as the major component (data not shown).

RESULTS

Selection of Carbon Source and Nitrogen Source. Among the carbon sources used in this experiment, the use of glucose yielded highest surfactin production, i.e. 0.320 g L^{-1}. Furthermore, from the experiments result to screen the nitrogen source showed that the use of urea yielded the highest surfactin production, i.e. 0.436 g L^{-1}. Therefore, glucose and urea were used as carbon and nitrogen source for the next study.

Plackett-Burman Experimental. The experiments for selection of nine critical factors affected surfactin production by *B. amyloliquefaciens* MD4-12 namely medium composition (seven factors) and environment conditions (initial medium pH and fermentation temperature) were conducted using PBD. The experimental design and results were presented in Table 2. The ANOVA of the experiments (Table 3) showed that the p-value (Prob>F) of the model was 0.0402, implied that the model was significant. The magnitude of each factor affecting on surfactin production was indicated by p-value. Among the factors screened, the concentration of glucose, urea, $MgSO_4.7H_2O$, and KCl showed significantly effecting on surfactin production with p-values, 0.0438, 0.0100, 0.0221, and 0.0262 with the coefficient of the model 0.056, 0.12, -0.080, and 0.074 respectively. The other factors have p-value (Prob>F) > 0.05 that means no significantly affecting on surfactin production.

Optimization Using Response Surface Methodology. Optimization of four significant factors on surfactin production was conducted using RSM and the results were presented in Table 4. The other factors were kept at their low level. The experiments result was modeled with a second-order polynomial equation to explain the effect of each factor on surfactin production, as follows:

$$Y = 1.18 - 0.034\,X_1 - 4.758E\text{-}003\,X_2 + 0.069\,X_3 - 0.080\,X_4 + 0.057\,X_1X_2 + 0.018\,X_1X_3 - 0.11\,X_1X_4 - 2.814E\text{-}003\,X_2X_3 + 0.047\,X_2X_4 + 8.887E\text{-}005\,X_3X_4 - 0.041\,X_1^2 - 0.077\,X_2^2 - 0.065\,X_3^2 + 0.023\,X_4^2$$

Where Y was the estimated surfactin production and X_1, X_2, X_3 and X_4 were coded value for glucose, urea, $MgSO_4.7H_2O$ and KCl concentration, respectively. The results of the second-order response surface model in the form of analysis of variance (ANOVA) are given in Table 5. It can be seen that two linear (X_3 and X_4) and three quadratic (X_1, X_2, and X_3) terms were significant, with the p-values being very small ($p<0.05$). The quadratic effect of X_4 was not significant ($p>0.05$). The significant interaction of variables was shown by $X_1.X_2$; $X_1.X_4$, and $X_2.X_4$ terms ($p<0.05$). The other interaction of factors was insignificantly effected on surfactin production. By considering the only significant factors, the model for surfactin production could be written as following equation:

$$Y = +1.18 - 0.069\,X_3 - 0.080\,X_4 + 0.057\,X_1X_2 - 0.11\,X_1X_4 - + 0.047\,X_2X_4 - 0.041\,X_1^2 - 0.077\,X_2^2 - 0.065\,X_3^2$$

According to the equation, the yield of surfactin could be estimated as a linear effect of magnesium sulphate and potassium chloride concentration and squared effect of glucose, urea and magnesium

Table 1 Plackett-Burman design for screening of significant parameters on surfactin production by *Bacillus amyloliquefaciens* MD4-12

Variable	Code	Unit	Level	
			-1	+1
Carbon source	A	g L^{-1}	15	45
Nitrogen source	B	g L^{-1}	1	9
MSG	C	g L^{-1}	1	4
$MgSO_4.7H_2O$	D	g L^{-1}	0.5	2
K_2HPO_4	E	g L^{-1}	0.5	2.5
KCl	F	g L^{-1}	0.3	1
Trace element	G	mL	0.5	2
pH	H		6	8
Temperature	I	°C	25	35

Table 2 The Plackett-Burman design and the experimental results for the selection of important parameters on the surfactin production

Std	Variables									Surfactin
	A	B	C	D	E	F	G	H	I	
	g L^{-1}	g L^{-1}	g L^{-1}	g L^{-1}	g L^{-1}	g L^{-1}	mL		°C	g L^{-1}
1	45	9	1	2	2.5	1	0.5	6	25	0.366
2	15	9	4	0.5	2.5	1	2	6	25	0.413
3	45	1	4	2	0.5	1	2	8	25	0.133
4	15	9	1	2	2.5	0.3	2	8	35	0.048
5	15	1	4	0.5	2.5	1	0.5	8	35	0.207
6	15	1	1	2	0.5	1	2	6	35	0.116
7	45	1	1	0.5	2.5	0.3	2	8	25	0.085
8	45	9	1	0.5	0.5	1	0.5	8	35	0.572
9	45	9	4	0.5	0.5	0.3	2	6	35	0.508
10	15	9	4	2	0.5	0.3	0.5	8	25	0.180
11	45	1	4	2	2.5	0.3	0.5	6	35	0.039
12	15	1	1	0.5	0.5	0.3	0.5	6	25	0.064

Table 3 ANOVA of the factors affecting on surfactin production using Plackett-Burman design

Source	Coeficient model	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model		0.39	9	0.043	24.26	0.0402*
Glucose	0.056	0.038	1	0.038	21.32	0.0438*
Urea		0.17	1	0.17	98.32	0.0100*
Yeast Extract	0.019	4.376E-003	1	4.376E-003	2.48	0.2563
$MgSO_4.7H_2O$	-0.080	0.077	1	0.077	43.83	0.0221*
K_2HPO_4	-0.035	0.014	1	0.014	8.15	0.1039
KCl	0.074	0.065	1	0.065	36.72	0.0262*
Trace element	-0.011	1.355E-003	1	1.355E-003	0.77	0.4737
Initial pH	-0.024	6.651E-003	1	6.651E-003	3.76	0.1920
Temperature	0.021	5.243E-003	1	5.243E-003	2.97	0.2272

*) significant at the level > 95.0% (for p-value < 0.05).

sulphate concentration.

ANOVA of the model for surfactin production showed that the regression model was highly significant and the lack of fit was insignificant (Table 5). The high determination coefficient (R^2) showed the goodness of fit of the second order model, and it implied that only 9.58% the total variations were not explained by the model. The high adjusted determination coefficient (adj R^2) was also satisfactory to confirm the significance of the model. A lower value of the coefficient of variation (CV) indicates that experiments were precise and reliable (Box *et al*. 1978). The signal to noise ratio (adequate precision) for the model was higher than 4, indicating a good fit.

Visualization of the interaction between the response and experimental levels of each variable and the type of interactions between test variables in order to deduce the optimum conditions provided by the 3D response surface. Fig 1 depicted the 3D response surface showing the interactive effect of the significant variable interaction. The other insignificant variables, i.e. concentration of monosodium glutamate, K_2HPO_4, and trace element were kept at a minimum level (1 g L^{-1}, 0.5 g L^{-1}, and 0.5 mL L^{-1}, respectively) and initial pH of medium and temperature were 7.0 and 30 °C, respectively.

Validation of the Optimized Condition. Based on the model, the optimized medium composition consists of (g L^{-1}): 45.00 glucose, 6.33 urea, 1 monosodium glutamate, 1.85 $MgSO_4.7H_2O$, 0.40 KCl, 0.5 K_2HPO_4, and 0.5 mL trace element. The model predicts the maximum yield of surfactin as 1.35 g L^{-1}. Under optimized condition, validation experiments were conducted in triplicates. The result of experiments showed that the yield of average surfactin was 1.25 g L^{-1}, suggesting that experimental and predicted values of surfactin yield were in a good agreement. The usage of optimized medium determined by response surface methodology for surfactin production could increase 2.4-fold over the yield in non-optimized medium.

DISCUSSION

B. amyloliquefaciens MD4-12 is a potential strain for surfactin production. The selection of potential strain is necessary for future mutation or bioengineering studies. The significant influence factors on surfactin production by *B. amyloliquefaciens* MD4-12 consist of glucose, urea, $MgSO_4.7H_2O$ and KCl selected by PBD experiments. *B. amyloliquefaciens* MD4-12 can use

Table 4 Central composite design and the experimental results for optimization of important parameters on surfactin production

Std	X_1:Glucose ($g\ L^{-1}$)	X_2:Urea ($g\ L^{-1}$)	X_3:$MgSO_4.7H_2O$ ($g\ L^{-1}$)	X_4:KCl ($g\ L^{-1}$)	Surfactin ($g\ L^{-1}$)
1	30.00	5.00	0.70	0.40	1.088
2	45.00	5.00	0.70	0.40	1.261
3	30.00	9.00	0.70	0.40	0.922
4	45.00	9.00	0.70	0.40	1.064
5	30.00	5.00	2.00	0.40	1.170
6	45.00	5.00	2.00	0.40	1.313
7	30.00	9.00	2.00	0.40	1.033
8	45.00	9.00	2.00	0.40	1.175
9	30.00	5.00	0.70	1.00	1.190
10	45.00	5.00	0.70	1.00	0.556
11	30.00	9.00	0.70	1.00	1.000
12	45.00	9.00	0.70	1.00	0.868
13	30.00	5.00	2.00	1.00	1.180
14	45.00	5.00	2.00	1.00	0.790
15	30.00	9.00	2.00	1.00	1.103
16	45.00	9.00	2.00	1.00	0.988
17	22.50	7.00	1.35	0.70	0.953
18	52.50	7.00	1.35	0.70	1.020
19	37.50	3.00	1.35	0.70	0.783
20	37.50	11.00	1.35	0.70	0.864
21	37.50	7.00	0.05	0.70	0.630
22	37.50	7.00	2.65	0.70	1.008
23	37.50	7.00	1.35	0.10	1.388
24	37.50	7.00	1.35	1.30	1.061
25	37.50	7.00	1.35	0.70	1.169
26	37.50	7.00	1.35	0.70	1.079
27	37.50	7.00	1.35	0.70	1.159

Table 5 ANOVA for respon surface with quadratic regression model

Source	Coeficient Estimates	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model		0.91	14	0.065	10.11	< 0.0001*
Intercept	1.18					
X_1-Glucose	-0.0337	0.027	1	0.027	4.26	0.0567
X_2-Urea	-0.0048	5.433E-004	1	5.433E-004	0.085	0.7747
X_3-$MgSO_4.7H_2O$	0.06930	0.12	1	0.12	18.02	0.0007*
X_4-KCl	-0.0798	0.15	1	0.15	23.89	0.0002*
$X_1.X_2$	0.05693	0.052	1	0.052	8.11	0.0122*
$X_1.X_3$	0.01790	5.127E-003	1	5.127E-003	0.80	0.3847
$X_1.X_4$	-0.1136	0.21	1	0.21	32.29	< 0.0001*
$X_2.X_3$	-0.0028	1.267E-004	1	1.267E-004	0.020	0.8899
$X_2.X_4$	0.04682	0.035	1	0.035	5.48	0.0334*
$X_3.X_4$	8.89E -15	1.264E-007	1	1.264E-007	1.976E-05	0.9965
X_1^2	-0.0412	0.046	1	0.046	7.26	0.0166*
X_2^2	-0.0767	0.16	1	0.16	25.23	0.0002*
X_3^2	-0.0647	0.11	1	0.11	17.97	0.0007*
X_4^2	0.0225	0.014	1	0.014	2.17	0.1610
Residual		0.096	15	6.396E-003		
Lack of Fit		0.082	10	8.195E-003	2.93	0.1236
Pure Error		0.014	5	2.798E-003		

*) significant at the level > 95.0% (for p value < 0.05); R^2 = 0.9042; Adj R^2 = 0.8148; Coeficient variation (C.V. %) = 7.63; Signal to noise ratio (adequate precision) = 14.605

several carbon sources for surfactin production. However, the nature and concentration of carbon source influenced on surfactin yield (Abdel-Mawgoud *et al*. 2008; Yi *et al*. 2013; Singh et al. 2014b). The best carbon source for surfactin production by *B. amyloliquefaciens* MD4-12 is glucose since this carbon source is easy to assimilate. The concentration of glucose for maximum surfactin production was reported at 40 g L^{-1} and at glucose concentration higher than 40 g L^{-1}, the surfactin yield decreased (Abdel-Mawgoud *et al*. 2008; Ghribi *et al*. 2011). In this study, the usage of glucose concentration higher than 40 g L^{-1} still showed increasing of surfactin production and at glucose concentration 45 g L^{-1} reached maximum surfactin yield. Although in this study the concentration of glucose required higher but keep in mind that surfactin produced was also higher than previous reported.

The nature of nitrogen source also plays an important role in the production of surface-active

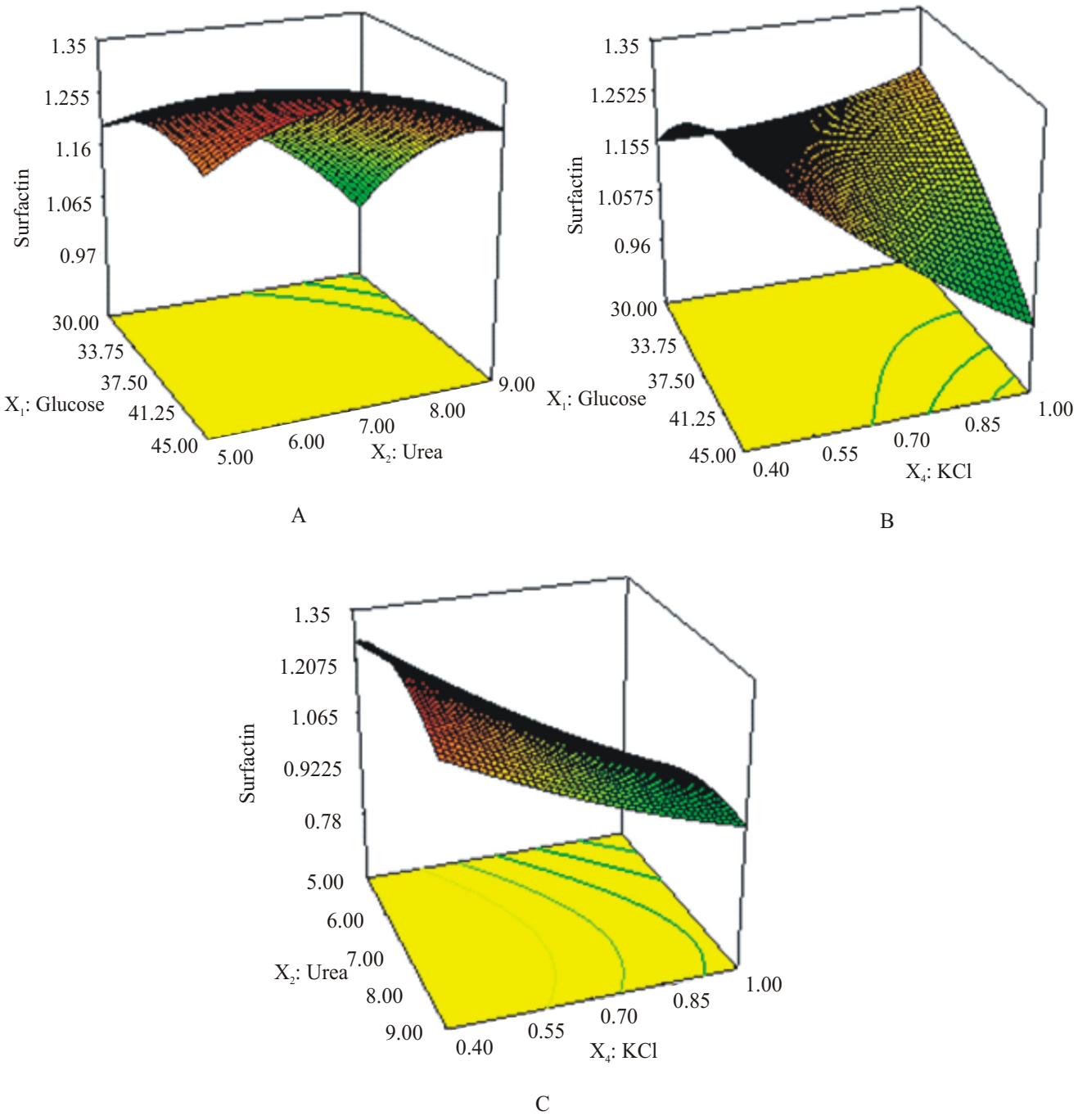

Fig 1 3D respon surface plot for effect combination of (a) glucose and urea (b) glucose and KCl and (c) urea and KCl on surfactin production.

compounds by microorganisms (Singh *et al*. 2014b). In this study the use of urea showed highest surfactin production. The optimum urea concentration was 6.33 g L^{-1}. The adequate C/N ratio is an important factor for efficiently surfactin synthesis.

The other significant factors effecting surfactin production in this study were $MgSO_4.7H_2O$ and KCl. These results correspond to those reported in literature (Wei *et al*. 2007). Under optimal condition of the metal ion Mg^{2+} and K^+ (2.4 and 10 mM, respectively), surfactin yield was improved significantly. In this study the optimum condition of $MgSO_4.7H_2O$ and KCl were 1.85 and 0.4 g L^{-1}, respectively, which is equal to concentration of Mg^{2+} and K^+ of 7.5 and 5.3 mM, respectively. MgSO4 concentration influenced cell growth as well as the surfactin production, while KCl was reported to relate with the permeation pressure by offering a buffer environment in cooperation with KH_2PO_4 for cell growth (Yi *et al*. 2013). Maintaining the environmental condition is important to support biomass growth and surfactin production as well as inhibit byproduct formation in the fermentation process.

In this study, after optimization of significant factors by RSM the surfactin yield could be markedly enhanced (2.4-fold). Using glucose, urea, $MgSO_4.7H_2O$ and KCl at optimal concentration and keeping other factors at their minimum value, the yield of surfactin reached 1.25 g L^{-1}, which closely corresponds to the model estimates (1.35 g L^{-1}). The surfactin yields obtained in this study was higher than some of reported yields in literature and also have shorter fermentation cycle (Sen 1997; Abdel-Mawgoud *et al*. 2008; Al-Ajlani *et al*. 2007).

REFERENCES

Abdel-Mawgoud AM, Aboulwafa MM,Hassouna NAH. 2008. Optimization of surfactin production by *Bacillus subtilis* isolate BS5. Appl Biochem Biotechnol. 150(5):305-25. doi:10.1007/s12010-008-8155-x.

Al-Ajlani MM, Sheikh MA, Ahmad Z,Hasnain S. 2007. Production of surfactin from *Bacillus subtilis* MZ-7 grown on pharmamedia commercial medium. Microb Cell Fact. 6(17). doi:10.1186/1475-2859-6-17.

Amani H,Haghighi M,Keshtkar MJ. 2013. Production and optimization of microbial surfactin by *Bacillus subtilis* for ex situ enhanced oil recovery. Petroleum SciTechnol. 31(12):1249-58. doi: 10.1080/10916466.2010.542416.

Banat IM, Franzetti A, Gandolfi I, Bestetti G, Martinotti MG, Fracchia L, Smyth TJ, Marchant R. 2010. Microbial biosurfactants production, applications and future potential. Appl Microb Biotechnol. 87(2):427-44.

Box GEP, Hunter WG, Hunter JS. 1978. Statistics for experimenters. New York: John Wiley and Sons 291-334.

Cooper DG, Macdonald CR, Duff SJB, Kosaric N. 1981. Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions. Appl Environ Microbiol. 42(3):408-12.

Ghribi D, Ellouze-Chaabouni S. 2011. Enhancement of *Bacillus subtilis* lipopeptide biosurfactants production through optimization of medium composition and adequate control of aeration. Biotechnol Res Int. doi:10.4061/2011/653654.

Huang X, Lu Z, Zhao H, Bie X, Lü F, Yang S. 2006. Antiviral activity of antimicrobial lipopeptide from *Bacillus subtilis* FMBJ against Pseudorabies Virus, Porcine Parvovirus, Newcastle Disease Virus and Infectious Bursal Disease Virus in vitro. Int J Peptide Res Therapeut. 12(14):373-377. doi:10.1007/s10989-006-9041-4.

Kim SY, Kim JY, Kim SH, Bae HJ, Yi H, Yoon SH, Koo BS, Kwon M, Cho JY, Lee CE, Hong S. 2007.Surfactin from *Bacillus subtilis* displays anti-proliferative effect via apoptosis induction, cell cycle arrest and survival signaling suppression. FEBS Let. 581(5):865-71. doi:10.1016/j/febslet.207.01.059.

Lim JH, Park BK, Kim MS, Hwang MH, Rhee MH, Park SC, Yun HI. 2005. The anti-thrombotic activity of surfactins. J Vet Sci. 6(4):353-5.

Liu X, Ren B, Gao H, Liu M, Dai H, Song F, Yu Z, Wang S, Hu J, Kokare CR, Zhang L. 2012. Optimization for the production of surfactin with a new ynergisticantifungal activity. PLoS ONE7(5). doi:10.1371/journal.pone.003 4430.

Meena KR, Kanwar SS. 2015. Lipopeptides as the antifungal and antibacterial agents: applications in food afety and therapeutics. BioMed Res Int. doi:10.1155/2015/473050.

Mnif I, Chaabouni-Ellouze S, Ghribi D. 2012. Optimization of the nutritional parameters for enhanced production of *B. subtilis* SPB1 biosurfactant in submerged culture using response surface methodology. Biotechnol Res Int. doi:10.1155/2012/795430

Sabate DC, Audisio MC. 2013. Inhibitory activity of surfactin, produced by different *Bacillus subtilis subsp. subtilis* strains, against *Listeria monocytogenes* sensitive and bacteriocin-resistant strains. Microbiol Res. 168(3):125-29. doi:10.1016/j.micres.2012.11.004.

Sen R. 1997. Response surface optimization of the critical media components for the production of surfactin. J Chem Tech Biotechnol. 68(3):263-70. doi:10.1002/(SI CI)1097-4660(199703)68:3<263::AID-JCTB631>3. 0.CO;2-8.

Sen R, Swaminathan T. 1997. Application of response surface methodology to evaluate the optimum environmental conditions for the enhanced production of surfactin. Appl Microbiol Biotechnol. 47(4):358-63. doi:10.1007/s002530050940.

Singh AK, Rautela R, Cameotra SS. 2014a. Substrate dependent in vitro antifungal activity of *Bacillus* sp. strain AR2. Microb Cell Fact. 13:67. doi:10.1186/1475-2859-13-67.

Wei YH, Lai CC, Chang JS. 2007 Using Taguchi experimental design methods to optimize trace element composition for enhanced surfactin production by *Bacillus subtilis* ATCC 21332. Proc Biochem. 42(1):40-5. doi:10.1016/j.procbio.2006.07.025.

Yi L, Zhang G, Zhu Z, Wang X, Ran W, Shen Q. 2013. Optimization of medium composition for lipopeptide production from *Bacillus subtilis* N7 using response surface methodology. Korean J Microbiol Biotechnol. 41(1):52-9.doi:10.4014/kjmb.1207.07020.

Zeraik AE, Nitschke M. 2010. Biosurfactants as agents to reduce adhesion of pathogenic bacteria to polystyrene surfaces: effect of temperature and hydrophobicity. Curr Microbiol. 61(6):554-9. doi:10.1007/s00284-010-9652-z.

Zhang Y, Liu C, Dong B, Ma X, Hou L, Cao X, Wang C. 2015. Anti-inflammatory activity and mechanism of surfactin in lipopolysaccharide-activated macrophages. Inflammation 38(2):756-64. doi:10.1007/s10753-014-9986-y.