Bioproduction of Valued Chemicals from Waste Mangosteen Peel Using the *Halomonas* Cell Factory

Thanawadee Sungnun[1], Jakkapon Phanthuwongpakdee[1], Hanna Mitsunaga[2], Pulla Kaothien-Nakayama[2], and Hideki Nakayama[2*]**

[1]Faculty of Environment and Resource Studies, Mahidol University, Nakhon Pathom 73170, Thailand
[2]Graduate School of Integrated Science and Technology, Nagasaki University, Nagasaki City, Nagasaki 852-8521, Japan
[*]Corresponding Author E-mail: nakayamah@nagasaki-u.ac.jp
[**]Co-corresponding Author E-mail: jakkapon.pha@mahidol.ac.th

ABSTRACT

Mangosteen is one of Thailand's economically significant agricultural products. However, its processing and consumption generate substantial agricultural waste, with the peel accounting for approximately 50% of the fruit's total weight. Mangosteen peel (MP) is a valuable source of bioactive compounds, including tannins, xanthones, and anthocyanins. Following the extraction of these high-value compounds, the remaining solid residue still contains complex carbohydrate structures that may be further utilized. This study aimed to hydrolyze macromolecules in MP and use the resulting product as a medium for cultivating cell factories to produce valuable chemicals. Raw MP and MP subjected to bioactive compound extraction were both used for comparative analysis. The cell factories selected for this study were *Halomonas elongata* OUT30018 and *H. elongata* Δ*doeA*Δ*ectD*, halophilic bacterial strains that produce the high-value chemical ectoine (Ect). First, high-salinity media derived from MP were prepared through acid hydrolysis. After neutralization and salt concentration adjustment, the media were used to culture the *H.* elongata strains. The results showed that both MP-derived media—prepared from raw MP and from MP following ethanol extraction—supported the growth of both *H. elongata* strains and the accumulation of Ect in their cells. Future studies will use MP-derived media to cultivate other *Halomonas* cell factories for the bioproduction of high-value chemicals, including γ-aminobutyric acid (GABA). These findings may help mitigate environmental issues associated with agricultural waste, in keeping with the concept of circular economy, while maximizing the value of MP by upcycling MP-derived carbon into valuable chemicals.

Keywords: Waste valorization/ Mangosteen peel/ Circular economy/ Hydrolysis/ Bioactive compounds/ *Halomonas elongata*/ Ectoine

1. INTRODUCTION

Thailand's agricultural sector generates millions of tons of crop residues annually, posing significant environmental management challenges nationwide. According to national agricultural statistics for 2024, approximately 65,818 ha of land in Thailand was devoted to mangosteen cultivation. In 2024, this area produced 301,649 tons of mangosteen, with production concentrated in eastern and southern agricultural hubs, including Chanthaburi, Trat, Chumphon, and Nakhon Si Thammarat [6]. Consumer demand is limited to the edible flesh, which constitutes only about 38% of the fruit's total weight; the remainder is discarded [2]. The thick purple pericarp accounts for approximately 50% of the total fruit weight, while the calyx comprises a further 12% [2]. Consequently, hundreds of thousands of tons of mangosteen peel (MP) are deposited in open landfills or burned in fields during each harvest season, contributing to air pollution, greenhouse gas emissions, and substantial waste-management burdens.

Despite being treated as agricultural waste, MP represents an exceptionally rich source of high-value bioactive compounds, including tannins, xanthones, and anthocyanins (ACNs) [6]. ACNs are water-soluble flavonoid pigments that give MP its distinctive deep purple-red color [6]. Quantitative studies indicate that dried MP contains total ACN concentrations exceeding 25 mg per 100 g of dried sample, substantially higher than those reported for other recognized agricultural waste streams, such as red onion peel and red dragon fruit peel [6]. ACNs extracted from MP have considerable commercial

potential across multiple sectors. In the food and beverage industry, they can be used as natural colorants to replace synthetic dyes and support the development of functional foods. In pharmaceuticals and dietary supplements, they offer valuable antioxidant, anti-inflammatory, anti-obesity, anti-neuroinflammatory, and antimicrobial benefits, while also modulating the gut microbiota. In cosmetics, they serve as premium active ingredients due to their potent antioxidant properties [4].

Moderately halophilic bacteria, particularly *Halomonas elongata*, can synthesize high-value compatible osmolytes, such as ectoine (Ect) and 5-hydroxyectoine (HydEct), under hyperosmotic stress induced by high salinity (Figure 1). *H. elongata* OUT30018 is a robust wild-type strain known for its high salinity tolerance and efficient metabolic machinery, which assimilates various types of biomass-derived carbon and nitrogen sources and biosynthesize the L-aspartate derivatives Ect and HydEct [1], [4], [9], [13]. To improve Ect yield, *H. elongata* OUT30018 was genetically modified to generate *H. elongata* Δ*doeA*Δ*ectD*, a double-deletion mutant lacking the *doeA* gene, which encodes Ect hydrolase and whose deletion blocks the primary pathway for Ect catabolism, and the *ectD* gene, which encodes Ect hydroxylase and whose deletion prevents the enzymatic conversion of Ect into HydEct [3], [5], [12].

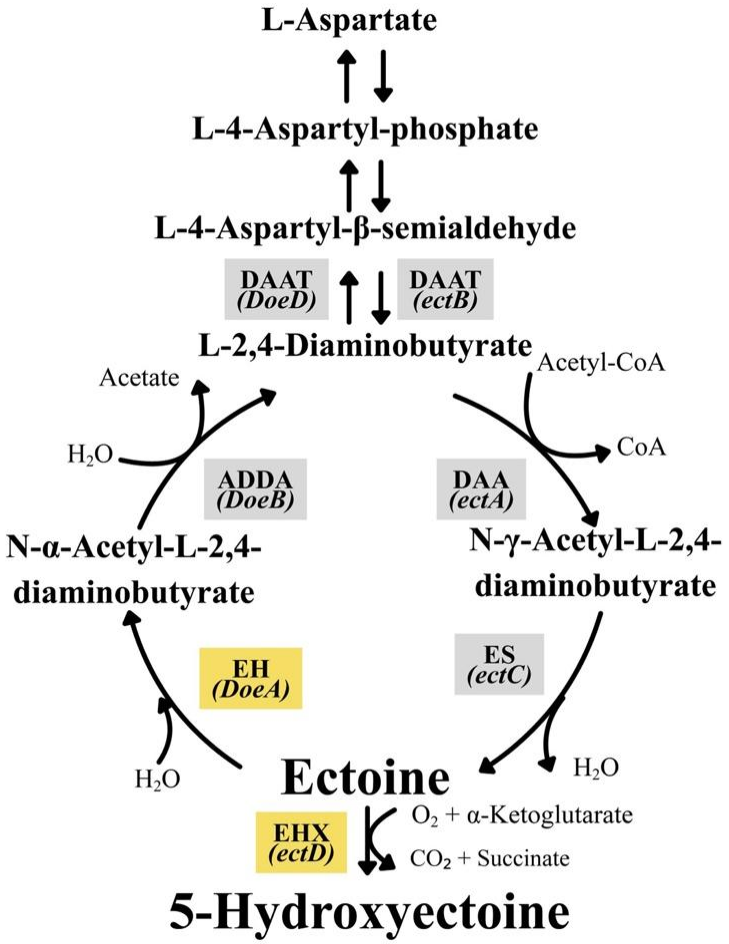


Figure 1. Biosynthetic and degradation pathways of ectoine (Ect) and 5-hydroxyectoine (HydEct) in *H. elongata* OUT30018. The strain synthesizes Ect in response to high-salinity stress through the activities of the three enzymes in the ectoine biosynthetic pathway: L-2,4-diaminobutyrate 4-aminotransferase (DAAT), L-2,4-diaminobutyrate acetyltransferase (DAA), and ectoine synthase (ES). Ect is further hydroxylated to HydEct by ectoine hydroxylase (EHX). Ectoine can also be recycled to L-aspartate through the Ect catabolic pathway. DAAT is encoded by the *ectB* or *doeD* genes; DAA is encoded by the *ectA* gene; ES is encoded by the *ectC* gene; EHX is encoded by the *ectD* gene; ectoine hydrolase (EH) is encoded by the **doeA** gene; and N-α-acetyl-L-2,4-diaminobutanoate deacetylase (ADDA) is encoded by the *doeB* gene.

To date, no studies have investigated the use of MP hydrolysis products as a medium for cultivating beneficial bacteria. This study evaluates the potential of MP hydrolysate as a low-cost culture medium for cultivating *H. elongata* strains to produce the valuable chemical Ect. MP was first hydrolyzed using sulfuric acid to depolymerize its polysaccharides into monosaccharides, which served as a carbon source. The resulting acidic MP hydrolysates were then neutralized with an ammonium solution, which also supplied nitrogen to the MP hydrolysate medium. Finally, the MP hydrolysate medium was used to cultivate *H. elongata* cell factories for Ect production. By applying green technology to valorize waste, this research advances sustainable production and directly supports the Circular Economy (CE) framework by converting discarded biomass into high-value biochemicals, such as Ect, while mitigating environmental pollution associated with biomass waste, including MP.

2. METHODOLOGY

2.1 Chemicals

Ethanol (99.5%, analytical grade; Nacalai Tesque, Inc., Kyoto, Japan) was diluted with distilled water to prepare a 50% (v/v) ethanol solution. Sulfuric acid (H_2SO_4, 1 mol/L), Ammonia solution (NH_3), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), ferrous sulfate ($FeSO_4$), potassium hydroxide solution (KOH), and sarcosine were obtained from Nacalai Tesque, Inc., Kyoto, Japan. Potassium dihydrogen phosphate (KH_2PO_4) and sodium chloride (NaCl) were obtained from Fujifilm Wako Pure Chemical Corporation, Japan. A derivatizing solution containing 18-crown-6 (Nacalai Tesque, Inc., Kyoto, Japan) and 4-bromophenacyl bromide (purity ≥98.0%, CAS RN: 99-73-0; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was prepared [7]. A 2×-concentrated, C- and N-free M63 salt stock containing 3 M NaCl was also prepared, and its pH was adjusted to 7.2 using KOH [10].

2.2 Preparation of freeze-dried mangosteen peel (MP) powder

Fresh mangosteens imported from Thailand were purchased from a fruit retailer in Japan. The fruits were washed with tap water, and their peels were collected. The mangosteen peels (MP) were frozen at -80°C overnight, then freeze-dried for 3 days using a freeze dryer (FDL-1000, EYELA, Tokyo, Japan) and ground into a fine powder using a millser (IFM-CLS-50, Iwatani Corporation, Tokyo, Japan).

2.3 Preparation of Anthocyanine-extracted mangosteen peel (MP-ACN) powder

MP powder was weighed into a 50 mL conical tube and mixed with 50% (v/v) ethanol at a solid-to-liquid ratio of 1:8.5 (w/v). The mixture was sonicated for 60 min in an ultrasonic cleaner (IWAKI Glass Co., Ltd., Tokyo, Japan) [11]. To separate the anthocyanin (ACN) extract from the solid residue, the mixture was centrifuged at 4,000 rpm for 20 min using an S700TR centrifuge (KUBOTA Corporation, Tokyo, Japan), and the supernatant was transferred to a new conical tube. The remaining pellet was washed with 20 mL of Milli-Q water, centrifuged at 10,000 rpm for 5 min using a centrifuge (Model 3740; KUBOTA Corporation, Tokyo, Japan), and dried to obtain ACN-extracted MP (MP-ACN) powder.

2.4 Acid hydrolysis of mangosteen peel and preparation of M63-derived mangosteen peel media

Freeze-dried MP and MP-ACN powders were separately added to a 1 M H_2SO_4 solution at a 10% (w/v) ratio in a 1 L plastic beaker (TPX® Graduated Beaker with Handle, SANPLAEC Co., Osaka, Japan). The mixtures were stirred with a plastic spatula in a 90 °C water bath for 30 min and then autoclaved at 121 °C for 60 min. After cooling to room temperature, each mixture was neutralized with aqueous NH_3, and a neutral pH was confirmed using litmus paper. The solution was filtered through Qualitative Filter Paper (No. 2, 400 mm diameter, ADVANTEC, Tokyo, Japan). NaCl was added to the filtrate to a final concentration of 1 M. The ammonia-neutralized sulfuric acid hydrolysates of MP or MP-ACN were then combined at a 1:1 (v/v) ratio with a 2×-concentrated, C- and N-free M63 salt stock

containing 3 M NaCl. The hydrolysates served as the sole carbon (C) and nitrogen (N) sources, resulting in a final NaCl concentration of 2 M in the MP-based media (MP+M63 and MP-ACN+M63 media).

2.5 Bacterial strain and growth conditions

The wild-type (WT) strain *Halomonas elongata* OUT30018 [9] and its derivative mutant (MT), *H. elongata* Δ*doe*AΔ*ectD*, were used in this study. *H. elongata* strains were precultured in M63 medium containing 3% NaCl and 4% glucose until the OD_{600} reached 1.0. The preculture was then used as a 10% inoculum for the main test cultures. All cultures were incubated in an air incubator at 35 °C with agitation at 150 rpm.

2.6 Cultures and harvest of H. elongata cells

Pre-cultures of the *H. elongata* OUT30018 and Δ*doe*AΔ*ectD* strains were grown in M63 salt medium supplemented with 4% glucose and 1 M NaCl in a shaking incubator at 150 rpm and 35°C for 24 h. Fifty mL of each experimental culture medium (C, N-free M63 medium, MP+M63 medium, and MP-ACN+M63 medium) were prepared in 300 mL baffled flasks. Each flask was inoculated with a 10% (v/v) inoculum of pre-cultured *H. elongata* OUT30018 or Δ*doe*AΔ*ectD* strains. The cultures were incubated for 24 h at 35°C with agitation at 150 rpm. Cell growth was monitored by measuring optical density at 600 nm (OD_{600}) every 4 h, and cell samples were harvested at 12, 16, and 24 h. The cultures were then centrifuged (4,000 × *g*, 20 min, 24°C), and the supernatants were discarded. The resulting cell pellets were subjected to hypo-osmotic treatment with Milli-Q water to induce the release of intracellular osmolytes into the aqueous phase.

2.7 Derivatization for HPLC analysis

A 20 µL aliquot of the sample was transferred to a microtube, followed by the addition of 25 µL of 100 mM KH_2PO_4, 5 µL of 2.5 mM sarcosine, and 450 µL of derivatization solution. The mixture was incubated at 80 °C for 60 min [7] to ensure completion of the derivatization reaction. Following incubation, the mixture was centrifuged, and a 400 µL aliquot of the resulting supernatant was filtered through a polytetrafluoroethylene membrane filter vial with 0.2 µm pore size (SEPARA syringeless filter, GVS Japan K.K., Tokyo, Japan) prior to high-performance liquid chromatography (HPLC) analysis (Shimadzu, Kyoto, Japan).

3. RESULTS AND DISCUSSION

3.1 Ammonia-neutralized sulfuric acid hydrolysates of mangosteen peels can serve as carbon and nitrogen sources in culture media for H. elongata.

As shown in Figure 2, growth tests of wild-type *H. elongata* OUT30018 (WT) under varying nutritional conditions demonstrate a direct relationship among substrate availability, cell proliferation, and osmolyte biosynthesis. To determine whether MP or MP-ACN can serve as a feedstock for a *Halomonas* cell factory, three media were prepared for culturing wild-type H. elongata OUT30018, which produces Ect in high-salinity media containing diverse carbon and nitrogen sources [8], [13]: carbon- and nitrogen-free M63 medium; MP+M63 medium, containing ammonia-neutralized sulfuric acid hydrolysate of MP; and MP-ACN+M63 medium, containing ammonia-neutralized sulfuric acid hydrolysate of ACN-extracted MP. *H. elongata* OUT30018 grew well in MP+M63 and MP-ACN+M63 media, reaching maximum OD_{600} values of approximately 5.0 and 4.2, respectively, after 24 hours (Figure 2A). By contrast, growth in carbon- and nitrogen-free M63 medium was severely restricted because the cells became carbon- and nitrogen-starved after consuming nutrients carried over from the preculture medium in the 10% inoculum. Growth ceased after 4 hours, and OD_{600} plateaued at approximately 0.8–0.9 (Figure 2A). HPLC analysis showed that Ect and HydEct were biosynthesized and accumulated as the major osmolytes in *H. elongata* OUT30018 cells cultured for 16 hours (Figure 2B). These results confirm that both MP and ACN-extracted MP can serve as feedstocks to support cell

growth and the biosynthesis of Ect and HydEct under high-salinity conditions. Notably, a small amount of glycine betaine (GB) was detected as an additional osmolyte in *H. elongata* OUT30018 cultured in MP+M63 and MP-ACN+M63 media, but not in carbon- and nitrogen-free M63 medium. This finding suggests that MP contains a small amount of GB, which cells can import and accumulate as an osmolyte.

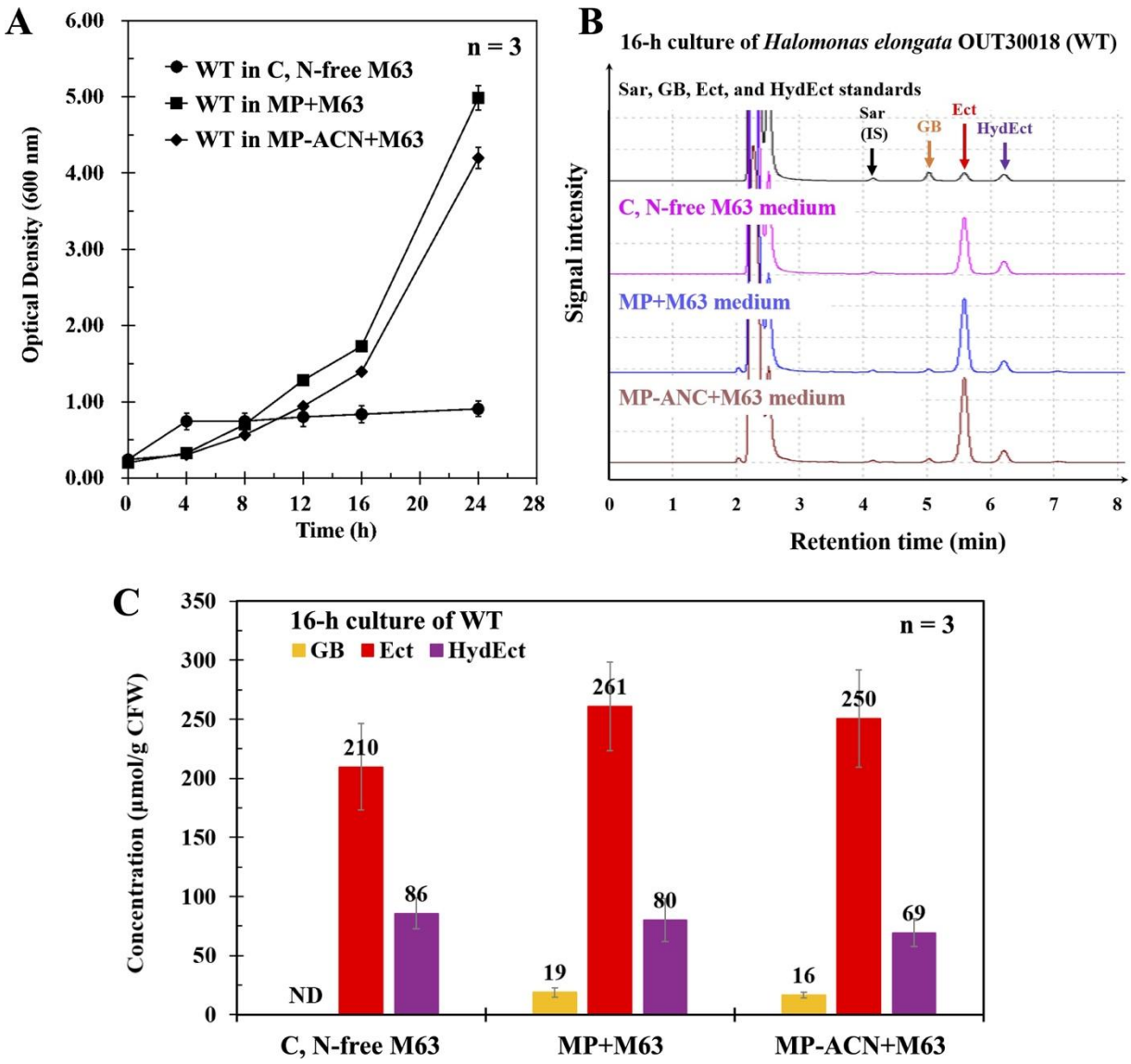


Figure 2. Growth and osmolyte profiles of wild-type *H. elongata* OUT30018 (WT) cultured in different media containing 2 M NaCl. **(A)** Growth curves of WT cells, measured by optical density at 600 nm (OD_{600}) every 4 hours over 24 hours in carbon (C)- and nitrogen (N)-free M63 medium (closed circle), MP+M63 medium (closed square), or MP-ACN+M63 medium (closed diamond) (n = 3). **(B)** HPLC chromatograms of the major osmolytes released from WT cells collected from 16-hour cultures, compared with a standard reference. GB, glycine betaine; Ect, ectoine; HydEct, 5-hydroxyectoine. Sarcosine (Sar) was used as an internal standard (IS). **(C)** Profiles of the major osmolytes in 16-hour cultures of WT cells grown in different culture media (n = 3).

3.2 Comparison of growth and osmolyte profiles between H. elongata OUT30018 and H. elongata ΔdoeAΔectD strains cultured in MP-CAN+M63 medium containing 2 M NaCl

As shown in Figure 3, genome engineering of *H. elongata* OUT30018 to generate the double-deletion mutant *H. elongata* Δ*doe*AΔ*ectD* (MT) significantly altered its metabolic profile without compromising its growth kinetics. The underlying biochemical basis for this phenotypic shift is the

targeted inactivation of key enzymatic branch points in the Ect metabolic network. In wild-type cells, Ect hydroxylase (*ectD*), a 2-oxoglutarate-dependent dioxygenase, catalyzes the addition of a hydroxyl group to Ect, converting a portion of the intracellular pool into HydEct, particularly under environmental stress conditions [14]. Deletion of the *ectD* gene eliminates this conversion step, thereby preventing byproduct contamination. Concurrently, deletion of the *doeA* gene inactivates Ect hydrolase, disrupting the primary pathway for Ect degradation back into N-α-acetyl-L-2,4-diaminobutyrate [12]. By eliminating both degradation and secondary modification pathways, metabolic flux is directed exclusively toward and retained within the Ect biosynthetic sink. Because Ect alone provides sufficient zwitterionic osmoprotective capacity to maintain intracellular turgor, cell viability is not compromised. Consequently, the *H. elongata* Δ*doe*AΔ*ectD* strain (MT) functions as a highly efficient cell factory for producing high-purity Ect, substantially simplifying downstream purification for industrial applications.

When cultivated in MP-ACN+M63 medium, the WT and MT strains exhibited nearly identical growth curves, reaching OD_{600} values of approximately 4.2 and 3.8, respectively, after 24 hours. Notably, HydEct was undetectable in MT cells, whereas WT cells produced and accumulated HydEct (Figures 3B, 3C, and 3D). Accordingly, MT cells yielded more Ect than WT cells (Figures 3C and 3D). HPLC chromatograms of major osmolyte samples collected after 16 hours of cultivation provided definitive evidence, showing a single prominent Ect peak and no detectable HydEct peak in the MT cell sample (Figure 3B). Consequently, particularly during the exponential growth phase at 12 and 16 hours, MT cells exhibited higher cellular Ect content than WT cells, accumulating 366 and 289 µmol/g CFW, respectively, compared with 325 and 250 µmol/g CFW in WT cells (Figures 3C and 3D). Overall Ect yields (Figure 3E) and productivities (Figure 3F) were slightly higher in the MT culture than in the WT.

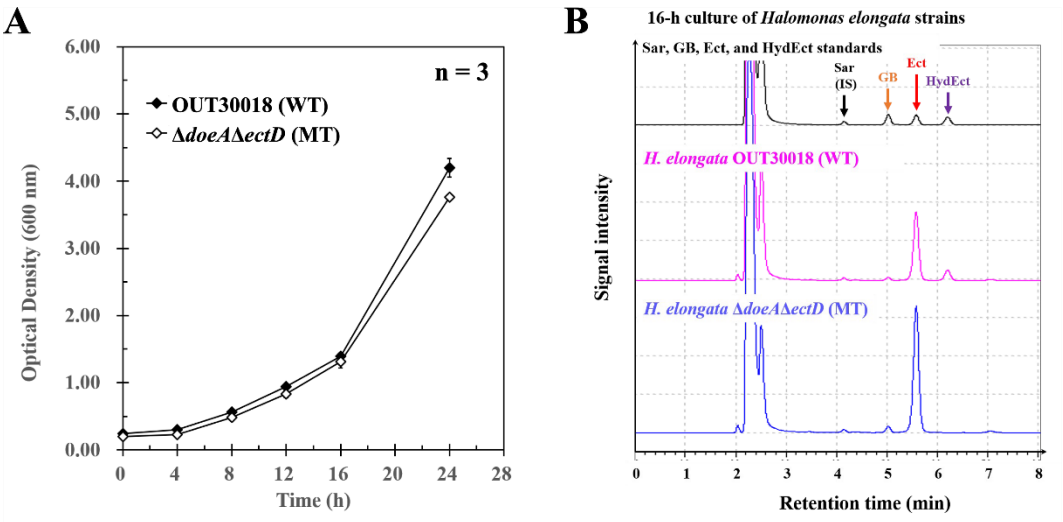
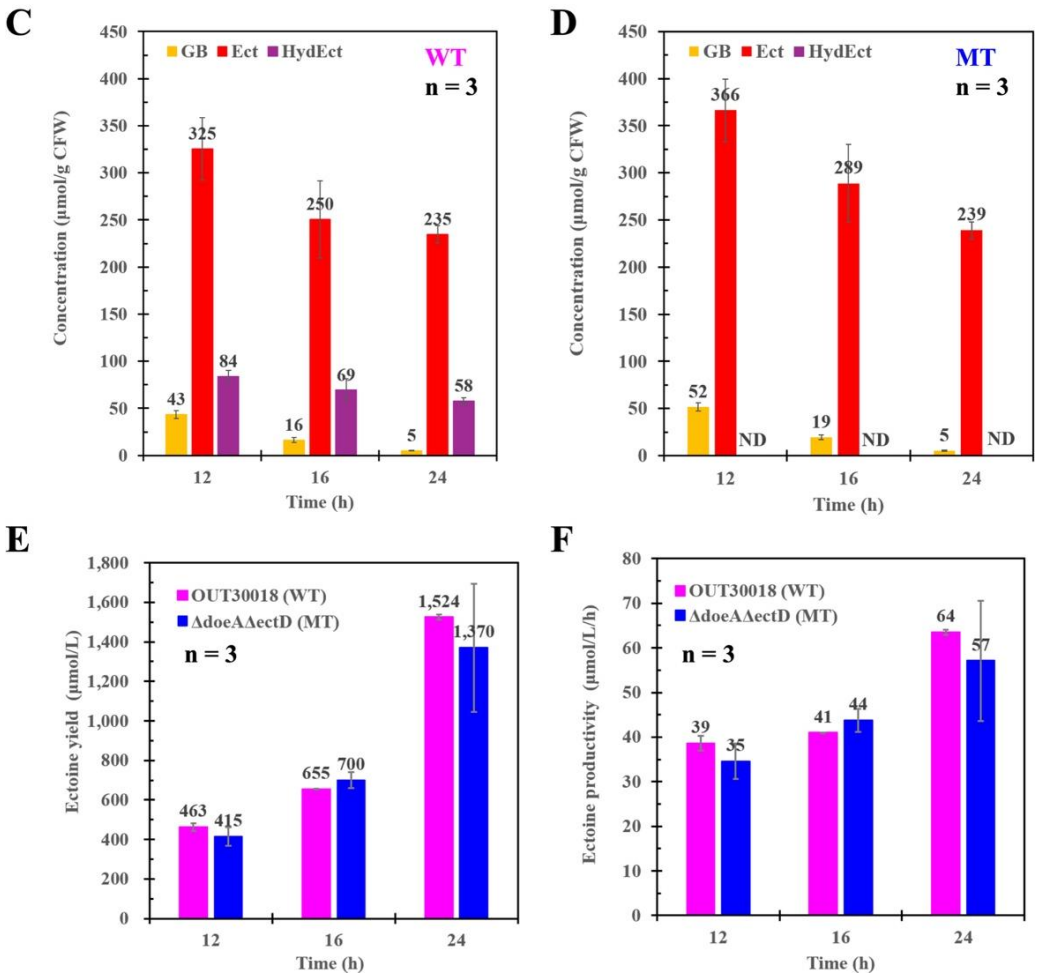


Figure 3. Comparison of growth, osmolyte profiles, and ectoine production between the wild-type *H. elongata* OUT30018 (WT) and the mutant *H. elongata* Δ*doeA*Δ*ectD* (MT). *H. elongata* strains were cultured under high-salinity conditions (2M NaCl) in MP-ACN+M63 medium **(A)** Growth curves of *H. elongata* strains. Optical density at 600 nm (OD_{600}) was measured every 4 hours over 24 hours in MP-ACN+M63 medium containing 2 M NaCl (n = 3). **(B)** HPLC chromatograms of major osmolyte released from WT and MT cells cultured for 16 hours and standard reference compounds (GB, glycine betaine; Ect, ectoine; HydEct, 5-hydroxyectoine). Sarcosine (Sar) was used as an internal standard (IS). **(C)** Major osmolyte profiles of WT cells cultured in MP-ACN+M63 medium for 12, 16, or 24 hours (n = 3). **(D)** Major osmolyte profiles of MT cells cultured in MP-ACN+M63 medium for 12, 16, or 24 hours (n = 3). **(E)** Ectoine yield (µmol/L) of WT and MT cells cultivated in MP-ACN+M63 medium containing 2 M NaCl (n = 3). **(F)** Ectoine productivity (µmol/L/h) of WT and MT cells cultivated in MP-ACN+M63 medium containing 2 M NaCl (n = 3).



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4. CONCLUSIONS

This study demonstrates the successful valorization of MP, an agricultural waste byproduct, into high-value chemicals, Ect [5],[13], using *H. elongata* cell factories. Instead of relying on costly synthetic media, ammonia-neutralized sulfuric acid hydrolysate derived from crude ACN-extracted MP serves as an abundant, cost-effective source of essential carbon and nitrogen.

Acknowledgement

This research was conducted with support from the JST Networked Exchange, United Strength for Stronger Partnerships between Japan and ASEAN (NEXUS), Young Talents Exchange and Capacity Development Program (Y-tec) FY2025, entitled “Young Researcher Exchange Program in the Field of Environmental Resource Circulation for Co-creating a Sustainable Future between Japan and ASEAN” (Grant No. Y2025L1015056).

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