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# Exploring useful fermentation strategies for the production of hydroxyectoine with a halophilic strain, *Halomonas salina* BCRC 17875

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**Hydroxyectoine, an ectoine derivative, is the most common compatible solute in halophilic microorganisms for resisting harsh environments. Compatible solutes can be utilized in fields such as cosmetics, medicine, and biochemistry. Moderately halophilic microorganisms produce much less hydroxyectoine as compared with ectoine. In this study, we first evaluate the effect of medium formulation (i.e., yeast extract (YE) medium and high yeast extract (HYE) medium) on hydroxyectoine production. In addition, an investigation of hydroxyectoine production by *Halomonas salina* under optimal conditions for vital factors (i.e., iron and α-ketoglutarate) and hydroxylase activity was also carried out. As a result, hydroxyectoine production was obviously elevated (0.9 g/L to 1.8 g/L) when the HYE medium was utilized. Furthermore, hydroxyectoine production further increased to 2.4 g/L when both the α-ketoglutarate and iron factors were added to the HYE medium in the early stationary phase. In addition, we found that ectoine hydroxylase activity increased more when a combination of iron and α-ketoglutarate was used than when either was used alone. The results showed that the alteration of iron and α-ketoglutarate clearly stimulated the expression of ectoine hydroxylase, which in turn affected hydroxyectoine synthesis. This study also showed that hydroxyectoine production was further raised from 2.4 g/L to 2.9 g/L when 50 mM of α-ketoglutarate and 1 mM of iron were added to the HYE medium. Ultimately, the experimental results showed using the optimal conditions further elevated the hydroxyectoine production yield to 2.90 g/L, which was over 3-fold higher than the best results obtained from the original medium.**



[**Key words:** Hydroxyectoine; *Halomonas salina*; Iron; α-Ketoglutarate; Divalent ions]

Sodium chloride-induced hypersaline environments are mainly inhabited by microorganisms (1). To survive in and resist the difficult surroundings, microorganisms activate an organic-osmolyte mechanism to synthesize and accumulate polar, highly water-soluble, low-molecular weight organic compatible solutes such as ectoine and sugars (2). To accumulate the compatible solutes in microorganisms, these compounds are synthesized *de novo* or directly taken up from the environment to promote survival in an osmotically challenging environment. These compatible solutes not only act as osmoprotectants but also help to stabilize enzymes, DNA, cytoplasmic membranes, and whole cells against different kinds of stress such as heating, drying, and freezing (3–6). Ectoine ((4*S*)-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid]) is a natural compatible solute that serves as a protectant in many bacterial cells (5). In addition to the stabilizing effects of ectoine in the biochemical, medical, cosmetic, or skin care fields, it has also been functionalized as a protectant for healthy cells during chemotherapy (5).

This seminal discovery was followed by the detection of a hydroxylated derivative of ectoine and hydroxyectoine [(4*S*,5*S*)-2-methyl-5-hydroxy-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid] in the gram-positive soil bacterium *Streptomyces parvulus* (7). The OH group of hydroxyectoine can partly replace the loss of water molecules from the hydrate shell and can stabilize the native structure of biopolymers to a higher degree than ectoine (8). Reports showed three enzymes are involved in ectoine synthesis: L-2,4-diaminobutyrate (DABA) transaminase, L-2,4-diaminobutyrate acetyltransferase, and ectoine synthase (9,10). 5-Hydroxyectoine is formed in a subgroup of ectoine producers in a process that is catalyzed by the ectoine hydroxylase. The ectoine hydroxylase is a member of the non-heme-containing iron (II) and α-ketoglutarate-dependent dioxygenase superfamily. Widderich et al. (11) produced a crystal structure to mimic the possible positioning of ectoine and α-ketoglutarate ligands at active sites. Their results indicated the positioning of the iron, ectoine, and α-ketoglutarate ligands in ectoine hydroxylase in close proximity to each other and in spatial orientation that allow the region-selective and stereo-specific hydroxylation of ectoine to hydroxyectoine (11).

*Halomonas salina* is a gram-negative, rod-shaped halophilic bacterium that lives in saline environments (7). *Halomonas* sp. can produce a mixture of both ectoine and hydroxyectoine (10). In

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addition, reports have shown that divalent ions and α-ketoglutarate may prompt the production of hydroxyectoine by microorganisms (8,9). Also, one study showed hydroxyectoine to have more biomedical applications than ectoine (10). Therefore, this study was an attempt to utilize *H. salina* to produce hydroxyectoine by optimizing growth conditions. Effects of growth conditions such as media salinity, carbon source, and nitrogen source on ectoine biosynthesis by *H. salina* microorganisms were reviewed (7,12). Therefore, the aim of this study was to evaluate and optimize the medium formulations, such as divalent ions and α-ketoglutarate.

## MATERIALS AND METHODS

**Bacterial strain** *H. salina* BCRC17875 was purchased from the Bioresource Collection and Research Centre, Hsinchu city, Taiwan. It can synthesize and accumulate intracellular ectoine and hydroxyectoine as compatible solutes in a hypersaline environment to resist osmotic stress.

**Culture medium and growth conditions** The precultivation medium for *H. salina* BCRC17875 consisted of yeast extract (5 g/L, Sigma-Aldrich, St. Louis, MO, USA), $(NH_4)_2SO_4$ (5 g/L), Na-glutamate (1 g/L, Sigma), $FeCl_2{\cdot}4H_2O$ (0.2 mM, Sigma), $MnSO_4{\cdot}H_2O$ (2 μM, Sigma), KCl (2 g/L, Sigma), $MgSO_4{\cdot}7H_2O$ (80 mM, Sigma), and sodium chloride (3 g/L, Sigma). The hydroxyectoine production medium (i.e., high yeast extract (HYE) medium) consisted of yeast extract (YE, 84 g/L, Sigma), $(NH_4)_2SO_4$ (28 g/L, Sigma), Na-glutamate (74.4 g/L, Sigma), $FeCl_2{\cdot}4H_2O$ (0.5 mM, Sigma), $MnSO_4{\cdot}H_2O$ (10 μM, Sigma), KCl (1.2 g/L, Sigma), $MgSO_4{\cdot}7H_2O$ (100 mM, Sigma), and sodium chloride (6.2 g/L, Sigma) (12). To evaluate how divalent ions and α-ketoglutarate affect hydroxyectoine production using *H. salina*, various concentrations and formulations of the ions and α-ketoglutarate were examined. *H. salina* was precultivated in flasks at 30 °C at 200 rpm and a pH 7.6. The main cultivation medium was inoculated with 10% of the cultivation broth for testing the various conditions. In addition, the production of hydroxyectoine was also carried out with the YE medium from our previous study (12) to evaluate how the medium affects the production of hydroxyectoine using *H. salina* BCRC17875, including yeast extract (56 g/L, Sigma), ammonium acetate (14 g/L, Sigma), $FeCl_2{\cdot}4H_2O$ (0.5 mM, Sigma), $MnSO_4{\cdot}H_2O$ (10 μM, Sigma), KCl (2 g/L, Sigma), $MgSO_4{\cdot}7H_2O$ (100 mM, Sigma), and sodium chloride (6.2 g/L, Sigma). All experiments were repeated three times to ensure reproducibility.

**Determination of cell concentration** Biomass was determined turbidometrically at 600 nm ($OD_{600}$), and values were converted to cell dry weight (CDW) using an appropriate calibration formula as follows: $CDW = 0.72 \times OD_{600} + 1.31$.

**Ectoine and hydroxyectoine assay** *H. salina* BCRC17875 was cultured and then harvested through centrifugation at 8000 ×*g*. The pellets were then resuspended in ethanol (Sigma) through rigorous shaking for 30 min. The ethanol extract was filtrated through a 0.45-μm filter to analyze ectoine and hydroxyectoine production. Twenty microliters of the extract were analyzed using high performance liquid chromatography (HPLC) (Hitachi, Tokyo, Japan) on an RP-18 column (Merck, Darmstadt, Germany) modified from that used by Malin et al. (13). Chromatography was carried out isocratically at a flow rate of 1 mL/min with acetonitrile/trifluoroacetate (4/1, v/v; pH = 2.5) (Sigma) as the mobile phase. Ectoine and hydroxyectoine were monitored at 210 nm by using a UV/Vis detector (Hitachi). Ectoine and hydroxyectoine purchased from Sigma were used as the standard.

**Ectoine hydroxylase assay** Ectoine hydroxylase activity in the extracts of *H. salina* BCRC17875 was assayed according to the method proposed by Zhu et al. (14). Briefly, 70-μL protein fractions were suspended in 100 μL of the reaction mixture, which contained 10 mM 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl) amino]ethanesulfonic acid (TES) buffer (pH 7.5, Sigma), 10 mM α-ketoglutarate (Sigma), 5 mM ectoine (Sigma), 1 mM FeSO4·7H2O (Sigma), and 1300 units of beef liver catalase (Roche, Mannheim, Germany). The reaction mixture was incubated at 42 °C for 20 min, and the reaction was stopped by adding 100 μL of 100% of acetonitrile. The supernatant was analyzed using HPLC. One unit of ectoine hydroxylase activity is defined as the conversion of 1 μmol of ectoine to 1 μmol of hydroxyectoine per minute.

## RESULTS AND DISCUSSION

**Effect of medium formulation on hydroxyectoine production using *H. salina*** Our previous study showed that the production of ectoine using *H. salina* could produce 13.96 g/L when the optimal YE medium was utilized (12). Meanwhile, the production of hydroxyectoine was also evaluated using the YE medium and HYE medium. The experimental results in Fig. 1 indicate that using the HYE medium under specific operating conditions further elevated the hydroxyectoine production yield to 1.86 g/L, which is more than double the best yield obtained using the YE medium (12). The biomass concentration in the HYE medium under specified operating conditions reached its highest level (around 244 mg/L) at 32 h, exceeding that obtained using the YE medium. The experimental results suggested that modifying the medium formulation not only affects the production of ectoine, but also the production of hydroxyectoine.

**Effects of divalent ions on ectoine and hydroxyectoine production using *H. salina*** Previous reports have shown that iron, molecular oxygen, and α-ketoglutarate can affect the direct synthesis of hydroxylate ectoine by using the protein EctD (ectoine hydroxylase) (10,14). Therefore, we attempted to determine how other divalent ions affect ectoine and hydroxyectoine production when using *H. salina*. Five divalent ions—$Zn^{2+}$, $Cu^{2+}$, $Co^{2+}$, $Ni^{2+}$, and $Ca^{2+}$—were added to the medium at concentrations ranging from 0 to 40 μM to observe their effect on hydroxyectoine production with *H. salina*. As shown in Fig. 2, hydroxyectoine production reached 1.7–1.8 g/L when $Cu^{2+}$, $Co^{2+}$, and $Ca^{2+}$ were added to the medium. However, this hydroxyectoine production could not surpass that obtained in the control group (~2.9 g/L). These results indicated that divalent ions might not be a major factor affecting ectoine and hydroxyectoine production by *H. salina*.

**Effect of the timing of iron and α-ketoglutarate addition on hydroxyectoine production using *H. salina*** Microorganisms

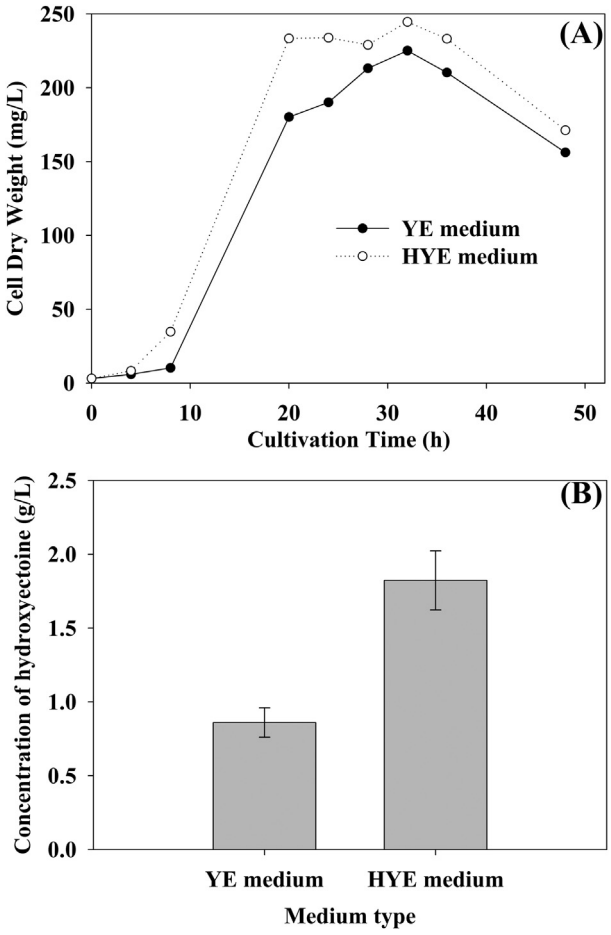


FIG. 1. Effect of (A) growth curves and (B) production of hydroxyectoine in different medium type.

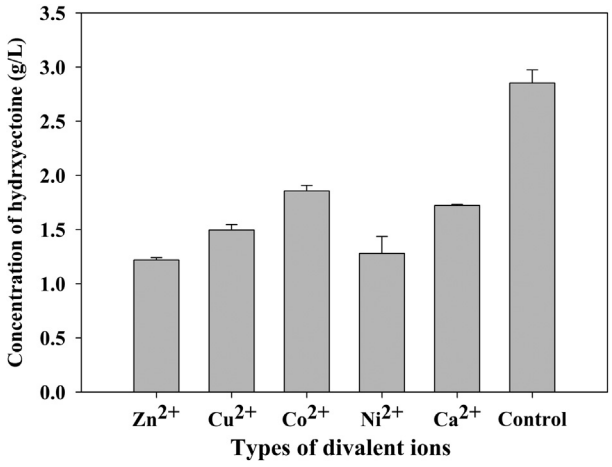


FIG. 2. Production of compatible solutes hydroxyectoine upon the addition of various divalent ions at different concentrations (0–40 μM): $Zn^{2+}$, $Cu^{2+}$, $Co^{2+}$, $Ni^{2+}$, and $Ca^{2+}$.

are capable of synthesizing ectoine and hydroxyectoine, and both of these compatible solutes are frequently found in microorganisms (15). In addition, the hydroxyectoine content in some microorganisms increases substantially in response to osmotic challenges when their cells enter the stationary phase (16,17). According to the hydroxyectoine synthesis pathway, iron and α-ketoglutarate play a key role in its production. Furthermore, in our previous study, we found that *H. salina* produces ectoine on the basis of a growth-associated model (12). Hydroxyectoine production using *H. salina* can be varied by adding iron and α-ketoglutarate at various time points. Thus, it is important to investigate the effect of the timing of the addition of iron and α-ketoglutarate on hydroxyectoine production using *H. salina*. The investigation was started by using a medium containing only iron (without α-ketoglutarate). Four time points, two in the exponential phase (12 h cultivation and 20 h cultivation) and two in the stationary phase (24 h cultivation and 28 h cultivation), were chosen to observe hydroxyectoine production using *H. salina* after the addition of 0.5 mM iron to the medium.

The addition of iron to the medium at the aforementioned time points strongly affected bacterial growth (Fig. 3A). Iron addition after 24 h of cultivation (early stationary phase) resulted in the highest bacterial growth (~265.4 mg/L, Fig. 3A) and the highest hydroxyectoine production (~1.95 g/L) when compared with the results obtained for the control group (~1.81 g/L) and other groups (Fig. 3B). Therefore, in subsequent experiments, iron addition to the medium was performed after 24 h of cultivation (early stationary phase). However, iron addition reduced bacterial growth and hydroxyectoine production in the early and the late exponential phases (Fig. 3B). In the late stationary phase, there were no significant variations in either the bacterial growth or the hydroxyectoine production. This observation might be attributed to the microorganisms' response to variations in the medium formulation in the exponential phase leading to their readjusting and readapting to the cultivation environment in terms of bacterial growth and hydroxyectoine production.

Next, the effect of α-ketoglutarate addition on hydroxyectoine production was investigated. It was added to the medium in the exponential (between 12 and 20 h of cultivation) and stationary phases (between 24 and 28 h of cultivation). As depicted in Fig. 4A, α-ketoglutarate addition in either of the phases resulted in more bacterial growth compared with the control group (iron addition in the early stationary phase). Moreover, early addition of α-ketoglutarate resulted in more bacterial growth (~277 mg/L, Fig. 4A)

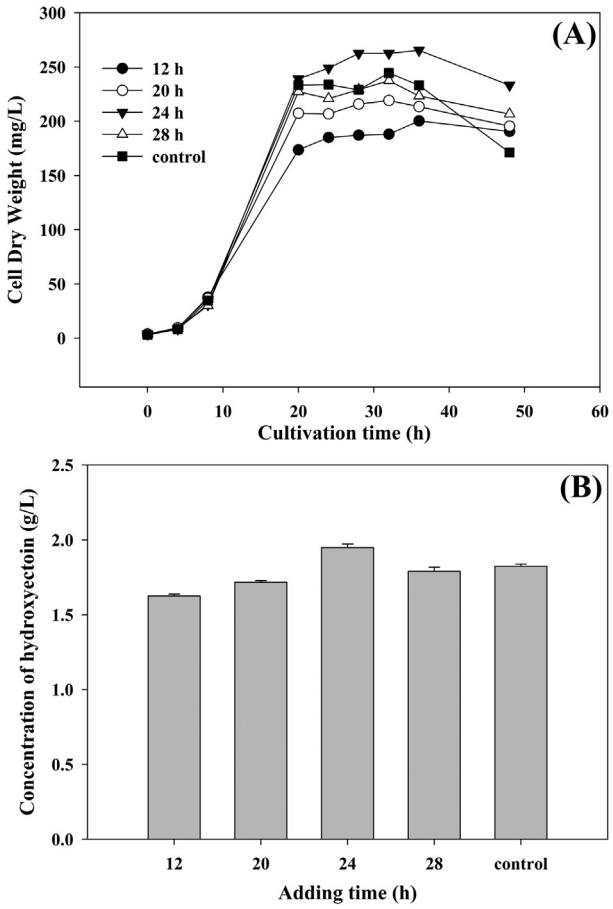


FIG. 3. (A) Growth curves and (B) production of hydroxyectoine upon the addition of 0.5 mM iron in the exponential phase (between 12 and 20 h of cultivation) and stationary phase (between 24 and 28 h of cultivation).

compared with late addition (~235 mg/L, Fig. 4A). Reports have indicated that α-ketoglutarate is an intermediate in the tricarboxylic acid cycle and a major contributor to amino acid formation. In other words, the addition of α-ketoglutarate can facilitate bacterial growth.

The results in Fig. 4B reveal a different trend from that shown in Fig. 4A. Hydroxyectoine production increased from 1.81 g/L to 2.37 g/L when both α-ketoglutarate and iron were added to the medium in the early stationary phase. These results indicated that hydroxyectoine production effectively increased when α-ketoglutarate and iron were added to the medium in the stationary phase. Interestingly, although bacterial growth for groups with iron and α-ketoglutarate addition in the exponential phase was greater than for groups with addition in the stationary phase, hydroxyectoine production showed an opposite trend.

**Effect of adding various amounts of iron and α-ketoglutarate on hydroxyectoine production by *H. salina*** To determine the effect of iron and α-ketoglutarate addition on hydroxyectoine production using *H. salina*, various amounts of iron and α-ketoglutarate were added in the exponential phase (after 24 h of cultivation). First, the iron concentration was fixed to observe how various concentrations of α-ketoglutarate (0, 25, 50, 75, 100, and 125 mM) affected bacterial growth and hydroxyectoine production using *H. salina*. As shown in Fig. 5A, bacterial growth increased with increasing α-ketoglutarate concentration. However, bacterial growth decreased when the α-ketoglutarate concentration exceeded 50 mM. Hydroxyectoine production

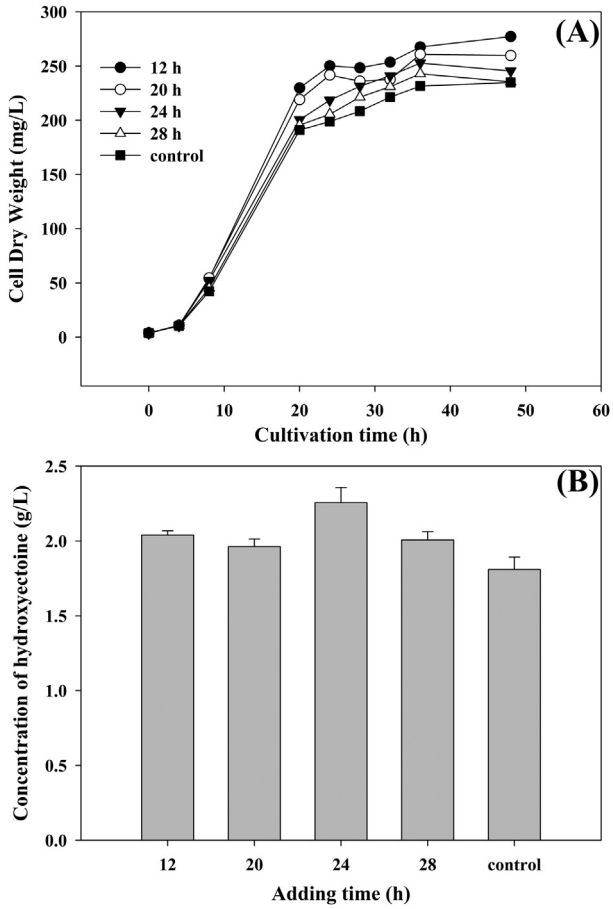


FIG. 4. (A) Growth curves and (B) production of hydroxyectoine upon the simultaneous addition of 1 mM iron and 50 mM α-ketoglutarate in the exponential phase (between 12 and 20 h of cultivation) and stationary phase (between 24 and 28 h of cultivation).

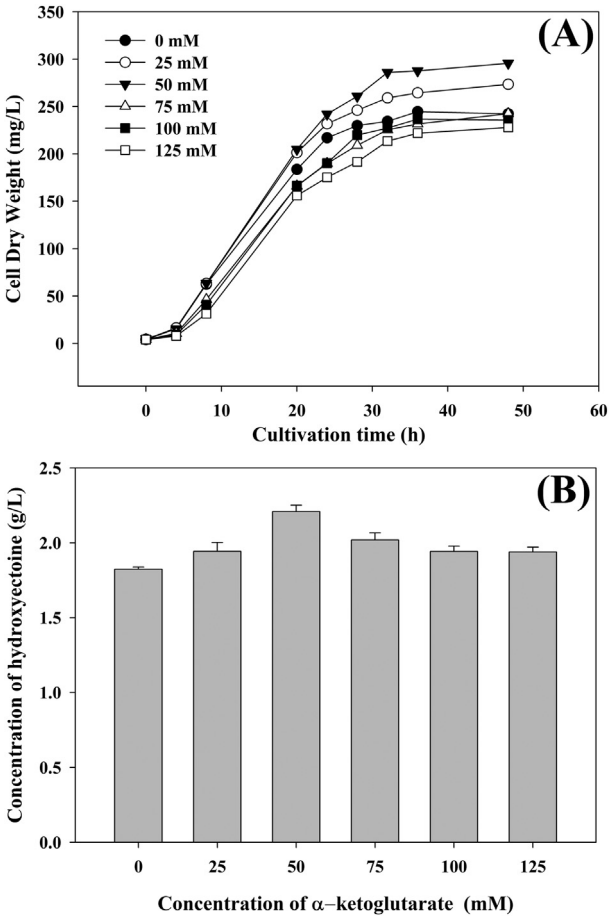


FIG. 5. (A) Growth curves and (B) production of hydroxyectoine upon the addition of α-ketoglutarate at different concentrations (0–125 mM).

showed a similar trend when varying the α-ketoglutarate concentration. Fig. 5B shows that the highest hydroxyectoine production was observed (~2.25 g/L) when 50 mM of α-ketoglutarate was added to the medium. Similar to bacterial growth, hydroxyectoine production began to decrease when the α-ketoglutarate concentration exceeded 50 mM. This might be attributable to the inhibition of bacterial growth or the inhibition of synthetic enzymes of compatible solutes. These results suggested that the addition of α-ketoglutarate facilitated hydroxyectoine production (Fig. 5B). In subsequent experiments, the α-ketoglutarate concentration was maintained at 50 mM.

Subsequently, the effects of adding various amounts of iron on hydroxyectoine production were investigated by adding 50 mM α-ketoglutarate to the medium in the exponential phase (24 h cultivation). The results shown in Fig. 6A indicate that the addition of iron at various concentrations (0–1.25 mM) prompted bacterial growth. Furthermore, the addition of 1 mM iron resulted in the highest bacterial growth (~295.9 mg/L; Fig. 6A) and the highest hydroxyectoine production (~2.9 g/L; Fig. 6B). Moreover, the production of hydroxyectoine by adding the optimal concentrations of iron and α-ketoglutarate at a specific time increased by around 2.37 folds when compared to the YE medium and HYE medium. Thus, in subsequent experiments, the concentration of iron was kept at 1 mM.

**Effects of iron and α-ketoglutarate addition on ectoine hydroxylase activity** On the basis of the results presented in Figs. 3–6, it was posited that the addition of an optimal concentration of iron or α-ketoglutarate to the medium facilitated hydroxyectoine production. In other words, an optimal concentration of iron or α-ketoglutarate might affect ectoine hydroxylase activity, thereby resulting in variations in ectoine or hydroxyectoine production. To prove that ectoine hydroxylase activity could be affected by iron or α-ketoglutarate, a hydroxylase activity assay was performed. Fig. 7 shows that the ectoine hydroxylase activity at 1 mM iron was higher than that at 0.5 mM iron. Upon further comparing the ectoine hydroxylase activity among the four cultivation groups, the results showed that the combination of iron and α-ketoglutarate resulted in greater hydroxylase activity than that obtained with either iron or α-ketoglutarate only. Previous studies have revealed that to increase ectoine hydroxylase activity, iron and α-ketoglutarate must be added simultaneously (9,18). Moreover, studies have reported that the ectoine hydroxylase crystal structure binds to nonheme iron and α-ketoglutarate (9,18,19). The results found in this study correspond to those reported in these studies. In addition, we were able to determine the optimal concentrations of iron and α-ketoglutarate for addition to the cultivation medium.

So far, the reports on hydroxyectoine production are mainly focused on the role and mechanism of ectoine hydroxylase in the process of ectoine transformed to hydroxyectoine. In this study, we attempted to determine the optimal concentration and time for the addition of iron and α-ketoglutarate to increase hydroxyectoine production with the use of *H. salina*. Our results showed that the simultaneous addition of 1 mM iron and 50 mM α-ketoglutarate in the early stationary phase effectively increased hydroxyectoine production using *H. salina*. The production of hydroxyectoine under

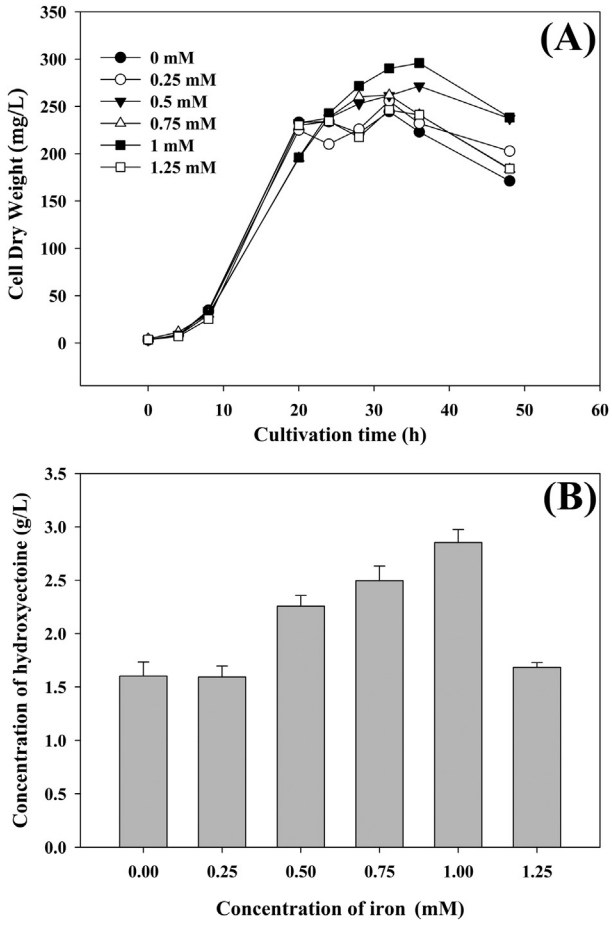


FIG. 6. (A) Growth curves and (B) production of hydroxyectoine upon the addition of iron at different concentrations (0–1.25 mM).

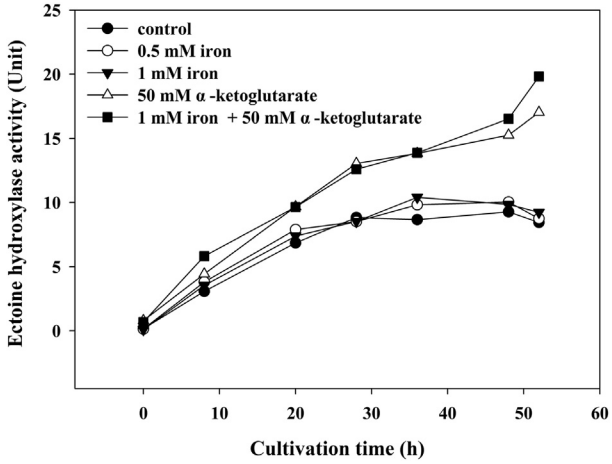


FIG. 7. Variation in ectoine hydroxylase activity upon the addition of iron and α-ketoglutarate, iron alone, and α-ketoglutarate alone.

these conditions increased around 2.37 folds when compared to the YE medium and HYE medium. In addition, we determined that a mixture of iron and α-ketoglutarate increased the activity of ectoine hydroxylase more than either of the other two when used alone. This study will provide a roadmap for future research, such as that attempting to further increasing hydroxyectoine production by increasing ectoine hydroxylase activity.

## ACKNOWLEDGMENTS

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