

# Production of ectoine by *Halomonas elongata* BK-AG25 using osmotic shock technique

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**Abstract.** The production of ectoine from a halophilic bacteria *Halomonas elongata* BK-AG25 was optimized using osmotic shock technique. At first, the bacteria were grown in MM63 media containing optimal level of salt to gain high biomass yield. The bacteria were then inoculated in the same media with high concentration of salt (osmotic upshock) to produce ectoine. Subsequently, the bacteria were transferred into distilled water containing lower concentration of salt (osmotic downshock) to release ectoine produced. The two process is known as “bacterial milking”. These process were repeated several cycles to gain a maximum yield of ectoine. The survival of the cells and ectoine released by the bacteria after osmotic downshock were determined. The results showed that the survival bacteria were more than 70% after the serial osmotic downshock, which was from 17.5%(w/v) to 1.5%(w/v) and then to 3% (w/v) NaCl. However, only 9% of the bacteria were survive when the same process was occurred from 17.5% to 0% (w/v) NaCl. By using the former downshock process, the yield of ectoine released by the bacteria was relatively high, which was about 88%. The productivity of the bacteria in producing ectoine was high, which was about 206.4 mg extracellular per g cell dry weight.

## 1. Introduction

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) is one of the osmolytes synthesized by halophilic microorganism to counter osmotic stress caused by high concentration of salt in their habitats. In addition, ectoine is also known as biomolecule's protector against various environmental stresses, such as heating, drought, freezing, and contact with UV light or toxic materials [1,2,3]. These superior characteristics lead to the widespread use of ectoine, especially in pharmaceuticals and cosmetics.

Ectoine-producing bacteria *Halomonas elongata* BK-AG25, moderate halophilic bacteria, has been isolated from the mud crater of Bledug Kuwu located in Purwodadi Central Java, Indonesia. In the previous work, production of ectoine by the bacteria have been optimized using two steps cultivation, first to gain high biomass yield of the bacteria followed by the second step to produce high level of ectoine [4]. Several factors such as the level of salt and nutrients in media as well as the incubation temperature have been optimized. The optimization was performed for both biomass and ectoine



production. The productivity of the bacteria after the optimization was as high as 179.9 mg ectoine per g cell dry weight (cdw). Ectoine produced by the bacteria was about 1.17 g/L of bacterial culture after 2 days incubation. Ectoines produced by the bacteria intracellularly, accordingly it requires extraction steps to release the molecules from the cell, thereby increasing the production cost.

In this study, the ectoine production of *Halomonas elongata* BK-AG25 was optimized using a combination of osmotic upshock and downshock techniques. Initially, the bacteria were grown in the media containing high concentration of salt (osmotic upshock) to stimulate ectoine production. Ectoine was released from the cell by incubating the bacteria in the lower concentration of salt solution (osmotic downshock). The process was repeated several cycles to obtain high yield of ectoine. Such process that well-known as “bacterial milking” was applied to produce ectoine from *Halomonas elongata* [5] and *Halomonas boliviensis* [6]. This study will demonstrate that such process was also effective to be applied for ectoine production from *Halomonas elongata* BK-AG25.

## 2. Material and methods

### 2.1. Bacterial strain and media

*Halomonas elongata* BK-AG25 was maintained at 4 °C on solid MM63 medium composed of (per liter): 13.61 g  $\text{KH}_2\text{PO}_4$ , 4.21 g KOH, 1,98 g  $(\text{NH}_4)_2\text{SO}_4$ , 0.25 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.0011 g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 5 g Glucose. $\text{H}_2\text{O}$ , 100 g NaCl, and 20 g bacto agar. The production of biomass and ectoine by the bacteria were conducted in MM63 medium with the optimized nutrients composition of (per liter): 13.61 g  $\text{KH}_2\text{PO}_4$ , 4.21 g KOH, 3.7 g  $(\text{NH}_4)_2\text{SO}_4$ , 0.15 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.0011 g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and 10.7 g Glucose. $\text{H}_2\text{O}$ . The level of salt (NaCl) in the medium was 8.9% [w/v] for bacterial biomass production and 17.5% [w/v] for ectoine production. Glucose was sterilized separately, pH was adjusted to 7,1 by using NaOH.

### 2.2. Determination of the ectoine released and the survival of cell after osmotic downshock

*Halomonas elongata* BK-AG25 was grown in 50 mL Erlenmeyer flasks containing 10 mL MM63 medium containing 8.9% [w/v] NaCl on a rotary shaker at 37 °C and 150 rpm for 18 hours. Subsequently, 0.4 mL of the culture was inoculated in 20 mL of fresh MM63 medium in 100 mL Erlenmeyer flasks and incubated at 37 °C with agitation of 150 rpm for 22 hours for biomass production. The bacterial cell was separated from the culture by cold centrifugation at 6,000 x g for 20 minutes and suspended aseptically in 20 mL fresh MM63 medium containing 17.5% [w/v] NaCl. The culture was incubated in a rotary shaker at 33 °C and 150 rpm for 24 hours for ectoine production. At the end of incubation, the bacterial cell was separated from the culture and then was incubated in distilled water containing varied level of NaCl (0%, 1.5% and 3% [w/v]) at 200 rpm and 27 °C for 30 minutes to release ectoine. The suspensions were then centrifuged at 6000 x g for 20 minutes and the ectoine content in the supernatants were analyzed. The yield of ectoine production was calculated as the percentage of ectoine excreted by the bacteria with respect to that stored in the cell before the osmotic downshock.

The cell pellets obtained after osmotic downshock were suspended in a sterile water containing 17.5% [w/v] NaCl. After serial dilution, the suspension was subsequently plated on solid Luria Bertani media containing 8.9% [w/v] NaCl. The bacteria colonies on the plates were counted after 2 days of cultivation at 37 °C. The cell survival was determined based on the ratio of the number of colonies after the osmotic downshock with respect to those that were not treated by the process.

### 2.3. Production of ectoine using “bacterial milking” technique

Further optimization of ectoine production was conducted by “bacterial milking” technique. The osmotic shock process (upshock and downshock) was repeated alternately for several cycles to obtain maximum ectoine yield. The concentration of ectoines released for each cycle were determined.

#### 2.4. Analytical methods

Bacterial cells were separated by cold centrifugation at 6,000 x *g* for 20 minute and then were lyophilized. Intracellular ectoines were extracted following the procedure presented by Bligh and Dyer [7]. The cell pellets from 1 mL bacterial cultures were extracted with 400 mL of methanol/chloroform/water (10/5/4, [vol/vol/vol]) by vigorous shaking for 90 minutes. Subsequently, equal volumes (130  $\mu$ L) of chloroform and water were added to the mixtures and shaken for 30 min. Ectoines in water phase were recovered by centrifugation at 10,000 x *g* for 30 minutes and lyophilized. In order to separate ectoine from hydroxyectoine, the dried ectoine was resuspended in methanol. Due to the solubility differences, ectoine can be separated from hydroxyectoine by centrifugation. Ectoines were collected by lyophilization and resuspended with water for HPLC analysis. Ectoines excreted in to the medium were directly analyzed using HPLC.

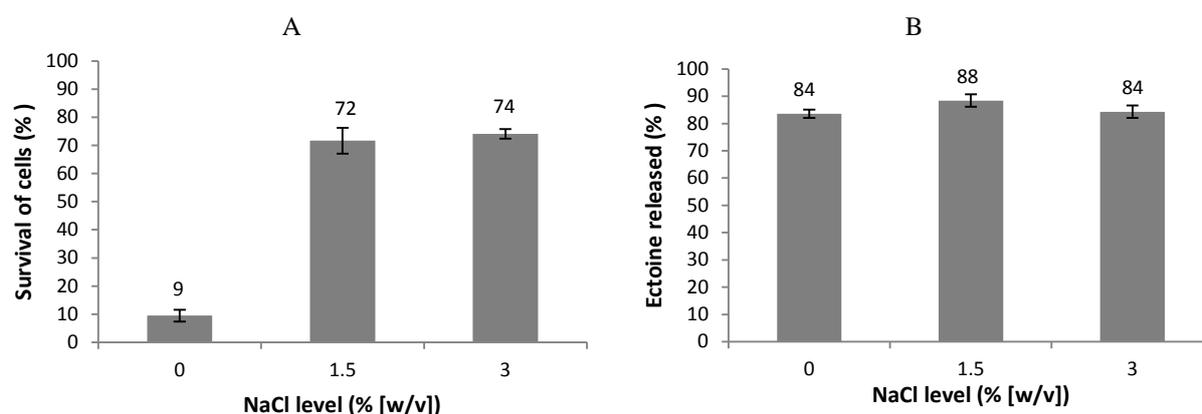
The extracellular and intracellular ectoines were subjected to 0.2  $\mu$ m filter membrane and analyzed using Agilent Technologies 1260 Infinity high-performance liquid chromatography (Germany). Twenty microliter of ectoine solutions were injected to Nucleosyl 100-5 C18, 25cm by 3.2 mm (5  $\mu$ m) column (Sigma-Aldrich, USA) and separated isocratically by water/acetonitrile (95/5 [vol/vol]) at flow rate of 1 mL/min and 20 °C. Ectoine concentration was monitored by measuring its absorbance at 210 nm using a UV/VIS detector. Commercial ectoine from sigma Aldrich was used to determine retention time of the sample.

### 3. Results and Discussion

#### 3.1. The survival of cells and ectoine released by bacterial osmotic downshock

As halophilic bacteria, a minimum level of salt was required by *Halomonas elongata* BK-AG2 to survive in a non halophilic environment. Most of the bacterial cells (up to 90%) were die after osmotic downshock using distilled water without addition of NaCl (figure 1A). This result suggested that *Halomonas elongata* BK-AG25 was not able to maintain its cell viability in a medium without salt. However, most of the bacterial cells (above 70%) still survived after osmotic downshock in distilled water containing 1.5% and 3% [w/v] NaCl and for subsequent hyperosmotic upshock (back to 17.5% NaCl). The level of bacterial cell survivals after the osmotic downshock by using water containing 1.5% and 3% NaCl were comparable, indicating that the minimum salt content for the bacterial survival was 1.5% [w/v]. The cell survival of the bacteria was slightly lower than *Halomonas elongata* DSM 142<sup>T</sup> which was able to maintain its cell viability up to 86% after the osmotic downshock from 15% to 3% [w/v] NaCl [5]. This probably due to the stiffer osmotic downshock, which was from 17.5% to 3% [w/v] NaCl experienced by the bacteria in this study.

The percentages of ectoine released by *Halomonas elongata* BK-AG25 after the osmotic downshock were relatively high (figure 1B). The osmotic downshock using distilled water containing NaCl level of 0%, 1.5% and 3% [w/v] released ectoines with significant yield, which was 84% (SE= 1.5), 88% (SE= 2.3) and 84% (SE= 2.3) respectively, in accordance with *H. elongata* DSM 142<sup>T</sup> that yield about 90% ectoine after the downshock in 3% NaCl [5]. *H. elongata* BK-AG25 maintained its intracellular ectoine up to 15% after the osmotic downshock. Interestingly, the bacteria still keep a small fraction of intracellular ectoine after the incubation in distilled water without NaCl. It is likely that the bacteria excreted more ectoine after the incubation in pure distilled water due to the higher osmotic downshock effect compared to that of *H. elongata* DSM 142<sup>T</sup>. However the level of ectoine excreted was comparable for the three NaCl level (figure 1B). The mechanism of ectoine excretion by halophilic bacteria has not been understood yet. Grammann et al reported that mutation of TeaABC, a specific transporter responsible for ectoine uptake of *Halomonas elongata*, caused overproduction and leaking of ectoine [8].



**Figure 1.** The survival of cells (A) and ectoine released (B) by *Halomonas elongata* BK-AG25 after the osmotic downshock

*Halomonas elongata* BK-AG25 showed a high productivity on ectoine released (table 1). The bacteria excreted a maximum of 206.4 mg ectoine per g cdw after the osmotic downshock process using distilled water containing 1.5% [w/v] NaCl. Comparably, the productivities of the bacteria by the same process using 0% and 3% NaCl were 195.5 and 196.9 mg/g cdw, respectively. However, the concentration of ectoine produced were not as expected due to low densities of the bacteria. Two steps cultivation of the bacteria in MM65 medium for the first 22 hours followed by the second 24 hours were only able to yield 5.4 g cell dry weight per litre bacterial culture. The maximum ectoine released by the cultivated bacteria after osmotic downshock in 1.5% NaCl was 1.11 g/L.

**Table 1.** Ectoine concentration and bacterial productivity after the osmotic downshock of *Halomonas elongata* BK-AG25

NaCl (% [w/v])	Ectoine concentration (g/L)				
	E1	E2	E3	Mean	Standard error
0	1.01	1.07	1.06	1.05	0.019
1.5	1.08	1.07	1.16	1.11	0.029
3	1.02	1.03	1.11	1.05	0.028
NaCl (% [w/v])	Productivity (mg ectoine/g cdw)				
	E1	E2	E3	Mean	Standard error
0	179.7	213.2	193.7	195.5	9.7
1.5	192.7	214.3	212.2	206.4	6.9
3	181.8	206.3	202.7	196.9	7.6

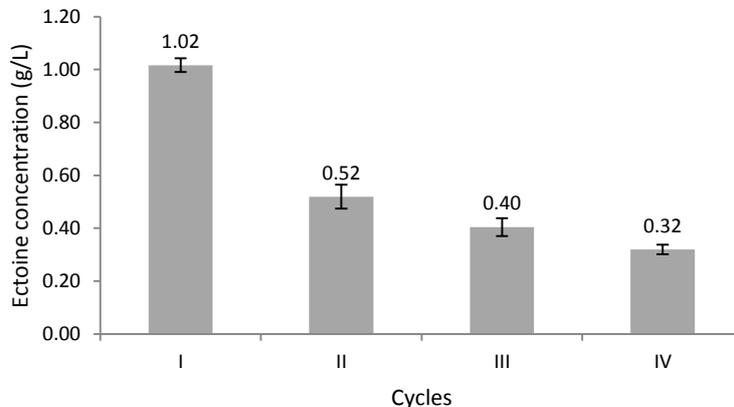
The productivity of *Halomonas elongata* BK-AG25 in this study (206.4 mg ectoine/g cdw) was fairly higher than *Halomonas elongata* DSM 142<sup>T</sup> (155 mg/g cdw) [5]; *Brevibacterium epidermis* DSM 20659 (160 mg/g cdw) [9]; *Alkalibacillus haloalkaliphiles* (170 mg/g) [10]; and *Halomonas boliviensis* DSM 15516 (145 mg/g) [6]. However, the level of ectoine produced by the bacteria (1.11 g/L/ two days) was lower than other bacteria as reported by Pastor et al [11], such as *H. elongata* DSM 142<sup>T</sup> (1.3 g/L/day); *B. epidermis* (2 g/L/day); and *H. boliviensis* (9.1 g/L/day). The low level of ectoine obtained in this study was caused by the low density of the bacterial cells (5.4 g/L) cultivated by using batch reactor system. Meanwhile, a fed-batch cultivation of bacteria was reported produce higher cell density, which was about 48, 62.4, and 50 g/L for *H. elongata* DSM 142<sup>T</sup>, *H. boliviensis* DSM 15516, and *B. epidermis* DSM 20659, respectively. The level of ectoine produced by *Halomonas*

*elongata* BK-AG25, however, slightly higher than *Brevibacterium sp.* JCM 6894 which produced 0.34 g ectoine/L/day in batch reactor [12]. Accordingly, ectoine production may vary depending on the bacterial species and the level of the downshock used to release ectoine from cells.

### 3.2. Production of ectoine by “bacterial milking” technique

The production of ectoine by *Halomonas elongata* BK-AG25 using “bacterial milking” technique is shown in figure 2. In the first cycle, the bacteria released ectoine with the highest concentration of 1.02 g/L (SE= 0.026). Entering the second cycle, the ectoine excreted by the bacteria was declined significantly to 0.52 g/L (SE= 0.045). Subsequently, the concentrations of excreted ectoine were reduced gradually to 0.40 g/L (SE= 0.034) and 0.32 g/L (SE= 0.018) on the third and the fourth cycles. In the first cycle of the bacterial milking, the bacteria were cultivated in two steps, the first step for biomass production followed by the second step for ectoine production. Meanwhile, in the subsequent cycles, the bacteria were only cultivated in one step using medium for the production of ectoine. This lead to a significant difference of ectoine synthesized by the bacteria in the first cycle and the subsequent cycles.

A total four cycles of bacterial milking of *Halomonas elongata* BK-AG25 for 5 days were able to produce ectoine as much as 2.26 g/L. Meanwhile, ectoine produced by *Halomonas elongata* DSM 142<sup>T</sup> after 9 cycles of the bacterial milking was 1.3 g/L/day [5], which is significantly higher than that produced by the bacteria in this study, which is around 0.5 g/L/day. The low density of the bacteria was the main factor inhibiting the production of ectoine by *Halomonas elongata* BK-AG25. Using a combination of batch-fed batch bioreactor system, *Halomonas elongata* DSM 142<sup>T</sup> was able to provide biomass up to 48 g/L, nearly nine times higher than provided by the bacteria in this study (5.4 g/L). Thus, optimization of the cell density using bioreactor is the main target of our future work.



**Figure 2.** The concentration of ectoine released by *Halomonas elongata* BK-AG25 during “bacterial milking”

## 4. Conclusion

*Halomonas elongata* BK-AG25 is the potential halophilic bacteria to produce ectoine. The productivity of *H. elongata* BK-AG25 on ectoine production was about 206.4 mg for each gram of the cell dry weight. Bacterial milking technique is the effective way to extract ectoine produced by the bacteria, in which most of ectoine can be released out of the cells after multiple cycles of the osmotic upshock and osmotic downshock. Total ectoine produced was about 1.11 g/L after two days cultivation and the addition of about 2.26 g/L ectoine was obtained after four cycles of bacterial milking process.

## 5. Acknowledgment

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