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Efficient Cyclic System to Yield Ectoine Using *Brevibacterium* sp. JCM 6894 Subjected to Osmotic Downshock

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ABSTRACT: *Brevibacterium* sp. JCM 6894 cells grown in the presence of 1.5-2.5 M NaCl for 24 h at 30°C were subjected to the osmotic downshock. Downshocked cells after ectoine release were grown for further 24 h in the fresh medium with same salinity as before shock. When this cyclic system was applied to the strain JCM 6894, the amount of ectoine in the cells increased with an increase of incubation time, which indicates that the cells manipulated by the present conditions were enough active to survive and synthesize ectoine after several times of osmotic downshock. In the presence of 2 M NaCl, the highest yield of ectoine released was achieved in this cyclic system, more than 2.4 g/L during 7 days of incubation. ¹H and ¹³C-NMR analyses of solutes released from the cells by the osmotic downshock showed the presence of only ectoine with high purity. Release of ectoine from the cells was carried out within 5 min and its rates were increased by the dilution in the downshock treatment. For the convenience of operations, non-sterilized medium containing 2 M NaCl was examined for the cell growth in the present system, in which almost same level of ectoine yield, release rates, and cell viability were observed as those of sterilized medium.

KEYWORD: *Brevibacterium* sp. JCM 6894, ectoine, cyclic system, osmotic downshock

Introduction

To cope with the hyperosmotic conditions, microorganisms accumulate large quantities of a particular group of organic osmolytes (Da Costa et al., 1998; Nagata, 2001; Kuhlmann and Bremer, 2002). Cyclic amino acid ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) was first discovered as a minor component in the phototrophic sulfur bacterium *Ectothiorhodospira halochloris* (Galinski et al., 1985). Since then ectoines (ectoine and its hydroxyl derivative, hydroxyectoine) are well known as the representative of ubiquitous compatible solutes serving mainly as osmoprotectants for halophilic and halotolerant bacteria under high osmolarity. In recent years ectoines have been paid attention as one of the most useful compounds to show a variety of characteristics such as stimulation on the respiration as well as growth of *Escherichia coli* (Nagata et al., 2002) or heat resistance of enzyme activities (Zhang et al., 2006), regular PCR enhancers by decreasing the melting temperature of dsDNA (Schnoor et al., 2004), skin protection from UVA-induced cell damage (Buenger and Driller, 2004), and inhibition of aggregation and neurotoxicity of Alzheimer's β -amyloid (Kanapathipillai et al., 2005).

To achieve a large scale of supply for ectoines in compliance with the demands, bacterial milking method for ectoines has been examined by using osmotic downshock (Frings et al., 1995; Sauer and Galinski, 1998) or fed-batch process (Onraedt et al., 2005). The reason why utilization of bacteria on the production of compatible solutes including ectoines has been developed might be ascribed to be easier and higher purity of product in comparison with chemical synthesis which generally consists of several different types of reactions. Bacterial methods reported until now, however, require not only high amounts of nutrients in growth medium but also severe controls such as pH, aeration, and feeding during the operation of ectoine production (Sauer and Galinski, 1998; Onraedt et al., 2005). In this

connection, it is of necessity and importance to contrive the efficient and convenient method to achieve the high yield of ectoines using bacterial functions efficiently.

Organic osmolytes accumulated by microorganisms to cope with hyperosmotic conditions are released when they are exposed to hypoosmotic conditions (Galinski, 1995; Oren, 1999). Unlike *Ectothiorhodospira halochloris* (Tschichholz and Trüper, 1990) or *Vibrio alginolyticus* (Unemoto et al., 1973), the halotolerant *Brevibacterium* sp. JCM 6894 which is an efficient producer of ectoine (Nagata et al., 1996) does not burst after several times of downshock treatments as well as dilution processes (Nagata and Wang, 2006). Thus, it seems to be plausible to use the downshocked cells of *Brevibacterium* sp. JCM 6894 repeatedly for the synthesis of ectoine after they are transferred into the fresh medium with high salinity. Taking the efficient yield of ectoine by the use of low concentration of nutrients into account, we tried to invent the cyclic system using the combination of osmotic downshock and regrowth of *Brevibacterium* sp. JCM 6894. In addition, an attempt was made to examine whether the autoclaving process for growth medium containing 2 M NaCl can be omitted to simplify the procedure in this cyclic system.

Materials and Methods

Medium and Growth Conditions

A halotolerant strain *Brevibacterium* sp. JCM 6894 used in this study was grown aerobically at 30°C in a medium with low concentration of nutrients as follows ; Polypepton (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan), 5.0 gL⁻¹ and Dried yeast extract (Nihon Pharmaceutical Co., Ltd.), 3.0 gL⁻¹, and 1.5-2.5 M NaCl (pH 7.5). Growth was monitored by measuring turbidity at 650 nm (OD₆₅₀) using a Beckman DU 640 spectrophotometer (Beckman

Instruments, Fullerton, CA, USA). The turbidity of cells was measured after appropriate dilution of samples with the medium when OD₆₅₀ was more than 1.

Procedure of the Novel Cyclic System

The cells were precultured in the nutrient medium without NaCl supplemented for 24 h at 30°C. 1 % of preculture was added to 30 mL of each medium in a 300 mL flask. After 24 h of incubation at 30°C, each culture was centrifuged (12,000g, 5 min, 4°C) and the pellets were resuspended in distilled water with original volume to induce the release of ectoine by hypoosmotic shock. The cells were allowed to equilibrate for 10 min at 25°C, unless otherwise stated, and then harvested by centrifugation (12,000g, 5 min, 4°C). Each pellet resuspended in the fresh medium with respective NaCl concentrations was incubated for further 24 h. The procedure was repeatedly carried out, 7-8 times.

HPLC and NMR Analyses of Ectoine

After osmotic downshock for the cells, appropriate amount of cell suspension was sampled and centrifuged (12,000g, 5 min, 4°C). The supernatant was measured to quantify the extracellular concentration of ectoine, while the pellets were extracted with 80 % ethanol. The latter was provided for the measurement of intracellular ectoine concentration.

Ectoine was detected by high performance liquid chromatography (HPLC) system (L-5020; Hitachi, Tokyo, Japan) with a Bio-Sil C₈ 90-5S reversed phase column (Bio-Rad Laboratories, Hercules, CA, USA) as described previously (Nagata and Wang, 2001) and NMR spectroscopy (JEOL JNM-AL300, JOEL Ltd., Tokyo, Japan). For the former, 5 µL of samples were eluted with 50 mM KP_i buffer at pH 6.0 at a flow rate of 1 mL/min at 35°C and detected by UV absorbance at 210 nm. For the latter, intracellular and extracellular samples were evaporated after extraction with 80 % ethanol and then suspended in D₂O, both of which were provided for NMR

measurements. The identification and quantification of ectoine were carried out using authentic ectoine as a standard.

Release rates (RR) of ectoine by the cells subjected to osmotic downshock were calculated by the following equation:

$$RR = [C(E)/\{C(E) + C(I)\}] \times 100$$

where C(E) and C(I) indicate the extracellular and intracellular concentrations of ectoine (mg/L), respectively.

Determination of Viable Cell Numbers

To determine the viable cell numbers before and after osmotic downshock, cell culture was serially diluted by distilled water and subsequently plated on agar plates consisting of the same compositions as the growth medium without NaCl supplemented. Colonies were counted after 3 days of incubations at 30°C.

Chemicals

Ectoine, which was of > 97 % purity, was provided by Dainippon Sumitomo Pharma Co., Ltd, Tokyo, Japan. Other chemicals used were of reagent or higher grade.

Results and discussion

Yield of Ectoine during Cyclic System

The cells were grown in the presence of 1.5-2.5 M NaCl for 24 h at 30°C and then subjected to the osmotic downshock. As a consequence of the repeated procedure of growth and downshock treatment every 24 h, the amount of ectoine in the cells increased regardless of the external NaCl concentrations

(Fig. 1), which indicates that the cells put in the present conditions were enough active to survive and resynthesize ectoine after respective osmotic downshock. When the cells were grown in the presence of 1.5 or 2 M NaCl, ectoine concentrations in the cells attained to about 900 mg/L in the cells at 144-168 h of incubations, although about 660 mg/L of ectoine was obtained as the maximum for the cells manipulated in the presence of 2.5 M NaCl.

The amounts of ectoine synthesized and its release from the cells were quantitatively determined every 24 h and tabulated in Table 1. As realized from this table, cyclic system using cells grown the presence of 2 M NaCl exhibited the highest yield of ectoine during 7 days of incubation, 2469.8 mg. The cells grown in the presence of 2.5 M NaCl, on the contrary, showed the lowest yield of ectoine among the growth conditions examined, mainly due to the absolutely low amount of ectoine synthesized, as seen in Fig. 1. To clarify whether poor ectoine yield for the cells grown in the presence of 2.5 M NaCl was attributed to the insufficient growth at first 24 h of incubation, $OD_{650} \approx 1.5$, we tried to examine the effect of the elongation of incubation time, 48 h of incubation, to achieve the sufficient growth of cells at first stage and then downshock in the same manner as described above. As a consequence, cell growth was improved after 48 h of incubation, $OD_{650} \approx 2.6$ compared to that of 24 h of incubation where almost stationary phase of growth was attained (Nagata, 1988), but ectoine yield was still low as follows; 141, 208, 342, and 684 mg/L of ectoine at 48, 72, 120, and 168 h of incubations, respectively. Yield of total ectoine released from the cells during 7 days of incubation resulted in 1381 mg/L with average release rate of 62 %. For comparison, we also examined the cells grown in the presence of 2 M NaCl for 48 h of incubation without downshock, in which yield of total ectoine released resulted in 2014 mg/L, slightly lower than that of every 24 h of downshock treatment mentioned above.

Taking both synthetic amounts and release rates of ectoine into consideration, optimum condition for the present cyclic system to obtain

ectoine was attained by carrying out every 24 h of downshock treatment for the cells grown in the presence of 2 M NaCl. Based on this result, we will mainly discuss the case of 2 M NaCl in the following sections.

Release of Ectoine

Final yield of ectoine was not so much for the cells grown in the presence of 1.5 M NaCl compared with that of 2 M NaCl. This is mainly due to the insufficient release of ectoine, since intracellular amount of ectoine was almost same as that of 2 M NaCl (Fig. 1). These results suggest that the final amount of ectoine obtained was dependent on not only synthetic amount of ectoine but the efficient release of ectoine from the cells, the latter of which played an important role to yield high amount of ectoine finally. Thus, release rates of ectoine were quantitatively calculated (Fig. 2), in which they were gradually reduced as a function of the incubation time for every case examined. As a whole, release rates of ectoine were conceivably dependent on NaCl concentrations in the growth medium, *i.e.*, larger difference of osmotic shock for the cells led to more efficient release of ectoine, as expected. The fastest reduction in release rates of ectoine was observed for the cells grown in the presence of 1.5 M NaCl, where they decreased almost linearly until 4 days of incubation and reached to about 40 % after 7 days of incubation. Release rates of both cells grown in the presence of 2 M and 2.5 M NaCl, on the other hand, were reduced slightly with the incubation time but they showed still over 50 % after 7 days of incubation.

Downshock treatment of the cells was carried out by the addition of deionized water into the culture by 1:1 ratio and incubated for 10 min. To improve the release rates of ectoine, we tried to examine the effects of cell incubation time in deionized water, 5-60 min, as well as dilution rates, up to 20 folds. In terms of employing the cells at 144 h of incubation, highest accumulation of ectoine (Table 1), their release rates of ectoine were examined under different conditions. As a result, release of ectoine was

carried out within 5 min of incubation (Table 2), indicating that such a rapid release of compatible solute was conducted through not transporters but channels like MscL (Berrier et al., 2000). On the other hand, dilution procedure brought about the effective improvement on the release rates of ectoine in the strain JCM 6894. For instance, they increased from 52.4 % to 67.1 % for 10 min of incubation when eight fold dilution was applied.

Interrelation of Efficiency on Ectoine Yield

An attempt was made to examine the changes of viable cell numbers in the present cyclic system. As shown in Table 3, viable cell numbers in every case examined were slightly reduced just after osmotic downshock, but they resumed to grow again when transferred to the fresh medium with the same salinity as before, regardless of the external NaCl concentrations, 1.5-2.5 M. As a consequence, the viable cell numbers at first 24 h of incubation, 3.0×10^9 cfu/mL, increased almost 10 folds after 7 days of incubation, 2.4×10^{10} cfu/mL, when the cells were grown in the presence of 2 M NaCl.

To clarify the most efficient condition to yield ectoine, we tried to depict the interrelation between intracellular ectoine concentrations and viable cell numbers before and after downshock treatment. As shown in Fig. 3, every condition of cell growth examined showed the increase of ectoine concentrations in the cells accompanied with the increase of viable cell numbers both before and after osmotic downshock. For the cells grown in the presence of 2.5 M NaCl, the amount of intracellular ectoine increased linearly from 60 to 660 mg during 168 h of incubation (Table 1 and Fig. 3), but viable cell numbers were very low as already shown (Table 3). Therefore, the interrelation between intracellular ectoine concentrations and viable cell numbers was the sharpest and the difference between before and after downshock treatments was the smallest among every condition examined. On the other hand, internal concentrations of ectoine in cells grown in the presence of 1.5-2 M NaCl gradually increased with the increase of viable cell

numbers and showed logarithmic correlation, suggesting that the amount of ectoine reflects the cell growth faithfully regardless of the osmotic downshock treatment. Here, the difference between before and after osmotic shock is regarded as the indicator for efficient synthesis and release of ectoine in this cyclic system, *i.e.*, larger difference higher efficiency of ectoine yield. In this regard, we can conclude that the highest recovery of ectoine synthesis after osmotic downshock was attained for cells manipulated in the presence of 2 M NaCl rather than 1.5 M NaCl, especially in the region of cell viability with $>1.5 \times 10^{10}$ cfu/mL.

Use of Non-sterilized Medium for Cyclic System

From the actual and convenient point of view, it seems to be one of the important factors if we are able to operate this system without trouble. In this connection, we attempted to examine whether the sterilization procedure can be omitted or not, when 2 M NaCl-containing medium is used for this system. As shown in Table 1, there was little difference in the synthetic amounts as well as release rates of ectoine between the cells grown in the sterilized and non-sterilized media, indicating that the contamination of other bacteria might be reasonably neglected in this condition. In fact, contaminated bacteria did not appear on the agar medium during 7-8 days incubations in the present system, probably due to the high concentration of NaCl present in the medium. In addition, viable cell numbers observed in the medium containing 2 M NaCl did not show a remarkable difference irrespective of whether the medium was sterilized or not (Table 3). Furthermore, similar level of release rates of ectoine was observed for the cells manipulated in both media (Fig. 2). As a whole, the highest yield of ectoine was achieved when the non-sterilized medium containing 2 M NaCl was used for the present cyclic system (Table 1).

Next, we tried to examine whether the downshock treatment affects the cellular ability of ectoine synthesis. For this purpose we prepared the

following cells that were grown in the fresh medium containing 2 M NaCl and were transferred every 24 h without osmotic downshock. As shown in Table 1, intracellular ectoine concentration after 7 days of incubation afforded 895.9 mg/L that was almost same as that of downshocked cells, 867.8 mg/L. Here it seems to be valuable to note that the final amount of released ectoine for cells grown in the presence of 2 M NaCl with and without osmotic downshock resulted in about 2470 and 416 mg of ectoine, respectively. This fact suggests that the synthetic activity of ectoine in downshocked cells was rather stimulated by several times of osmotic treatments, since more than half of ectoine synthesized was lost by downshock treatment and continuously they resumed to synthesize ectoine to higher levels than before shock.

Downstream processing

Purity of released ectoine by the present cyclic system was examined by NMR for the samples before and after downshock treatment. As the representative, supernatant fraction after three times of osmotic downshock, 72 h of incubation, in the presence of 2 M NaCl was measured and shown in Figs. 4A and 4B, in which quite high purity of ectoine was indicated. Taking the appearance of only one peak in HPLC analysis (data not shown) into account, released solute from the cells consists of ectoine with high purity, suggesting that the further purification seems to be not required. It is of interest to point out that intracellular solutes before shock also seem to be mainly occupied by ectoine (Fig. 4C), suggesting that the present growth condition of strain JCM 6894 led to the exclusive synthesis of ectoine. Other samples after 24, 120, 168 h of incubations showed quite similar patterns of NMR spectra as that of 72 h of incubation (data not shown).

As the economical point of view, the cost to yield 1 g of ectoine in this cyclic system resulted in 140 Japanese yen, equivalent with 0.88 Euro or 1.18 US dollar, which is more expensive than that of Onraedt et al. (2005). Average of ectoine yield in the present cyclic system, however, afforded about

150 mg of ectoine per cycle per gram cell dry weight, which is almost same as that of *H. elongata* (Sauer and Galinski, 1998).

Present study is focused on the establishment of simple cyclic system to yield ectoine by the use of low concentrations of nutrients for the cell growth in combination with the osmotic downshock. Lower yield of ectoine compared with previous study (Onraedt et al., 2005) will be improved in terms of bioengineering techniques such as cell immobilization as well as effective gene insertion of ectoine synthesis, changes of osmolarity during this process, usage of other strains with higher ability to synthesize and release of ectoine than JCM 6894, usage of high concentrations of effective nutrients, trial of high cell density (Frings et al., 1995; Sauer and Galinski, 1998), etc. To gain more excellent procedure, further improvement of ectoine yield is now under progress.

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Figure captions

Figure 1.

Synthesis and release of ectoine by *Brevibacterium* sp. JCM 6894. The cells were grown in the medium containing 1.5-2.5 M NaCl at 30°C for 24 h. After centrifugation and washing the cells, they were subjected to the osmotic downshock by incubating in deionized water for 10 min at 30°C. Subsequently the downshocked cells were incubated for further 24 h in the fresh medium with the same concentrations of NaCl as before shock. This procedure was repeatedly carried out until 168-192 h of incubation. Open and shaded parts in each column indicate the amounts of released and remained ectoine in the cells, respectively. Analytical procedure of ectoine was described in Material and Methods Section. The values are the means ± SD from three independent experiments.

Figure 2.

Release rates (RR) of ectoine as a function of incubation time. The cells

were grown in the presence of 1.5 M (open triangles), 2 M (open squares), and 2.5 M NaCl (open circles) and were subjected to the osmotic downshock, as described in legend of Fig. 1. Dotted line with closed squares indicates the cells grown in the non-sterilized medium containing 2 M NaCl. RR values were calculated as described in Material and Methods Section. The values are the means \pm SD from three independent experiments.

Figure 3.

Interrelation between viable cell numbers and intracellular concentrations of ectoine before and after osmotic downshock. The cells were grown in the presence of 1.5 M (triangles), 2 M (squares), and 2.5 M NaCl (circles) and were subjected to the osmotic downshock, as described in legend of Fig. 1. Amount of intracellular ectoine and viable cell numbers were obtained before (closed symbols) and after (open symbols) downshock treatments. The values are the means \pm SD from three independent experiments.

Figure 4.

^1H and ^{13}C NMR spectra of solutes before and after osmotic downshock. The cell suspension subjected to the osmotic downshock at 72 h of incubations were centrifuged and separated. The supernatant fraction evaporated at 40°C and suspended in D_2O , for which ^1H (A) and ^{13}C (B) NMR spectra were measured. Intracellular solutes by extraction with 80 % ethanol of the cells mentioned above were also analyzed by ^{13}C NMR (C).

Table 1 Changes of intracellular ectoine concentrations before and after osmotic downshock (mg/L)

NaCl (M) in medium	Osmotic shock	24 h	48 h	72 h	96 h	120 h	144 h	168 h	Yield of total ectoine (mg) ^a
1.5	BS ^b	224.6 ± 7.8	414.7 ± 8.7	458.2 ± 5.5	574.0 ± 18.2	638.6 ± 30.3	791.6 ± 46.8	895.3 ± 6.1	1828.5
	AS ^c	75.9 ± 11.3	181.9 ± 9.9	222.3 ± 10.7	317.1 ± 14.2	362.9 ± 21.3	470.2 ± 22.8	538.2 ± 4.9	
2.0	BS	167.5 ± 12.2	393.8 ± 22.1	549.5 ± 16.2	713.5 ± 27.6	813.5 ± 16.2	908.6 ± 13.5	867.8 ± 7.3	2469.8
	AS	62.4 ± 0.9	142.0 ± 8.8	222.8 ± 12.1	309.5 ± 12.2	371.6 ± 7.6	432.1 ± 9.1	404.0 ± 8.6	
2.5	BS	57.8 ± 4.6	204.8 ± 1.9	347.2 ± 35.2	390.8 ± 19.0	471.8 ± 26.1	579.9 ± 19.5	663.6 ± 7.0	1652.9
	AS	18.0 ± 3.4	70.5 ± 7.6	134.8 ± 22.9	153.5 ± 16.7	186.1 ± 19.6	228.8 ± 15.0	271.3 ± 4.6	
2.0 ^d	BS	158.7 ± 13.5	412.9 ± 11.8	569.7 ± 10.4	740.7 ± 18.2	818.6 ± 14.9	905.2 ± 11.2	869.3 ± 12.8	2524.6
	AS	55.8 ± 12.2	145.5 ± 6.9	226.1 ± 11.2	321.9 ± 10.3	379.2 ± 21.3	434.9 ± 10.1	387.1 ± 7.4	
2.0 ^e	BS	157.9 ± 9.2	398.5 ± 20.2	561.1 ± 11.4	716.9 ± 41.9	814.4 ± 29.9	926.1 ± 10.7	895.9 ± 20.6	416.0 ^f

^a Total amounts of ectoine released by seven times of osmotic downshock are given.

^b Before osmotic downshock

^c After osmotic downshock

^d The cells were grown and manipulated in the non-steriled medium.

^e The cells were transferred into fresh medium every 24 h without osmotic downshock.

^f Final amount of ectoine released from the cells after 168 h of incubation is given.

Table 2 Release rates (RR) of ectoine in various dilutions (%)^a

Incubation time (min)	Dilution fold				
	1	2	4	8	20
5	52.4 ± 0.0	59.9 ± 0.1	64.3 ± 0.5	66.9 ± 0.6	68.1 ± 0.3
10	52.4 ± 0.8	59.9 ± 0.1	64.1 ± 0.0	67.1 ± 0.2	68.3 ± 0.2
60	52.4 ± 0.1	59.9 ± 0.2	64.1 ± 0.7	67.7 ± 0.0	68.4 ± 0.2

^a Dilution experiment was carried out for the cells after 144 h of incubation in the present cyclic system with 2 M NaCl.

Table 3 Changes of viable cell numbers before and after osmotic downshock (cfu/ml $\times 10^8$)

NaCl in Medium (M)	Medium Sterilization	Osmotic shock	24 h	48 h	72 h	96 h	120 h	144 h	168 h
1.5	+	BS ^a	56.8 \pm 1.3	84.5 \pm 2.7	103.5 \pm 7.8	155.0 \pm 12.7	215.4 \pm 30.2	226.4 \pm 3.7	270.2 \pm 42.1
		AS ^b	55.3 \pm 5.2	77.8 \pm 1.4	92.8 \pm 4.7	141.0 \pm 4.2	198.0 \pm 24.0	222.9 \pm 7.2	247.3 \pm 35.0
2.0	+	BS	30.4 \pm 1.3	74.1 \pm 6.7	103.0 \pm 21.4	130.4 \pm 18.8	209.7 \pm 31.7	255.1 \pm 16.9	238.1 \pm 11.1
		AS	27.1 \pm 1.4	62.4 \pm 9.1	101.7 \pm 17.3	121.0 \pm 36.4	196.7 \pm 10.6	233.9 \pm 5.7	222.2 \pm 9.7
	—	BS	29.7 \pm 1.8	73.9 \pm 5.1	113.0 \pm 12.5	140.4 \pm 24.3	200.3 \pm 11.5	249.5 \pm 27.7	229.7 \pm 7.7
		AS	28.9 \pm 1.3	66.7 \pm 0.9	99.7 \pm 3.6	130.5 \pm 8.8	186.9 \pm 29.1	232.3 \pm 21.6	210.5 \pm 14.3
	+	Non	29.7 \pm 4.7	74.4 \pm 1.6	106.9 \pm 22.1	121.9 \pm 24.4	199.0 \pm 21.7	265.1 \pm 13.8	251.8 \pm 21.2
2.5	+	BS	20.4 \pm 2.3	27.0 \pm 0.0	40.3 \pm 2.1	43.8 \pm 1.7	47.0 \pm 1.4	49.9 \pm 1.8	54.0 \pm 3.1
		AS	15.2 \pm 3.2	25.6 \pm 4.1	38.6 \pm 3.6	43.6 \pm 2.0	45.5 \pm 0.7	48.6 \pm 2.7	53.5 \pm 4.5

^a Before osmotic downshock

^b After osmotic downshock

Fig. 1

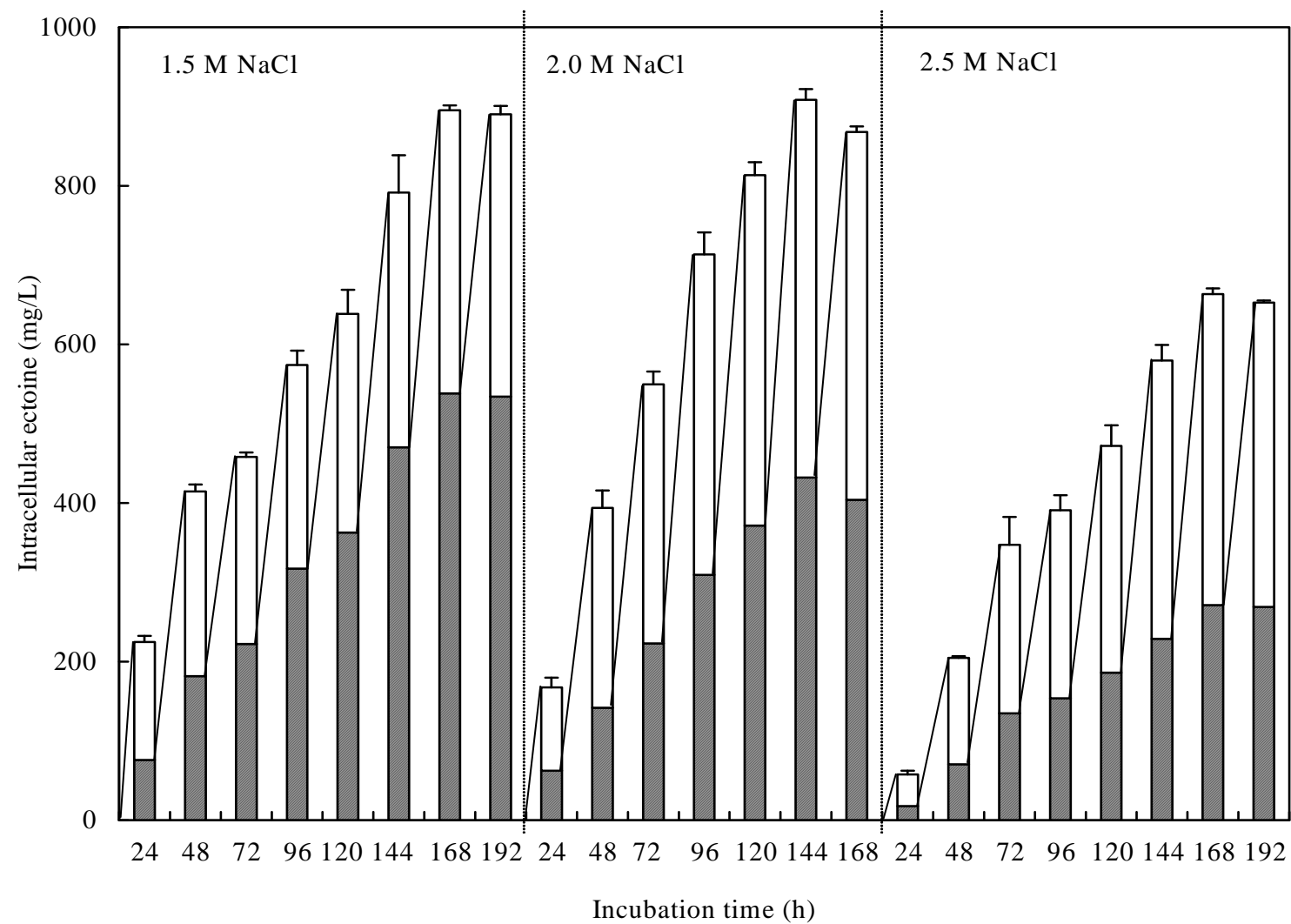


Fig. 2

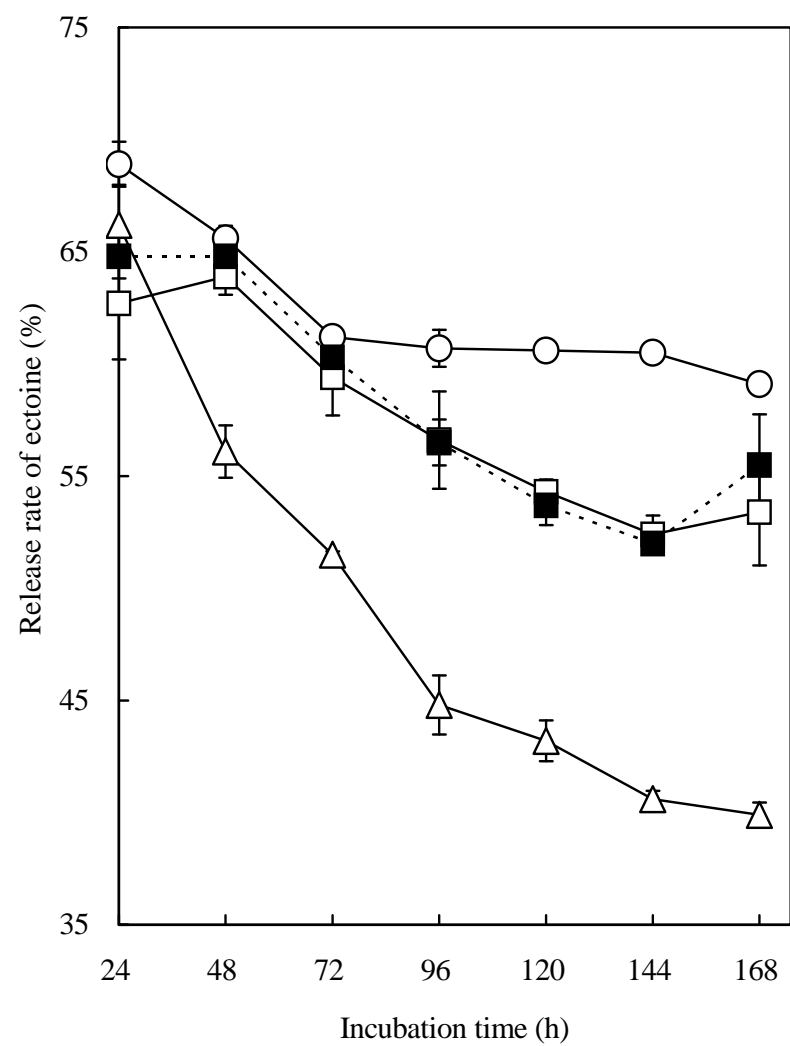
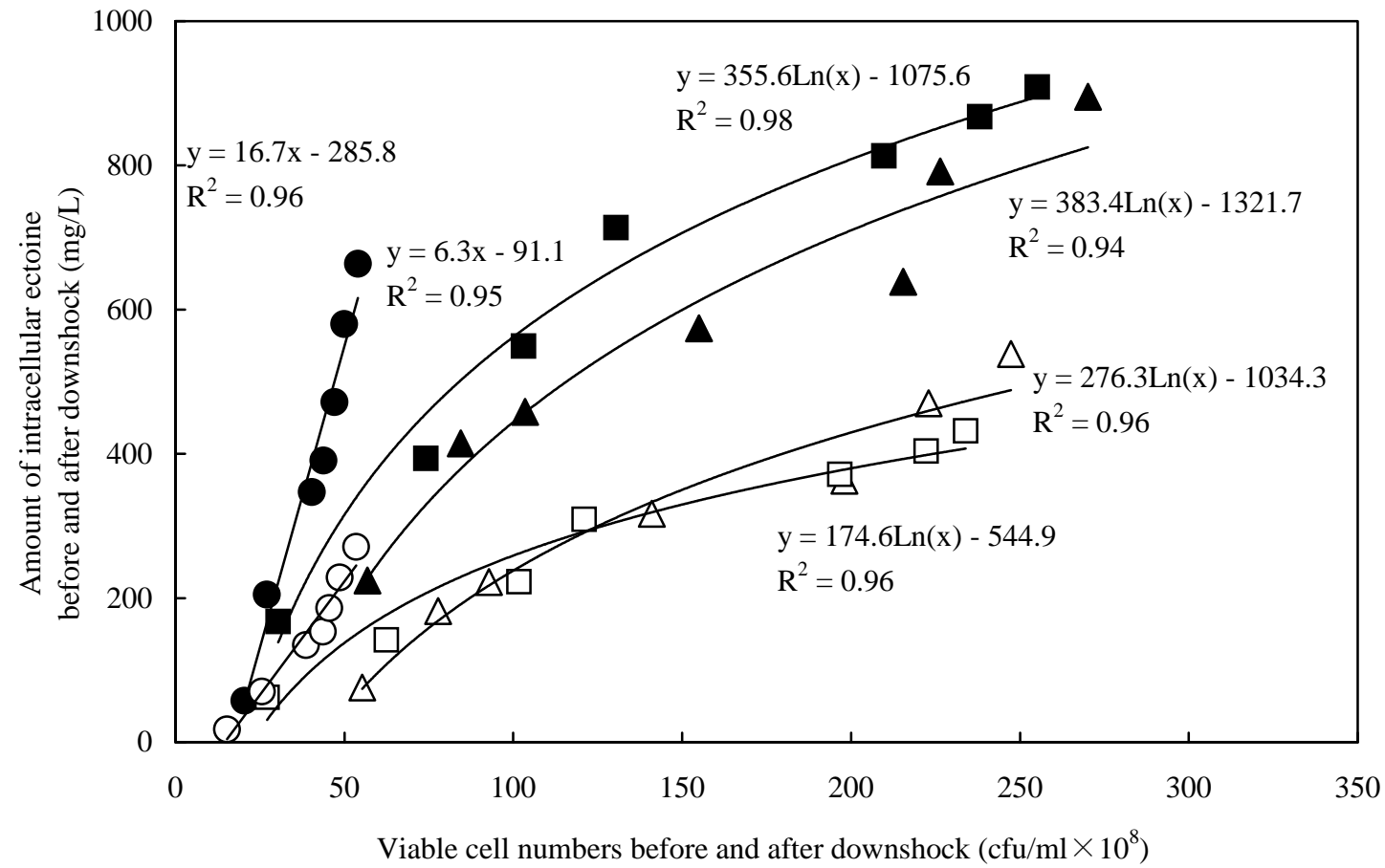


Fig. 3



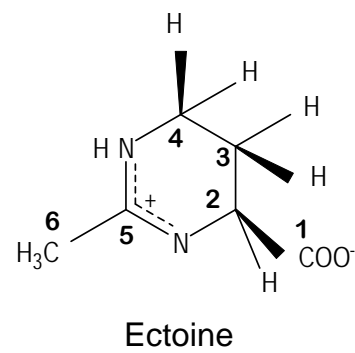
[illegible]

Fig. 4

