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ECTOINE PRODUCTION BY *HALOMONAS ELONGATA* BK-AG25: TWO-STEP OPTIMIZATION USING THE RESPONSE SURFACE METHODOLOGY

I PUTU PARWATA[1,4], DEANA WAHYUNINGRUM[2], SONY SUHANDONO[3] AND RUKMAN HERTADI[1*]

[1]Biochemistry Research Division, Faculty of Mathematics and Natural Sciences, Bandung Institute of Technology, Bandung 40132, West Java, Indonesia
[2]Organic Chemistry Research Division, Faculty of Mathematics and Natural Sciences, Bandung Institute of Technology, Bandung 40132, West Java, Indonesia
[3]Genetics and Molecular Biology Division, School of Biological Science and Technology, Bandung Institute of Technology, Bandung 40132, West Java, Indonesia
[4]Analytical Chemistry Department, Faculty of Mathematics and Natural Sciences, Universitas Pendidikan Ganesha, Singaraja 81116, Bali, Indonesia

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Abstract – This study is aimed to optimize ectoine production by *Halomonas elongata* BK-AG25 with a two-step cultivation method. First, the bacteria were cultivated in MM63 media containing a moderate level of salt to obtain optimal biomass, followed by cultivation using the same media with a higher level of salt to gain maximum ectoine production. The level of nutrients in MM63 media and the incubation temperature were optimized using the response surface methodology (RSM). Using a central composite design to attain the optimal conditions for biomass level and ectoine production. The RSM model predicted a maximum biomass level of about 5.04 mg/mL when the bacteria were first cultivated at 37.4 °C in MM63 media. The model also predicted that a maximum ectoine production will reach about 186.4 mg/g of cell dry weight after the second cultivation in MM63 media with an NaCl level of 18% [w/v] at 33 °C. The experimental result following this two-step cultivation obtained a biomass level of 4.92 mg/mL and ectoine production of 179.9 mg/g of cell dry weight. The results showed that two-step cultivation was an effective method for elevating both the biomass level and ectoine productions.

INTRODUCTION

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) is characterized by compatible organic molecules produced by halophilic microorganisms as a response and a protection against high salinity levels in their habitat. It has outstanding water-binding activity preventing water loss from inside the bacterial cell (Graf *et al.,* 2008). Some studies have obtained that ectoine has a critical function for the survival of halophilic bacteria against various environmental stresses other than high salinity, such as heating, freezing, drought, UV light, or contact with toxic materials (Knapp *et al.,* 1999; Lamosa *et al.,* 2000; Margesin and Shinner, 2001).

Ectoine is produced by halophilic bacteria intracellularly. Therefore, the production yield is related to the level of biomass. However, typically the biomass level is inversely proportional to the ectoine production yield in terms of the salinity level of the culture medium. The growth of the bacteria is generally inhibited by a high level of salinity, while ectoine production is stimulated by the high level of salinity of the culture medium. One way to overcome this problem is by performing a two-step cultivation method. The first cultivation is carried out in a medium containing a moderate level of salinity to gain a high level of biomass. The second cultivation is aimed to boost ectoine production by using a high-salinity culture medium (Van-Thuoc *et al.,* 2010).

In the previous work, we identified a potential halophilic bacteria, *Halomonas elongata* BK-AG25,

which is indigenous to a salty mud crater located in Bleduk Kuwu village, Purwodadi, Central Java, Indonesia. The initial attempt at ectoine production by the bacteria gave a relatively low production yield, which was about 61.7 mg/g of the cell dry weight (Parwata *et al.*, 2016). The production of ectoine from *Halomonas elongata* by using "bacterial milking" techniques to produce ectoine at about 155 mg/g of the cell dry weight (Sauer and Galinski, 1998). Therefore, this work is aimed to optimize ectoine production by *Halomonas elongata* BK-AG25. In the present study, the optimization is carried out using the two-step cultivation method described above. The level of nutrients in the production media and the incubation temperature of each cultivation step were optimized with the statistical approach using the response surface methodology (RSM). The experimental setup for the optimization was performed following the central composite design (CCD). The combination of RSM and CCD is an effective technique to identify critical factors to boost the production yield of biotechnological products (Van-Thuoc *et al.*, 2010; Barrington and Kim, 2008; Bergmann *et al.*, 2013; Dutka *et al.*, 2015; Huang *et al.*, 2007; Soni *et al.*, 2007). We show here that RMS and CCD can improve the ectoine production yield of *Halomonas elongata* BK-AG25 significantly.

MATERIALS AND METHODS

Bacterial strain and media

Halomonas elongata BK-AG25 was isolated from the mud crater of Bledug Kuwu located in Purwodadi Central Java, Indonesia. The bacteria was maintained at 4°C on solid MM63 medium composed of (per liter) 13.61 g KH_2PO_4, 4.21 g KOH, 1.98 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4.7H_2O$, 0.0011 g $FeSO_4.7H_2O$, 5 g glucose.H_2O, 100 g NaCl, and 20 g Bacto Agar. The culture medium used in this study was MM63 medium (Larsen *et al.*, 1987). Glucose was sterilized separately, and the pH was adjusted to 7.1 using NaOH. The concentrations of glucose, NaCl, $(NH_4)_2SO_4$, and $MgSO_4$ in the medium varied to obtain optimum conditions for bacterial growth and ectoine production.

Biomass production

Halomonas elongata BK-AG25 was grown in 50-mL Erlenmeyer flasks containing 10 mL of the MM63 medium on a rotary shaker at 37 °C and 150 rpm for 18 hours. Subsequently, 0.25 mL of the culture was then inoculated in 20 mL of fresh MM63 medium in 100-mL Erlenmeyer flasks. Cultivation was carried out on a rotary shaker at varied temperatures (28–45 °C) with agitation of 150 rpm for 22 hours for biomass production. The samples were then taken for biomass analysis. The composition of the MM63 medium used for biomass production varied (% [w/v]), i.e. glucose (0.1–1.6), $(NH_4)_2SO_4$ (0.05–0.75), $MgSO_4.7H_2O$ (0.015–0.15), and NaCl (1–13).

Ectoine production

Halomonas elongata BK-AG25 was grown in MM63 medium for 22 hours to generate the optimum biomass concentration. Subsequently, the bacterial cells were separated from the culture by cold centrifugation at 6,000 xg for 20 min. The cells were than suspended aseptically in 20 mL fresh MM63 medium containing varied concentrations of NaCl (9–20% [w/v]) in 100-mL Erlenmeyer flasks. The culture was incubated on a rotary shaker at varied temperatures (15–45 °C) and agitation of 150 rpm for 24 hours for ectoine production. After 24 hours of incubation, the samples were taken for biomass and ectoine analysis.

Analytical methods

Biomass concentration was determined following the procedure proposed by Van-Thuoc et al. (2010). Bacterial cells were separated by cold centrifugation at 6,000 rpm for 20 min. The cell pellet was washed with media (without glucose) containing NaCl at the same level as the previous medium. After centrifugation, the cells were dried in an oven at a temperature of 70 °C to obtain a constant weight. The biomass concentration is expressed as a gram of cell dry weight (cdw) per liter of inoculum.

Extraction of ectoine produced by each bacterium was conducted following the procedures presented by Bligh and Dyer (Kunte *et al.*, 1993). One mL of bacterial culture was centrifuged at 6,000 x g for 20 min at 4 °C. The cell pellets were separated from the media and lyophilized. The dried cells were then extracted with 400 mL of methanol/chloroform/water (10/5/4 [vol/vol/vol]) by vigorous shaking for 90 min. Equal volumes (130 μL) of chloroform and water were then added. The mixture was again shaken for 30 min, and phase separation was enhanced by centrifugation at 10,000 x g for 30 min. The water phase containing ectoine was recovered and lyophilized. The dried ectoine was resuspended in methanol. Due to solubility differences in

methanol, ectoine was separated from concomitant precipitation of hydroxyectoine by centrifugation. Ectoine was than recovered by lyophilization and resuspended in water. The suspension was diluted to suitable concentrations in water for HPLC analysis.

Twenty microliters of each sample were analyzed on a Nucleosil 100-5 C18, 25 cm by 3.2 mm (5 µm) column (Sigma-Aldrich, USA), and ectoine was monitored by its absorbance at 210 nm using a UV/VIS detector. The solvent employed for compatible solute separation was water/acetonitrile (95/5 [vol/vol]). Chromatography was carried out isocratically at a flow rate of 1 mL/min and at 20 °C using Agilent Technologies 1260 Infinity high-performance liquid chromatography (Germany). The ectoine retention time was determined using the standard purchased from Sigma Aldrich.

Experimental design

Biomass production of *Halomonas elongata* BK-AG25 was generated in two-step optimization. The first step was designed to gain the optimum condition of the incubation temperature and the level of salt (NaCl) in the culture medium. The second step was performed to optimize the composition of glucose, $(NH_4)_2SO_4$, and $MgSO_4.7H_2O$ in the medium. While the optimization of ectoine production by the bacteria was conducted by varying the level of NaCl in the culture medium and the incubation temperature.

Preliminary experiments have been generated using the "one variable at a time" methods to approximate the relevant range for the parameters above (data not shown). Subsequently, a CCD was performed to obtain a five-levels range of each parameter optimized (Table 1). The responses for biomass and ectoine production were fitted to a full quadratic optimization model using Minitab 17 software.

RESULTS

Optimization of nutrients in media and incubation temperature for biomass production

The first cultivation was performed to optimize the biomass production of the bacteria. The biomass optimization was conducted in two steps. A total of 26 experiments with several combinations of NaCl concentration and incubation temperature were performed for the first biomass optimization. The results obtained for biomass production by *Halomonas elongata* BK-AG25 are presented in Supplementary Table 1. The second biomass optimization involved a total of 40 experiments with different combinations of glucose, $(NH_4)_2SO_4$, and $MgSO_4$ concentrations; the results are showed in Supplementary Table 2. The response analysis of the experimental data resulted in a high coefficient of determination (R^2) for biomass production of the first and the second optimization of 0.907 and 0.949, respectively. Thus, there was good agreement between the experimental data and the predicted value for both optimizations. This means that 90.7% and 94.9% of the variability in the response could be explained by the model of the first and the second biomass optimization, respectively.

The regression equations in noncoded units for biomass concentration generated by both optimizations are given below. These models (equations 1 and 2) were used to calculate the predicted values of biomass concentration for the first and second optimization (data are showed in Supplementary Tables 1 and 2).

Biomass conc. =–26.34 + 0.322 NaCl + 1.571 Temp. –

Table 1. The value of parameters for optimization of biomass and ectoine production performed using central composite design

Optimization	Parameter	Level				
Biomass production (I)		-1.414	-1	0	1	1.414
	NaCl (% w/v)	1	2.8	7	11.2	13
	Temperature (°C)	28	30.5	36.5	42.5	45
Biomass production (II)		-1.682	-1	0	1	1.682
	Glucose (% w/v)	0.1	0.4	0.85	1.3	1.6
	$(NH_4)_2SO_4$ (% w/v)	0.05	0.19	0.4	0.61	0.75
	$MgSO_4.7H_2O$ (% w/v)	0.015	0.042	0.083	0.123	0.15
Ectoine production		-1.414	-1	0	1	1.414
	NaCl (% w/v)	9	10.6	14.5	18.4	20
	Temperature (°C)	15	19.4	30	40.6	45

0.0573 NaCl × Nacl–0.0231 Temp. × Temp.+0.0185 Nacl × Temp.

.. (1)

$$Biomass\ conc. = -0.249 + 8.504\ Glucose + 3.35\ (NH_4)_2SO_4 + 7.60\ MgSO_4 - 3.667\ Glucose \times Glucose - 3.161\ (NH_4)_2SO_4 \times (NH4)_2SO_4 - 28.5\ MgSO_4 \times MgSO_4 - 1.170\ Glucose * (NH_4)_2SO_4 - 6.70\ Glucose * MgSO_4 + 5.07\ (NH_4)_2SO_4 \times MgSO_4 \quad .. (2)$$

The significance of the regression coefficients was determined using Student's *t*-test with a confidence level of 5%. The calculated coded coefficient and the p-value of each variables optimized are showed in Table 2. It revealed that in linear terms, glucose and NaCl in the media had a significant effect on the biomass production of *Halomonas elongata* BK-AG25 (given by p-value below 0.05), while $(NH_4)_2SO_4$, $MgSO_4$, and incubation temperature were not significant (p-value above 0.05). In terms of quadratic regression, all variables except for $MgSO_4$ in the media significantly influenced the biomass production of the bacteria. Among the four nutrients, NaCl and sugar showed a crucial effect on the biomass production of the bacteria, given by high linear coefficients of 0.837 and 0.5574, respectively. However, $(NH_4)_2SO_4$ as a nitrogen source for bacterial growth had a low effect on biomass yield (quadratic coefficient of -0.1369). Meanwhile, $MgSO_4$ showed an insignificant impact on the biomass production of the bacteria at the level range conducted. Furthermore, incubation temperature showed a significant quadratic effect on the biomass production of the bacteria with a regression coefficient of -0.835. The high negative quadratic coefficients for NaCl, glucose, and incubation temperature suggest that the variables were in close range of the optimum value.

Table 2. Estimated coded coefficient and p-value for biomass concentration on optimization using response surface methodology

Factor	Coded Coefficient	p-Value
Linear		
NaCl	0.837	0.000
Temperature	0.077	0.476
Glucose	0.5574	0.000
$(NH_4)_2SO_4$	0.0517	0.208
$MgSO_4$	-0.0312	0.444
Square		
NaCl x NaCl	-1.031	0.000
Temperature x Temperature	-0.835	0.000
Glucose x Glucose	-0.7291	0.000
$(NH_4)_2SO_4$ x $(NH_4)_2SO_4$	-0.1369	0.001
$MgSO_4$ x $MgSO_4$	-0.0460	0.249
Interaction		
NaCl vs Temperature	0.473	0.005
Glucose vs $(NH_4)_2SO_4$	-0.1086	0.047
Glucose vs $MgSO_4$	-0.1200	0.030
$(NH_4)_2SO_4$ vs $MgSO_4$	0.0424	0.426

The statistical significance of the response model for the biomass production of the bacteria was conducted using Fisher's test for ANOVA. The F values of the model for the first and the second optimizations are 39.18 (p-value 0.000) and 61.74 (p-value 0.000), respectively, indicating that both models are very fit and can adequately explain the variation observed. The model showed a significant interaction between NaCl and incubation temperature in influencing the biomass production of the bacteria (given by p-value below 0.05 for interaction coefficient of 0.473). A high NaCl concentration along with low incubation temperature or vice versa or a low level of both could reduce the biomass production of the bacteria (Fig. 1a). Based on the model obtained, the optimum biomass production (above 4 mg/mL) resulted after incubation of the bacteria in MM63 media containing 6–12% [w/v] of NaCl at a temperature of 33–42 °C.

The model also showed a moderate interaction between glucose and $MgSO_4$ on the biomass production of the bacteria (interaction coefficient of -0.12). Based on the model, the optimum concentrations of glucose and $MgSO_4$ for biomass production are in the range of 1–1.2% and 0.015–0.08% [w/v], respectively (Fig. 1b).

The glucose level under 0.3% along with the $MgSO_4$ level under 0.08% could reduce the biomass production of the bacteria drastically. The same effect was obtained when using media with a glucose level above 1.5% and a $MgSO_4$ level above 0.06%. Furthermore, a weak interaction was observed between glucose and $(NH_4)_2SO_4$ (interaction coefficient of -0.1086). A low concentration of glucose (under 0.3%) combined with a low level of $(NH_4)_2SO_4$ (under 0.3%) dramatically lowered the biomass production. Similarly, a high concentration of glucose (above 1.5% [w/v]) and $(NH_4)_2SO_4$ (above 0.5%) could reduce the biomass production of the bacteria. The concentration of $(NH_4)_2SO_4$ for the optimum biomass production was 0.2–0.5% [w/v] (Fig. 1c). Based on fig. 1d showed a moderate interaction between $(NH_4)_2SO_4$ and $MgSO_4$ on the biomass

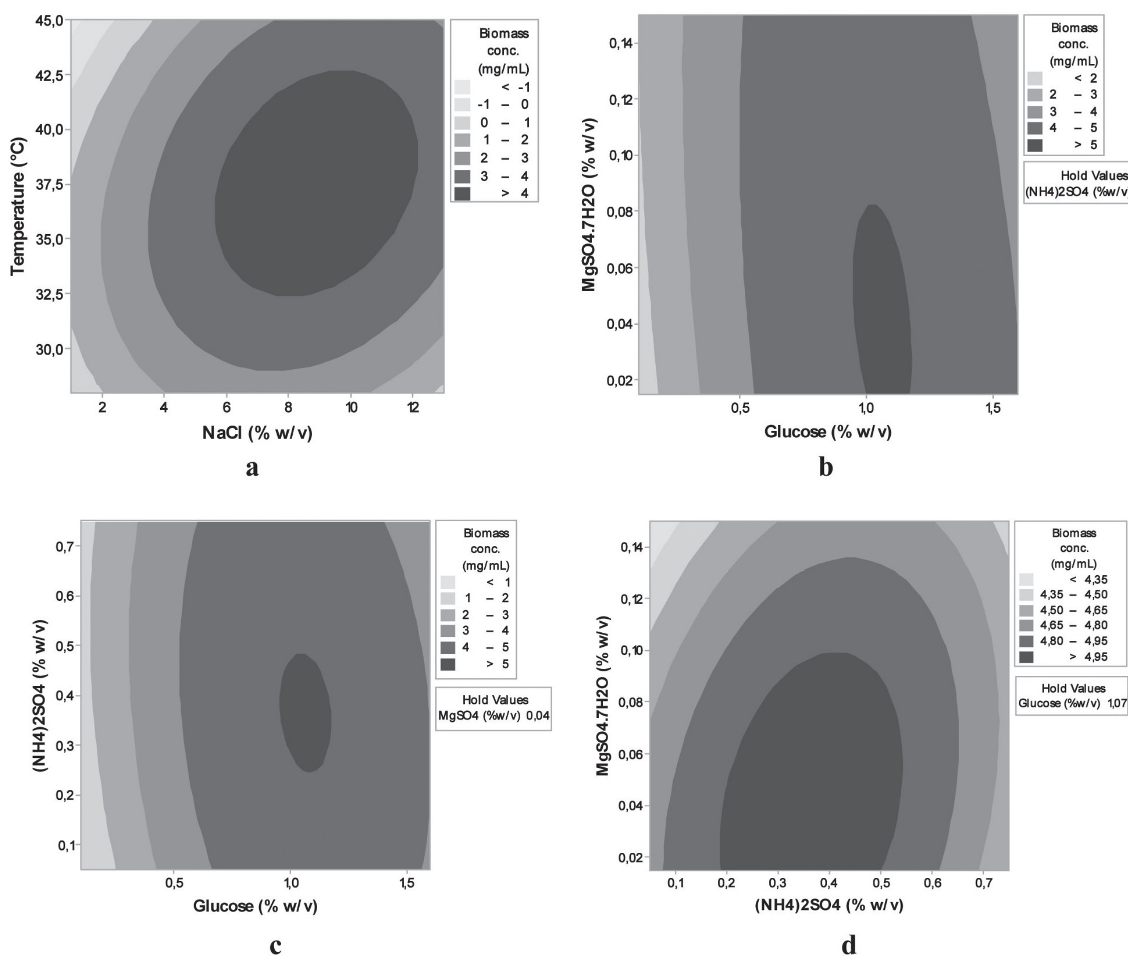

Fig. 1. Effect of nutrients in MM63 media and incubation temperature on biomass production of *Halomonas elongata* BK-AG25

production of the bacteria. Based on the model, the optimum biomass production concentration of $(NH_4)_2SO_4$ and $MgSO_4$ are in a range of 0.2 – 0.5 and 0.015 – 0.10% [w/v].

Based on the model obtained, the predicted value of biomass concentration of 5.04 mg/mL was gained after incubation of *Halomonas elongata* BK-AG25 in MM63 media containing the optimum levels of NaCl, glucose, $MgSO_4$, and $(NH_4)_2SO_4$ of 8.9%, 1.07%, 0.04%, and 0.37% [w/v], respectively, at an incubation temperature of 37.4 °C. The experimental value of the biomass concentration obtained using those optimum conditions was 4.92 mg/mL with a standard deviation (SD) of 0.028 (data not shown). The experimental value obtained was in good agreement with the predicted value.

Optimization of incubation temperature and the salt level of media for ectoine production

The second cultivation was conducted to optimize the ectoine production of the bacteria. Optimization for ectoine production included a total of 26 experiments with several combinations of NaCl concentration and incubation temperature. The results obtained for ectoine concentration and the productivity of *Halomonas elongata* BK-AG25 are presented in Table 3. The analysis of the experimental data resulted in good agreement between the experimental data and the predicted value, indicated by a high coefficient of determination (R^2) of 0.866 and 0.928 for ectoine concentration (g/L) and the productivity of the bacteria (mg ectoine/g cdw), respectively. The regression equations in noncoded units for ectoine concentration produced by the bacteria and its productivity are given below (equations 3 and 4) and used to calculate the predicted values of both (data are shown in Table 3).

$$\textit{Ectoine conc.} = -0.558 + 0.0363\ \textit{NaCl} + 0.0839\textit{Temp.} - 0.00302\ \textit{NaCl} \times \textit{NaCl}$$

-0.00192 *Temp.* × *Temp.* + 0.00235 *NaCl***Temp.* .. (3)

Productivity = 29.8 + 8.16 *NaCl* + 5.05 *Temp.* – 0.510 *NaCl* × *NaCl* – 0.1867 *Temp.* × *Temp.* + 0.3562 *NaCl** *Temp.* .. (4)

Using Student's *t*-test with a confidence level of 5%, the calculated coded coefficient and the p-value of each variable optimized were attained for ectoine concentration and the productivity of *Halomonas elongata* BK-AG25 (Table 3). It showed that the level of NaCl in the media, as well as the incubation temperature, had a vital effect on the ectoine production of the bacteria. Both factors showed a significant effect on the productivity of the bacteria in producing ectoine in both linear and quadratic terms. The incubation temperature and NaCl level also influenced the ectoine concentration significantly. However, the incubation temperature showed an insignificant linear effect on ectoine concentration (p-value 0.131), indicating that increasing or decreasing temperature in the range of 15–40 °C did not change the ectoine concentration produced by the bacteria.

Fisher's test for the response model of ectoine concentration and bacterial productivity resulted in a high value of 25.95 (p-value 0.000) and 51.75 (p-value 0.000), respectively, suggesting that the model is very fit and can adequately explain the variation observed. The model showed the range level of NaCl l, and the incubation temperature for the optimum ectoine concentration is wider than for the optimum bacterial productivity. The optimum ectoine concentration (above 1.0 g/L) was obtained at a NaCl level of 10–20% [w/v] and incubation temperature of 22–43 °C (Fig. 2a). The optimum productivity of the bacteria (above 180 mg ectoine/g cdw) was attained at a NaCl level of 15–20% [w/v] and temperature of 23–37 °C (Fig. 2b). This result showed that each cell of the bacteria produced ectoine optimally at a high level of NaCl. Since ectoine production is related to biomass yield, optimum ectoine production depended on both bacteria productivity and biomass yield. As a consequence, a high ectoine concentration was also obtained at a lower level of NaCl, which supported biomass production.

The model showed a significant interaction between NaCl level and incubation temperature on the productivity of *Halomonas elongata* BK-AG25

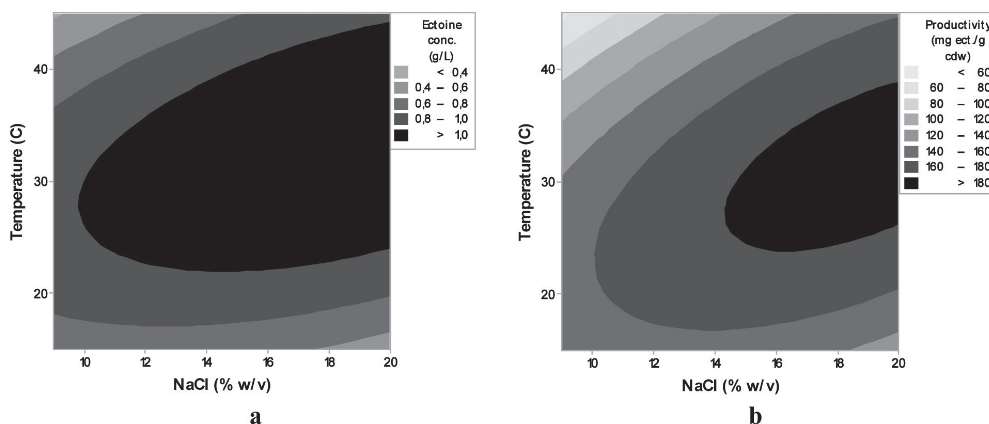

Fig. 2 Effect of salt level and incubation temperature on ectoine production of *Halomonas elongata* BK-AG25

Table 3. Estimated coded coeffisient and p-Value for ectoine concentration (g/L) and bacterial productivity in optimization using response surface methodology

Factor	Ectoine concentration (g/L)		Productivity (mg ectoine/g cdw)	
	Coded Coeffisient	p-Value	Coded Coeffisient	p-Value
Linear				
NaCl	0.0752	0.001	15.82	0.000
Temperature	0.0315	0.131	-10.51	0.000
Square				
NaCl x NaCl	-0.0457	0.046	-7.71	0.001
Temperature x Temperature	-0.2158	0.000	-21.01	0.000
Interaction				
NaCl vs Temperature	0.0971	0.003	14.69	0.000

(interaction coefficient of 14.69). Cultivation of the bacteria in MM63 media containing a NaCl level under 13% [w/v] at an incubation temperature above 40 °C dramatically reduced its production to be less than 80 mg ectoine/g cdw (Fig. 2b). Moreover, a combination of a high level of NaCl (> 18% [w/v]) and a low incubation temperature (< 17 °C) slightly lowered the productivity of the bacteria (< 140 mg ectoine/g cdw). A similar pattern of the interaction effect of NaCl and temperature interaction was observed in ectoine concentration with a moderate level of interaction coefficient of 0.0971 (Table 3).

Biomass production of *Halomonas elongata* BK-AG25 in the second cultivation was investigated. The analysis of the response model for the biomass increment of the bacteria resulted in a high coefficient of determination (R^2) of 0.947 (data not shown). Based on the model, the optimum increment of the biomass (above 50%) was obtained at a NaCl level under 12% [w/v] (Fig. 3).

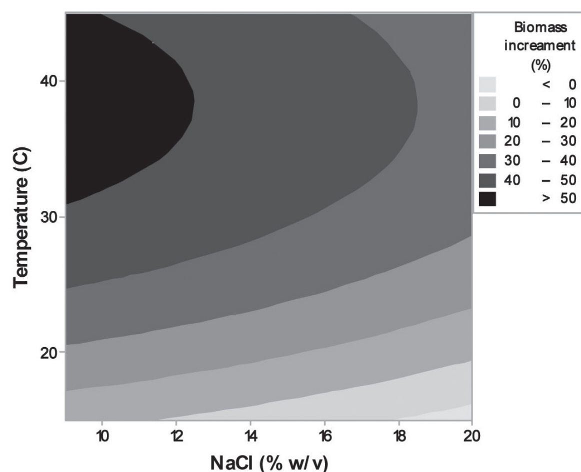

Fig. 3. Effect of salt level and incubation temperature on biomass increment of *Halomonas elongata* BK-AG25 on the second cultivation

According to the model obtained, the optimum concentration of ectoine (1.19 g/L) and the bacterial productivity (186.4 mg ectoine/g cdw) were predicted on the cultivation of *Halomonas elongata* BK-AG25 in MM63 media containing a NaCl level of 18% [w/v] at a temperature of 33 °C. The experimental value of ectoine concentration and the productivity of the bacteria obtained using those optimum conditions were 1.17 g/L (SD= 0.015) and 179.9 mg/g cdw (SD= 8.52), respectively (data not shown). The experimental value obtained was in good agreement with the predicted value.

DISCUSSION

Based on this result suggests that two-step cultivation successfully increased the biomass yield, thus increasing the ectoine production of the bacteria. The significant increase in biomass concentration in the second cultivation was most likely due to the removal of growth inhibitory byproducts formed during the first cultivation and is in accordance with the report on an increase in cell density obtained for Marinococcus M52 (Frings *et al.*, 1995) and *Halomonas boliviensis* (Van-Thuoc *et al.*, 2010).

The productivity of *Halomonas elongata* BK-AG25 in the ectoine production in this study (179.9 mg/g cdw) is comparable to that of *Brevibacterium epidermis* (160 mg/g cdw) (Onraedt *et al.*, 2005) and *Alkalibacillus haloalkaliphiles* (170 mg/g) (Bergmann *et al.*, 2013). However, it is slightly higher than *Halomonas boliviensis* (145 mg/g) (Van-Thuoc *et al.* 2010). Moreover, the production of ectoine from *Halomonas elongata* DSM 142^T reported by Sauer and Galinski (1998) yielded 155 mg of pure ectoine per gram cdw, comparable to the crude ectoine produced by *Halomonas elongata* in this study. However, the level of NaCl in the media used for ectoine production by *Halomonas elongata* DSM 142^T was 15%, lower than that used by *Halomonas elongata* BK-AG25 in this study (18% [w/v]).

CONCLUSION

The optimum level of NaCl for the biomass production of *Halomonas elongata* BK-AG25 was much lower than for ectoine production; thus, two-step cultivation of the bacteria effectively enhanced the biomass yield, consequently increasing ectoine production. The optimization using the RSM was very effective and efficient to attain the optimum conditions for the biomass and ectoine production of the bacteria. The production of ectoine by the bacteria (179.9 mg/g cdw) in this study was significantly high, comparable to that produced by other bacteria.

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