Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–7, 2017

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Production and characterization of ectoine using a moderately halophilic strain *Halomonas salina* BCRC17875

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Received 21 March 2017; accepted 13 December 2017
Available online xxx

This study attempted to utilize *Halomonas salina* BCRC17875 to produce ectoine by optimizing the agitation speed and medium composition. In addition, the chemical structure of ectoine produced by *H. salina* BCRC17875 was determined. The results indicate that ectoine production reached 3.65 g/L at 38 h of cultivation when the agitation rate and NaCl concentration were fixed at 200 rpm and 2.0 M, respectively. It reached 9.20 g/L at 44 h of cultivation when the major medium components were yeast extract (56 g/L), glutamate (74.40 g/L), and ammonium sulfate (14 g/L). After the nitrogen concentration had been evaluated, evaluation of the nitrogen concentration revealed that the ectoine production reached 11.80 g/L at 44 h of cultivation when 56 g/L of yeast extract and 28 g/L of ammonium sulfate were used. Ectoine production reached 13.96 g/L at 44 h of cultivation when the carbon/nitrogen ratio was fixed at 3/1 using 84 g/L of yeast extract and 28 g/L of ammonium sulfate. Furthermore, the identification of ectoine were identified and characterized by fast atom bombardment mass spectrometry (FAB-MS) and ^{1}H NMR. The results demonstrated a fermentation strategy was successful in increasing ectoine production, and that the fermentation medium of ectoine had commercialization potential.

[**Key words:** Ectoine; *Halomonas salina*; Medium optimization; Identification]

Hypersaline environments saturated by sodium chloride are mainly inhabited by microorganisms (1). To survive in and resist the hypersaline environments, a salt-in-cytoplasm mechanism from microorganisms is activated to synthesize and accumulate polar, highly water-soluble, low-molecular weight organic compatible solutes such as ectoine and sugars (2). The salt-in-cytoplasm mechanism can be found in many halobacteria (3). 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 (4–6). Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) is a natural compatible solute, which serves as a protectant in many bacterial cells (5). Ectoine was first identified in *Ectothiorhodospira halochloris*, an extremely halophilic phototrophic bacterium (6). In addition to the stabilizing effects of ectoine in biochemical, medical, cosmetic, or skin care fields, ectoine has also been functionalized as a protectant for healthy cells during chemotherapy (5).

Halomonas salina is a gram-negative, rod-shaped halophilic bacterium that lives in saline environments (7). This study attempted to utilize *H. salina* to produce ectoine by optimizing growth conditions. Effects of growth conditions such as media salinity, carbon source, and nitrogen source on ectoine biosynthesis by *H. salina* microorganisms have been reviewed (7,8). Therefore, the aim of this study was to evaluate and optimize the growth conditions including parameters such as agitation rate, types of carbon and nitrogen sources, and salinity. Furthermore, the chemical structure and molecular mass of purified ectoine from *H. salina* BCRC17875 were identified by ^{13}C NMR and fast atom bombardment mass spectrometry (FAB-MS) (3,9,10).

MATERIALS AND METHODS

Bacterial strain *H. salina* BCRC17875 was purchased from Bioresource Collection and Research Centre, Taiwan. *H. salina* BCRC17875 could synthesize and accumulate intracellular ectoine as a compatible solute to resist osmotic stress in a hypersaline environment.

Culture medium The medium for *H. salina* BCRC17875 cultivation consisted of yeast extract (YE, 56 g/L), ammonium acetate (14 g/L), $FeCl_2 \cdot 4H_2O$ (0.5 mM), $MnSO_4 \cdot H_2O$ (10 μM), KCl (2 g/L), $MgSO_4 \cdot 7H_2O$ (100 mM), and sodium chloride (6.2 g/L). This medium was utilized a basal medium to optimize the carbon sources and nitrogen sources in subsequent experiments.

Effects of carbon sources and nitrogen sources Previous studies showed that the type of carbon substrate markedly affected ectoine yield (11). The precise effect of carbon and nitrogen types on crude ectoine production was evaluated by

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https://doi.org/10.1016/j.jbiosc.2017.12.011

various carbon sources (including YE, glutamate, glucose, starch, sucrose, and fructose), nitrogen sources (including peptone, tryptone, ammonium acetate, ammonium sulfate, and ammonium chloride), and NaCl concentrations. Each carbon and nitrogen source was individually supplemented into the medium at a fixed concentration of 56 g/L and 14 g/L, respectively, to study its effect on crude ectoine production. Various ammonium sulfate concentrations (0–35 g/L) were also evaluated for crude ectoine production. In addition, the appropriate carbon/nitrogen ratio (i.e., 1/1, 1/2, 1/3, 1/4, 2/1, 3/1, and 4/1) was evaluated during medium optimization. Carbon/nitrogen ratios of 1/1, 1/2, 1/3 and 1/4 were obtained by using a fixed yeast extract concentration of 28 g/L and sulfate concentrations of 28, 56, 84 and 112 g/L. Carbon/nitrogen ratios of 2/1, 3/1 and 4/1 were obtained by using a fixed ammonium sulfate concentration of 28 g/L and yeast extract concentrations of 56, 84 and 112 g/L.

Growth conditions For liquid fermentation, a loopful of *H. salina* BCRC17875 cells was added into a 250-mL Erlenmeyer flask containing 50 mL fresh broth. *H. salina* BCRC17875 was grown in a rotary shaker at 30°C and 200 rpm for seed culture. After 12 h of cultivation, 1 mL inoculum was inoculated into 50 mL liquid medium and cultivated at 30°C and 200 rpm for experiments. For investigating the effects of agitation rate (100–250 rpm) and the effect of NaCl (0.5, 1.0, 1.5, 2.0 and 2.5 M) on ectoine production, batch cultures were performed. After 24 h of cultivation, samples were taken to analyze cell growth and ectoine concentration.

Determination of cell concentration Biomass was determined turbidimetrically at 600 nm (OD_{600}) and values were converted to cell dry weight (CDW) via an appropriate calibration ($CDW = 0.72 \times OD_{600} + 1.31$).

Ectoine assay *H. salina* BCRC17875 was cultured and then harvested by centrifugation at 8000 ×*g*. Then, the pellets were resuspended in ethanol (Sigma) with rigorous shaking for 30 min. The ethanol extract was filtrated through a 0.45-μm filter to analyze ectoine production. Twenty microliters of the extract were analyzed with HPLC (Hitachi, Tokyo, Japan) on an RP-18 column (Merck, Darmstadt, Germany) modified from Malin et al. (12). 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 was monitored at 210 nm by UV/VIS detector (Hitachi). Ectoine purchased from Sigma served as a standard.

Ectoine purification To purify ectoine from *H. salina* BCRC17875, cells were cultivated and harvested by centrifugation at 8000 ×*g*. The pellets were then mixed with ethanol/chloroform/water (1/1/1, v/v/v) (Sigma). The mixture was then re-centrifuged at 8000 ×*g*. The pellet was dried at 100°C in an oven, re-dissolved in ethanol, filtrated through a 0.45 μm filter, and passed through activated carbon for 30 min to absorb ectoine. After the absorption, the active carbon was placed into boiling ethanol for ectoine extraction. The ethanol with extracted ectoine was then dried at 100°C again to harvest the purified ectoine (3).

Characterization of purified ectoine The characterization of mass and chemical structure of the purified ectoine were determined by FAB-MS (JMS-700, JEOL, Tokyo, Japan), and nuclear magnetic resonance (NMR) spectrometry (Bruker, Rheinstetler, Germany).

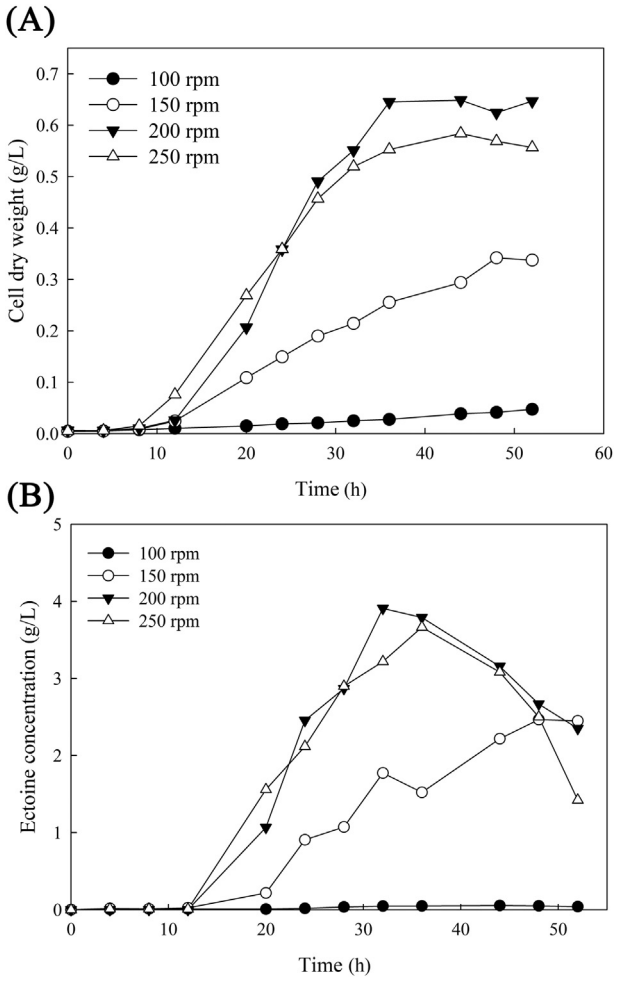

FIG. 1. Effect of agitation rate on (A) cell dry weight and (B) ectoine production in a batch culture of *H. salina* BCRC17875 grown in a medium containing 56 g/L yeast extract and 14 g/L ammonium acetate at 30°C and pH 7.0. The data shown are mean values of triplicates.

RESULTS AND DISCUSSION

Effect of agitation rate on ectoine production The agitation rate is regarded as a key parameter for product secretion and microbial growth in the fermentation field (13). In other words, the mass transfer between the dissolved oxygen and medium components in the medium is also a crucial factor for ectoine production. Therefore, batch cultures of *H. salina* BCRC17875 were cultured at agitation rates of 100, 150, 200, and 250 rpm to examine the effects of agitation rate on ectoine production. As revealed by the experimental results in Fig. 1, both ectoine production and microbial growth increased with cultivation time as the agitation rate increased from 100 rpm to 200 rpm. The highest ectoine production was achieved at 32 h cultivation, and slightly decreased to the end of the cultivation period. The ectoine production (3.10 g/L) at an agitation rate of 200 rpm was higher than that at 100 rpm (0.05 g/L) and 150 rpm (2.60 g/L). Agitation rate impacts oxygen transfer, in other words, faster agitation rate results in higher dissolved oxygen (DO) level (3,14). Owing to the high shear force, microbial growth at 250 rpm was less than at 200 rpm. Ectoine production decreased to 1.50 g/L by the end of the cultivation period. These results suggested that the mass transfer of oxygen deeply affected not only ectoine production but also microbial growth. In addition, mass transfer of medium components toward the cells might play a major role in the relationship between agitation rate and ectoine production (3,15). Hence, an agitation rate of 200 rpm was used for the rest of experiments.

Effect of sodium chloride concentration on ectoine production Since *H. salina* BCRC17875 is a halophilic bacterium and ectoine serves as a compatible solute for this bacterium, the concentration of NaCl in the medium might play an important role for ectoine synthesis (16). This study attempted to examine the optimal condition for NaCl supplementation to achieve the highest ectoine production by *H. salina* BCRC17875. *H. salina* BCRC17875 was grown in medium containing various concentrations of NaCl (0.5, 1.0, 1.5, 2.0 and 2.5 M) to investigate the effect on ectoine production. As depicted in Fig. 2, the microbial growth and ectoine production followed similar trends. The experimental results indicated that microbial growth and ectoine concentration (3.65 g/L) increased with NaCl concentration from 0.5 to 2.0 M, while the ectoine concentration declined sharply to 2.45 g/L as the NaCl concentration was further increased from 2.0 to 2.5 M. Thus, 2 M of NaCl appeared to be the optimal concentration of NaCl, giving the maximum ectoine yield. A previous report showed that *H. salina* is a moderately halophilic bacterium (17). Accordingly, the experimental results regarding NaCl concentration may be attributed to the effect of high osmotic stress on cell growth (17). This result also addresses the necessity of controlling salinity of

Please cite this article in press as: Chen, W.-C., et al., Production and characterization of ectoine using a moderately halophilic strain *Halomonas salina* BCRC17875, J. Biosci. Bioeng., (2017), https://doi.org/10.1016/j.jbiosc.2017.12.011