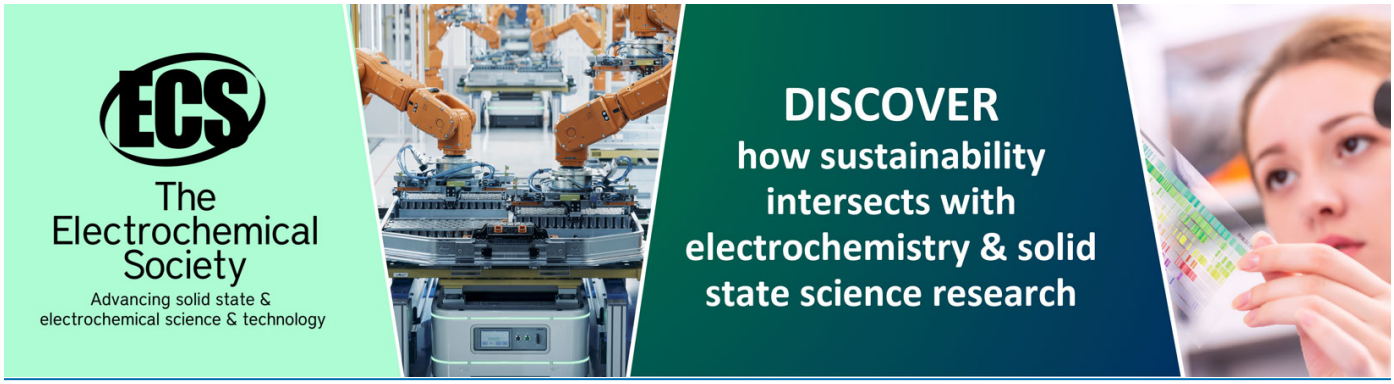
Ectoine production from putrefactive non-volatile amines in the moderate halophile *Halomonas elongata*



Ectoine production from putrefactive non-volatile amines in the moderate halophile *Halomonas elongata*

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Abstract. The moderate halophile *Halomonas elongata* can assimilate a variety of sugars and/or amino acids derived from bio-wastes as carbon and/or nitrogen sources. Thus, *H. elongata* could be a practical cell factory for bioproduction of fine chemicals such as ectoine. In protein-rich bio-wastes, putrefactive non-volatile amines could be formed as biogenic amines by decarboxylation of amino acids during biological rotten processes and be a considerable toxicological risk. Therefore, we investigated whether *H. elongata* can assimilate major putrefactive non-volatile amines, Histamine and Tyramine, as carbon and/or nitrogen sources or not. Comparative analysis using closely related *H. elongata* strains, OUT30018 and DSM2581^T, revealed that *H. elongata* OUT30018 can assimilate both Histamine and Tyramine, and produce ectoine under high salinity, while *H. elongata* DSM2581^T cannot. Thus, *H. elongata* OUT30018 would be a one of the most promising cell factories to produce fine chemicals such as ectoine from the putrefactive non-volatile amines in protein-rich bio-waste.

1. Introduction

In order to adapt high salinity environments, halophilic bacteria have evolved effective biosynthetic systems for bioproduction of osmolytes so-called compatible solutes [1]. One of the most well-known compatible solutes, ectoine (1,4,5,6-tetrahydro-2-methyl-4-primidinecarboxylic-acid) was originally identified as a major osmolyte of the extremely halophilic phototrophic bacterium *Ectothiorhodospira halochloris* [2]. Currently, ectoine is used in cosmetic moisturizers and other skin care products, as a chiral building block, and has also been shown to potentially protect healthy cells during chemotherapy [3, 4].

Among ectoine-producing bacteria, *Halomonas elongata* is ubiquitously isolated from various high-salinity origins including solar saltern [5], brine for meat fermentation [6] as well as salty soil [7], and recognized as one of useful fermentation microorganisms [8] and practically used as an ectoine producer strain in industry [4, 9, 10]. In *H. elongata*, ectoine is biosynthesized from carbon/nitrogen sources via L-4-aspartate-β-semialdehyde, which is a key intermediate for biosynthesis of aspartate-family amino acids such as lysine and threonine (Figure 1). L-4-aspartate-β-semialdehyde is further catalyzed to

ectoine by three enzymes (EctA, L-2,4-diaminobutyric acid Nγ-acetyltransferase; EctB, L-2,4-diaminobutyric acid transaminase; and EctC, ectoine synthase), that are encoded by *ectABC* gene cluster in *H. elongata* genome [11-13] (Figure 1). While ectoine is degraded and assimilated by four enzymes (DoeA, ectoine hydrolase; DoeB, Nα-acetyl-L-2,4-diaminobutyric acid deacetylase; DoeC, aspartate-semialdehyde dehydrogenase; and DoeD, L-2,4-diaminobutyric acid transaminase), encoded by *doeABCD* gene cluster in *H. elongata* genome [9] (Figure 1). The moderate halophile *H. elongata* can assimilate a variety of sugars and/or amino acids derived from bio-mass wastes including rice straw hydrolysates [14]. Thus, *H. elongata* could be a practical cell factory for bioproduction of fine chemicals such as ectoine using eco-friendly and cheap carbon and/or nitrogen sources derived from bio-wastes.

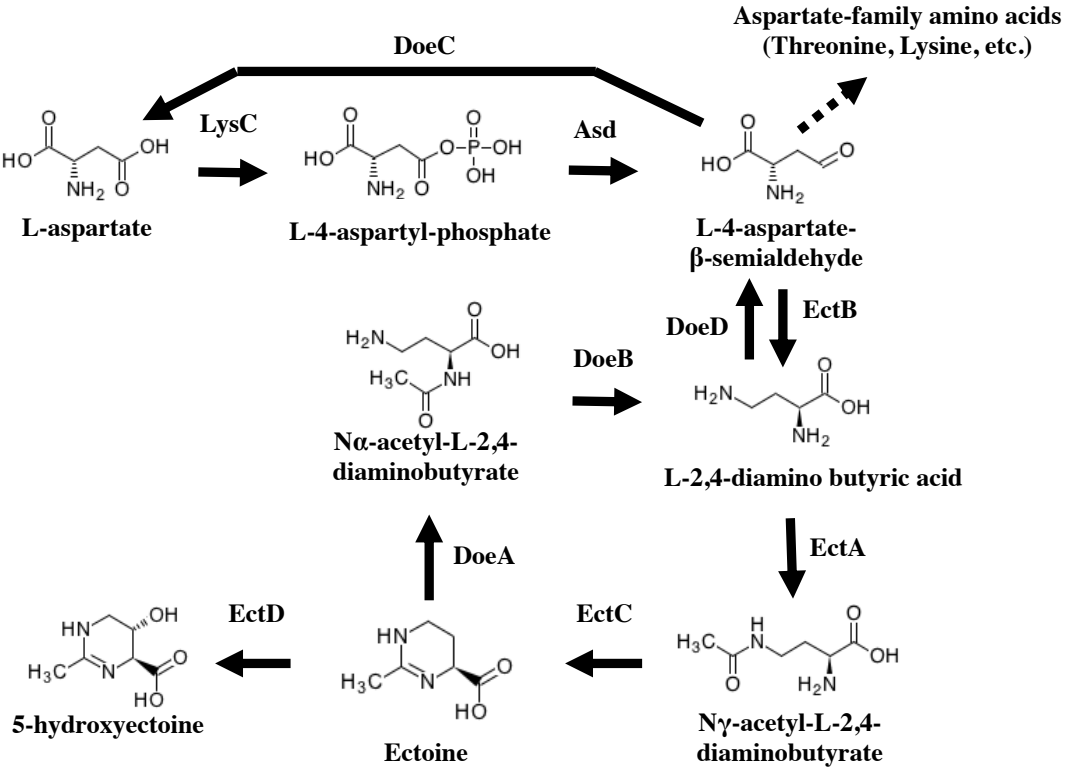

Figure 1. Metabolic pathway of ectoine from L-aspartate in *H. elongata*. LysC: aspartate kinase; Asd: β-aspartate-semialdehyde-dehydrogenase; EctB: L-2,4-diaminobutyric acid transaminase; EctA: L-2,4-diaminobutyric acid Nγ-acetyltransferase; EctC: ectoine synthase; EctD: ectoine hydroxylase; DoeA: ectoine hydrolase; DoeB: Nα-acetyl-L-2,4-diaminobutyric acid deacetylase; DoeD: L-2,4-diaminobutyric acid transaminase; DoeC: aspartate-semialdehyde dehydrogenase.

Biogenic amines (BAs) are organic bases, which can be formed during fermentation and/or spoilage process of foods (as well as protein-rich bio-wastes) by amino acid-decarboxylating microorganisms [15]. As BAs may cause intoxication symptoms in human, the presence of biogenic amines in fermented and non-fermented foods has become one of the most important food quality and safety issues [16]. The two most important BAs in food quality and safety are putrefactive non-volatile histamine derived from histidine and tyramine derived from the aromatic amino acid tyrosine (Figure 2). Recently, histamine- and/or tyrosine-degrading fermentation microorganisms have been studied for suppressing BAs formation during fermentation processes [17].

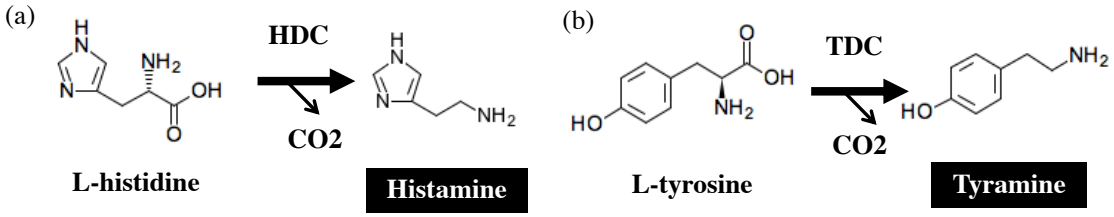

Figure 2. Biological formation of putrefactive non-volatile amines. L-histidine is decarboxylated by histidine decarboxylase (HDC) to histamine (a). L-tyrosine is decarboxylated by tyrosine decarboxylase (TDC) to tyramine (b).

In protein-rich bio-wastes, the putrefactive non-volatile amines, histamine and/or tyramine, could be formed as hazardous BAs during biological rotten processes. Therefore, in this study, we investigated whether *H. elongata* can assimilate histamine and tyramine as carbon and/or nitrogen sources for *de novo* biosynthesis of ectoine or not. Comparative analysis using closely related *H. elongata* strains, OUT30018 [7, 11] and DSM2581^T [5, 9], revealed that *H. elongata* OUT30018 can assimilate both histamine and tyramine, and produce ectoine under high salinity, while *H. elongata* DSM2581^T cannot. Thus, *H. elongata* OUT30018 would be a one of the most promising cell factories to produce fine chemicals such as ectoine from the putrefactive non-volatile amines in protein-rich bio-wastes.

2. Methods

2.1. Bacteria and growth conditions

The type strains *H. elongata* OUT30018 and DSM2581T were used in this study. Cells were grown in M63 medium [18], which consisted of 100 mM KH_2PO_4, 1 mM $MgSO_4$, 3.9 µM $FeSO_4$, 0.5 M or 1.5 M NaCl, 15 mM $(NH_4)_2SO_4$, and 0.4% Glucose. The modified C/N-free M63 medium is made by substitution the 15 mM $(NH_4)_2SO_4$ and 0.4% Glucose from M63 medium and added 15 mM K_2SO_4 and histamine or tyramine as sole carbon/nitrogen source. The pH was adjusted to 7.2 with KOH. For ectoine production, *H. elongata* was precultivated for 20 h at 37 °C in M63 medium containing 0.5 M NaCl, after which cells were collected by centrifugation at 3500 rpm for 15 min and the cells were inoculated into fresh M63 medium containing 1.5M NaCl. The cells were incubated at 37 °C and growth of the cells was monitored by optical density at 600 nm (OD600). A 2-mL aliquot was collected following centrifugation of the culture, and the resulting pellet was resuspended in distilled water (40 µL/mg fresh cell weight (FCW)) to extract ectoine from inside the cells. The extracted solutions were then examined by HPLC.

2.2. HPLC analysis of ectoine extract

The ectoine derivatizing solution was made by dissolving 2.5 mmol of 18-crown-6 and 50 mmol of 4-bromophenacyl bromide in acetonitrile. To 30 µl of ectoine extract sample was added 30 µl of 100 mM KH_2PO_4. After the solution was vortex-mixed, 540 µl of derivatizing solution was added. The tubes were vortex-mixed and heated to 80°C for 60 min [19]. The derivatized products were analyzed by HPLC as described below. The concentration of ectoine was determined using a Shimadzu HPLC system (Kyoto, Japan) equipped with a SUPELCOSILTM LC- SCX column (Sigma–Aldrich Corp. St. Louis, MO, USA). The analyses were carried out at 28 °C using 22 mM choline chloride dissolved in 90% acetonitrile as the mobile phase. The column was eluted at a flow rate of 1.5 mL/min, and the eluate was analyzed using an ultraviolet absorbance detector set at 254 nm.

3. Results and Discussion

3.1. Ability of H. elongata assimilate histamine or tyramine as sole carbon/nitrogen source

Growth of *H. elongata* strains OUT30118 and DSM2581^T in medium containing 15 mM histidine, histamine, tyrosine, or tyramine as the sole carbon/nitrogen source was evaluated. The initial OD600 was adjusted to 0.05. As shown in Figure 3, *H. elongata* OUT3018 can assimilate both histamine and tyramine as sole carbon/nitrogen source but DSM2581^T cannot. Further, analysis of the change in OD600 of *H. elongata* OUT30018 during cultivation at 37 °C is shown in Figure 4. These results revealed that *H. elongata* OUT30018 would be better strain than DSM2581^T for developing cell factory for bioproduction of fine chemicals such as ectoine from protein-rich bio-waste with the putrefactive non-volatile amines as feedstock.

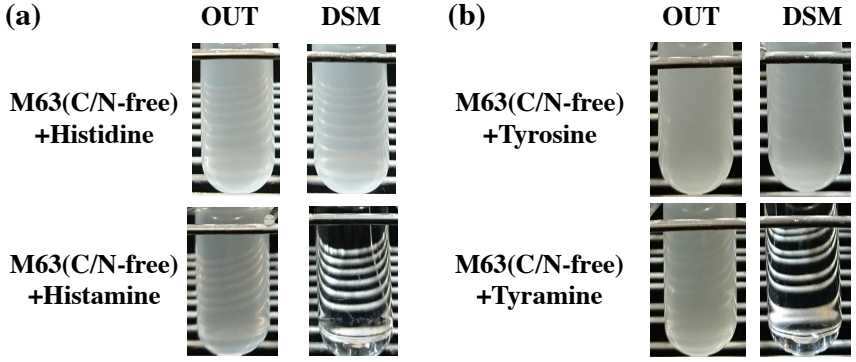

Figure 3. Growth test of *H. elongata* in the modified carbon/nitrogen-free M63 media containing putrefactive non-volatile amines. *H. elongata* strains OUT30018 (OUT) and DSM2581^T (DSM) were incubated for 24 h at 37°C using the liquid modified carbon/nitrogen-free M63 media, M63(C/N-free), containing 1.5 M NaCl and additional carbon and nitrogen sources. (a) Growth test of *H. elongata* strains supplemented with histidine or histamine as a sole carbon/nitrogen source. (b) Growth test of *H. elongata* strains supplemented with tyrosine or tyramine as a sole carbon/nitrogen source.

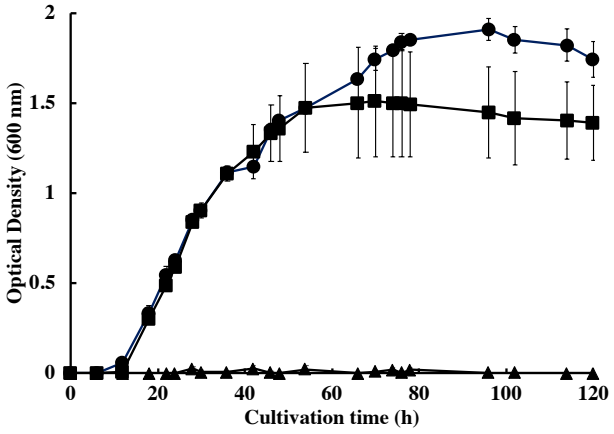

Figure 4. Growth of *H. elongata* OUT30018 in the carbon/nitrogen-free modified M63 minimal media containing putrefactive non-volatile amines as a sole carbon/nitrogen source. *H. elongata* OUT3018 was incubated at 37°C using the M63(C/N-free) medium containing 1.5 M NaCl and additional carbon/nitrogen source, histamine (closed squares) or tyramine (closed circles). As negative control, no carbon/nitrogen source was added in the media (closed triangles). Data are presented as the average of three independent experiments. Error bars represent the standard deviation.

3.2. H. elongata OUT30018 can produce ectoine from the putrefactive non-volatile amines

In order to confirm whether *H. elongata* OUT30118 can consume the putrefactive non-volatile amines and de novo biosynthesize ectoine in the cell, growth of *H. elongata* OUT30018 and change in amine concentrations of culture media with 1.5 M NaCl was investigated (Figure 5). Furthermore, after harvesting the cells, concentration of ectoine per fresh cell weight (FCW) was quantified under 0.5 M NaCl and 1.5 M NaCl conditions (Figure 6). These results suggested that *H. elongata* OUT30018 strain can effectively consume both histamine and tyramine as sole carbon/nitrogen source and utilize the putrefactive non-volatile amines as an energy source for growing and also as raw materials for bioproduction of amino-acids derivative fine-chemicals such as ectoine. These properties of *H. elongata* OUT30018 are suitable for developing superior cell factories producing fine chemicals including amino-acids derivatives from protein-rich bio-wastes.

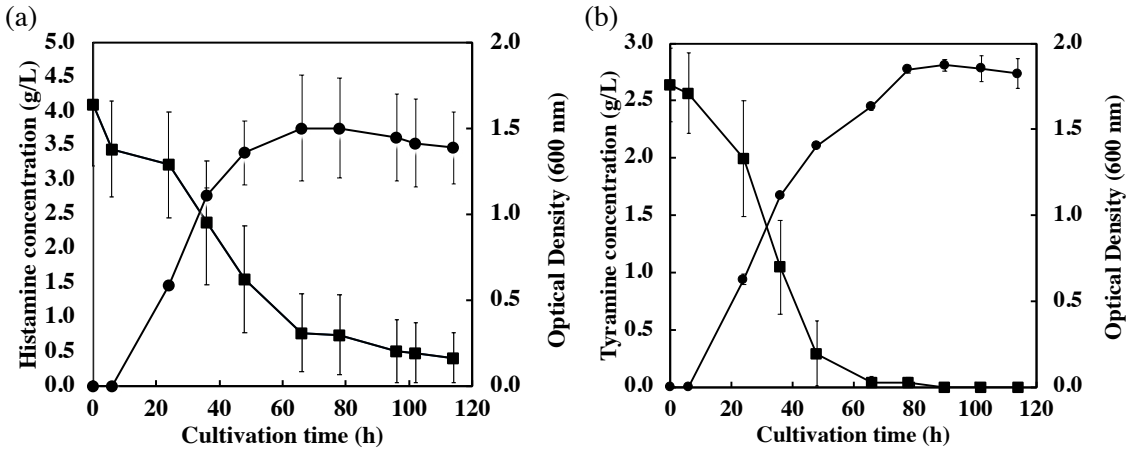

Figure 5. Growth of *H. elongata* OUT30018 and change in amine concentrations of culture media. *H. elongata* OUT30018 was incubated at 37℃ using the M63(C/N-free) media containing 1.5 M NaCl with then putrefactive non-volatile amines as a sole carbon/nitrogen source. (a) Growth of *H. elongata* OUT30018 supplemented with histamine was monitored using Optical Density at 600 nm (closed circles). Histamine concentration (closed square) was determined by HPLC analysis. (b) Growth of *H. elongata* OUT30018 supplemented with tyramine was monitored using Optical Density at 600 nm (closed circles). Concentration tyramine (closed square) was determined by HPLC analysis. Data are presented as the average of three independent experiments. Error bars represent the standard deviation.

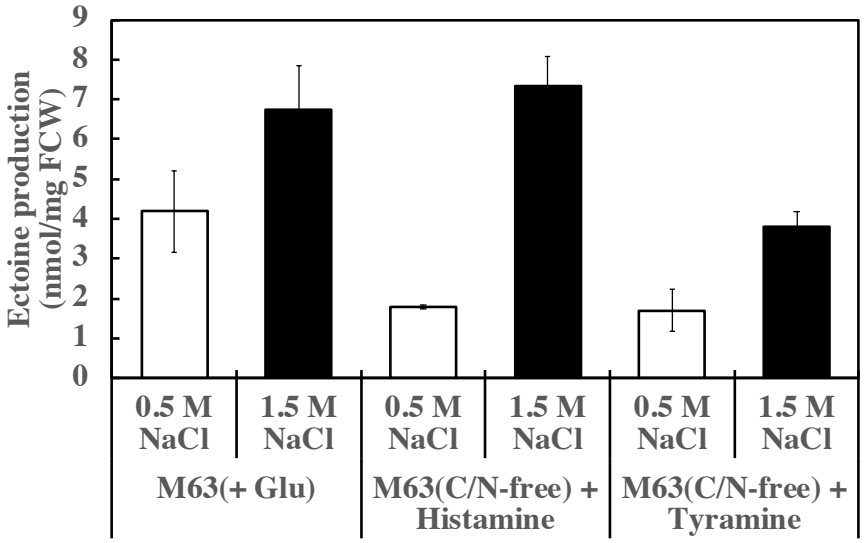

Figure 6. Ectoine production under high salinity in *H. elongata* OUT30018. *H. elongata* OUT30018 was incubated at 37°C using the M63 medium containing glucose as a sole carbon source (M63(+Glu)) and M63(C/N-free) medium with histamine or tyramine as a sole carbon/nitrogen source. Concentration of ectoine per fresh cell weight (FCW) was quantified under 0.5 M NaCl and 1.5 M NaCl conditions. Concentration ectoine was determined by HPLC analysis. Data are presented as the average of three independent experiments. Error bars represent the standard deviation.

4. Conclusions

The *H. elongata* OUT30018 can assimilate histamine and tyramine as sole carbon/nitrogen source and produce ectoine under high salinity conditions. Thus, *H. elongata* OUT30018 could be a practical cell factory for bioproduction of fine chemicals such as ectoine from protein-rich bio-waste.

5. Acknowledgments

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