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OPTIMUM OF MEDIUM COMPONENTS FOR THE PRODUCTION OF ECTOINE BY A HALOPHILIC BACTERIUM ISOLATED FROM CAN GIO MANGROVE

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Abstract. A fermentation process for ectoine production has been developed, using a halophilic bacterium strain D209 isolated from Can Gio mangrove as the producer strain. First, the effect of different carbon and nitrogen sources on growth of producer strain was investigated. Sucrose and glucose were found to be suitable carbon sources, and monosodium glutamate was favorable nitrogen source for bacterial cell growth. The effect of different NaCl concentrations on growth and ectoine accumulation was also evaluated. Optimum salt concentrations for bacterial growth was ranged from 4 to 6 %, whereas, NaCl concentrations from 12 to 15 % found to be good for ectoine accumulation. Two-step fed-batch fermentation was then designed, first step for biomass production and second step for ectoine synthesis. Biomass and ectoine content were significant increased by using this process, maximum CDW of 25 g/l and ectoine content of 10.3 % were obtained. Strain D209 was then characterized by using 16S rRNA gene sequence.Our results showed that strain D209 belonged to *Halomonas* genus and closed to *Halomonas organivorans* species.

Keywords: ectoine, mangrove, halophilic bacteria, D209.

Classification numbers: 1.1.5, 1.3.2, 1.5.3.

1. INTRODUCTION

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) is well known as the most abundant compatible solute, accumulated intracellularly by many halotolerant and halophilic bacteria for osmotic stress protection. Accumulation of ectoine helps to maintain the turgor pressure, cell volume, and electrolyte concentration, which are all important conditions for cell proliferation [1]. Apart from osmotic functions, ectoine has gained much attention in biotechnology as protective agent for enzymes, DNA, and even entire cells against stress conditions such as heating, drying and freezing [2-4]. Ectoine is not only a macromolecule protection, but it can be used as a protecting agent for skin. It has been demonstrated that ectoine protects the skin against stress factors that would normally lead to skin dehydration. Ectoine has served as a potent moisturizer and skin moisture can be maintained for long periods of time [5]. Furthermore, antiaging properties of ectoine was evaluated by prevention the activity of oxidative damage in skin. Ectoine thus is available as active ingredients in cosmetics, or skin

care products. An additional role for ectoine in general is in health benefits. Ectoine function as a therapeutic agent for certain diseases, for oral care, as adjuvants for vaccines among many other applications (https://www.bitop.de/). Previous investigations have also underlined the application of ectoine in antiviral drugs such as human immunodeficiency virus (HIV) by inhibition the interaction of the viral regulatory proteins [6].

Ectoine is currently produced on a large scale by using a moderately halophilic bacterium *Halomonaselongata*in a process called "bacterial milking", which repeats of three main steps: fed-batch cultivation of the bacterium at 15 % (w/v) NaCl; concentration of cell broth by membrane filtration; and ectoine release from the cells by hypoosmotic shock [7].

Recently, we have isolated an ectoine producer (strain D209) from Can Gio mangrove forest (Ho Chi Minh City, Vietnam), the isolated strain is a Gram-negative and halophilic bacterium.In the present study, optimum of medium components for production of biomass and ectoine by strain D209 was investigated. In addition, the strain D209 was also characterized based on 16S rRNA gene sequence.

2. MATERIALS AND METHODS

2.1. Bacterial strain, maintenance and cultivation media

Bacterial strain D209 was maintained at 4 ^{o}C on solid MPA medium containing (g/l): peptone, 5; meat extract, 5; NaCl, 40; and granulated agar, 20.

The basic culture medium (HM medium) [8] for production of biomass and ectoine contains (g/l): $MgSO_4$.$7H_2O$, 0.5; $CaCl_2$.$2H_2O$, 0.009; KCl, 0.05; K_2HPO_4, 0.5; $FeSO_4$.$7H_2O$, 0.005, Monosodium glutamate, 2; glucose, 10; and NaCl, 40.

2.2. Characterization of isolated strain

The genomic DNA of the isolated strains was extracted by DNA extraction kit "GeneJET Genomic DNA Purification Kit" according to the manufacturer's recommended procedure. The 16S rRNA gene was amplified using the universal primers: 27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R: 5'-GGTTACCTTGTTACGCTT-3'. Sequencing of the amplified DNA fragment was performed at 1st Base (Singapore). GenBank database was used to search for 16S rRNA gene similarities. Phylogenic analysis based on 16S rRNA gene was performed with the aid of the Mega 6 software using the neighbor-joining distance correlation method. Almost complete sequence (c.a. 1400 bp) of the 16S rRNA gene of the strain isolated from Can Gio mangrove forest was deposited at GenBank/EMBL/DDBJ databases and was used in the analysis.

2.3. Effect of medium components on growth of strain D209

2.3.1. Seed culture preparation

Strain D209 was first grown in 25 ml of MPA medium in 100 ml Erlenmeyer flask at 35 °C and 180 rpm in rotary shaker incubator for 15 h.

2.3.2. Effects of carbon sources

One milliliter of seed culture was inoculated in 20 ml of HM medium in 100 ml Erlenmeyer flasks containing different carbon sources: glucose, sucrose, xylose, galactose, and lactose. After 30 h of growing at 35 °C and 180 rpm, bacterial cells were harvested from the culture broth by centrifugation at 10000 x g for 5 min and used for cell dry weight (CDW) analysis.

2.3.3. Effects of nitrogen sources

One milliliter of seed culture was inoculated in 20 ml of HM medium in 100 ml Erlenmeyer flasks containing different nitrogen sources: yeast extract, monosodium glutamate, NH_4Cl, and KNO_3. After 30 h of growing at 35 °C and 180 rpm, bacterial cells were harvested from the culture broth by centrifugation at 10000 × g for 5 min and used for CDW analysis.

2.3.4. Ectoine production at different NaCl concentrations

One milliliter of seed culture was inoculated in 20 ml of HM medium in 100 ml Erlenmeyer flasks containing different NaCl concentrations: 0, 2 %, 3 %, 6 %, 9 %,12 %, 15 %, 18 %. After 30 h of growing at 35 °C and 180 rpm, bacterial cells were harvested from the culture broth by centrifugation at 10000 x g for 5 min and used for CDW and ectoine analysis.

2.4. Two cultivations steps in bioreactor

Four hundreds of seed culture were used to inoculate 3.6 L of HM medium containing 40 g/l NaCl in a 10-L bioreactor. After 54 h of cultivation, bacteria cells were harvested from the culture broth by filtration, and used to inoculate 4 L of HM medium containing 120 g/l NaCl. The samples were taken every 3 h for biomass and ectoine analysis.

2.5. Analytical methods

2.5.1. Determination of cell dry weight

Cell dry weight (CDW) was determined by centrifuging 3 ml of the culture samples at 5 000 g for 15 min in a pre-weighed centrifuge tube, the pellet washed quickly once with 3 ml distilled water, centrifuged and dried at 105 ^oC until constant weight was obtained. The centrifuge tube was weighed again to calculate the CDW.

2.5.2. Ectoine analysis

Extraction of ectoine for its analysis was performed as reported previously [9].Ten milligram cell mass was extracted with 570 µl extraction mixture (methanol/chloroform/water 10:5:4, by volume) by vigorous shaking for 5 min followed by the addition of equal volumes (170 µl) of chloroform and water. The mixture was shaken again for 10 min and phase separation was enhanced by centrifugation. The hydrophilic top layer containing compatible solutes was recovered. Concentrations of ectoines was determined by High performance liquid chromatography (HPLC) analysis [10], using a UltiMate 3000 Standard Dual System with an Aminex HPX-87C column (Biorad) and a UV detector at 65 ºC, and monitoring the compounds at 210 nm. Calcium chloride (5 mM) was used as mobile phase at a flow rate of 0.3 ml/min. Ectoine (Sigma) was used as a standard for calibration.

The intracellular ectoine content (ectoine per biomass, g/g of CDW) was calculated according to standard procedure [11].

3. RESULTS AND DISCUSSION

3.1. Effect of carbon sources on bacterial cell growth

All life forms are made up of organic compounds and carbon is the building block of organic compounds. Therefore, carbon as a part of an ingredient in the medium is required for bacterial cell growth. Ectoine is an intracellular product, so the productivity of ectoine is closely related to biomass. High cell density and ectoine content will reduce the production cost [11]. In order to investigate the effect of carbon sources on growth of strain D209, the strain was cultivated in HM medium containing 10 g/l of 5 different carbon sources. As shown in Figure 1, among five tested carbon sources, sucrose and glucose were the preferred carbon sources for bacterial cell growth, followed by galactose, lactose, and xylose. After 30h of cultivation, CDW of 3.5 and 3.2 g/l were obtained by the strain D209 when sucrose and glucose wererespectively used as carbon substrates.For further studies, both sucrose and glucose can be chosen as carbon substrate for strain D209.

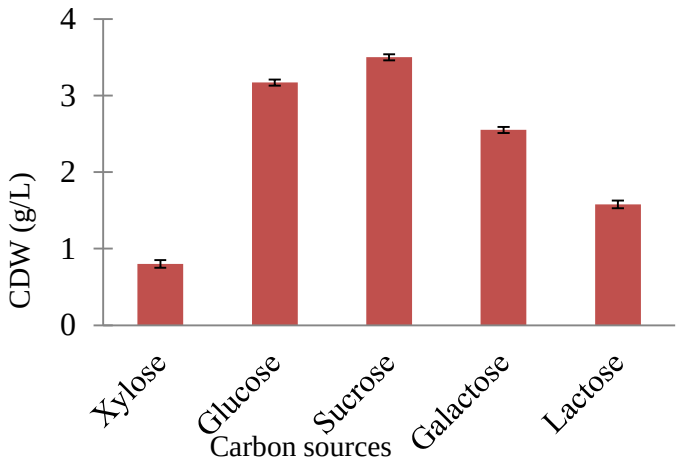

Figure 1. Effect of different carbon sources on bacterial cell growth.

3.2. Effect of nitrogen sources

Beside carbon source, nitrogen is a second major essential element for all organisms. The effect of four different nitrogen sources including NH_4Cl, KNO_3, monosodium glutamate (MSG), and yeast extract (YE) on growth of strain D209 was evaluated. The results showed that strain D209 can grow in all four tested nitrogen sources.The highest CDW of 3.9 g/l was obtained when monosodium glutamate was used as nitrogen source. Yeast extract and KNO_3 were also suitable nitrogen sources for bacterial cell growth, high CDW of 3.5 g/l and 2.9 g/l were obtained when yeast extract and KNO_3 were used as nitrogen sources, respectively. However, NH_4Cl was found to be poor nitrogen source for bacterial cell growth, CDW of only 0.7 g/l was achieved when NH_4Cl was used (Figure 2). Previous studies have demonstrated that monosodium glutamate is the key factor having extreme influence on cell growth and ectoine

synthesis by halotolerant and halophilic bacteria [10-12]. As compared to other substrates such as glucose and monosodium aspartate, CDW and ectoine content were significantly increased when MSG was used as both carbon- and nitrogen sources for ectoine production by *Brevibacterium epidermis* [10].

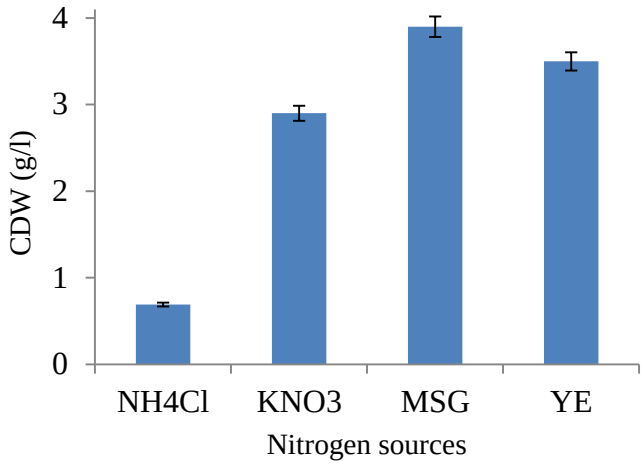

Figure 2. Effect of different nitrogen sources on bacterial cell growth.

3.3. Ectoine production by strain D209 at different NaCl concentrations

Normally, the concentrations of compatible solutes are regulated according to the salt concentration in which the cells live, and they can often be rapidly adjusted as required when the outside salinity is changed [1].

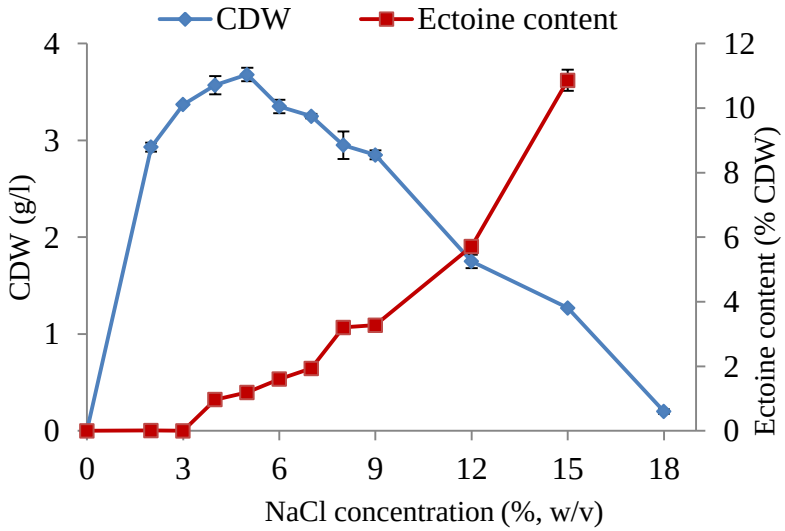

*Figure 3.*Bacterial cell growth and ectoine accumulation at different NaCl concentrations.

Hence, the presence of NaCl in the culture medium is expected to increase the ectoine content of the biomass. However, high concentration of NaCl can also reduce the growth rate and biomass yield. Therefore, the effect of different NaCl concentrations in the culture medium on growth and ectoine accumulation was investigated. Figure 3 showed that the no growth occurred in the absence of salt, NaCl concentrations of between 3 and 6 % were optimal condition for bacterial cell growth, the growth rate was rapidly reduced when salt concentration above 9 %. Maximum CDW of 3.7 g/l was obtained at NaCl concentration of 5 %. On the other hand, ectoine content in the bacterial cells was induced by salt concentration in the culture medium. Ectoine content was increased when salt concentration in the culture medium was increased, maximum ectoine content of 11 % of CDW was obtained at 15 % of NaCl. Strain D209 could grow at 18 % of NaCl, but the CDW was not high enough for ectoine analysis.

3.4. Two-step fed-batch fermentation for ectoine production

Ectoine accumulate in the bacterial cells, high ectoine productivity will be obtained when both biomass and ectoine content in the biomass are high. However, as showed in section 3.3 the optimal conditions for biomass and ectoine production are different with respect to the concentrations of salt in culture medium. Therefore, two-step fermentation was designed, the first for obtaining high biomass and the second for inducing ectoine accumulation. In this study, strain D209 was first grown in HM medium containing 5 % NaCl, bacterial cells were then collected by filtration, and transferred to new HM medium containing 12 % NaCl. The results in Figure 4 showed that CDW was increased during first step, high CDW of nearly 19 g/l was achieved after 54 h of cultivation. Ectoine content was changed during first step, ectoine content was increased from 5.2 % at 6 h to 7.3 % at 33 h, it was decreased after that and ectoine content of only 5.7 % was accumulated in the bacterial cells at the end of first step (after 54 h of cultivation). When transferred the bacterial cells to second step, the CDW was 22 g/l, higher than that obtained at the end of first step (19 g/l). It is due to the reduction of culture broth volume from 4.7 l at the end of first step to 4 l at the beginning of second step.In the second step, some of bacterial cells were died during the first hours of cultivation, for that reason CDW was decreased from 22 g/l to 20.5 g/l in first 6 h of cultivation. After that, when the bacterial cells adapted with new medium, CDW was increased and reached maximum value of 25 g/l after 15 h of cultivation. Ectoine content was slightly increased in first 6 h of cultivation, and then rapidly increased and obtained maximum value of 10.3 % after 12 h of cultivation in second step (Figure 4). Ectoine content obtained in second step was nearly two times higher than that obtained in the first step. However, biomass and ectoine content obtained here by strain D209 were still lower than those obtained by other ectoine producers such as *Halomonas elongata* [7], *Brevibacterium epidermis* [10], or *Halomonas boliviensis* [11]. Therefore, further studies need to be done to improve ectoine productivity of strain D209.

3.5. Phenogenetic studies based on 16S rRNA sequence of the isolated strain

Genomic DNA of the strain D209 was successfully isolated and used for PCR reaction with the universal primers: 27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R: 5'-GGTTACCTTGTTACGCTT-3'. The PCR product was then sequenced at 1st base company (Singapore). The partial sequence of the 16S rRNA gene (about 1400 bp) of the strain D209 was obtained and deposited at GenBank/EMBL/DDBJ databases with the registered number of MH715407. The sequence of strain D209 was used to search for 16S rRNA genes similarities on Genbank. Figure 5 showed that strain D209 belonged to *Halomonas* genus and shared a closest

relationship with *Halomonas organivorans* (99.5 %), followed by *Halomonas koreensis* (99 %), and *Halomonas beimenensis* (98 %). *Halomonas organivorans* is a halophilic bacterium isolated from hypersaline habitats in Spain. This strain is able to use wide range of toxic compounds such as benzoic acids, phenol, and salicylic acid [13]. However, there are no reports on the synthesis of ectoine by this strain.

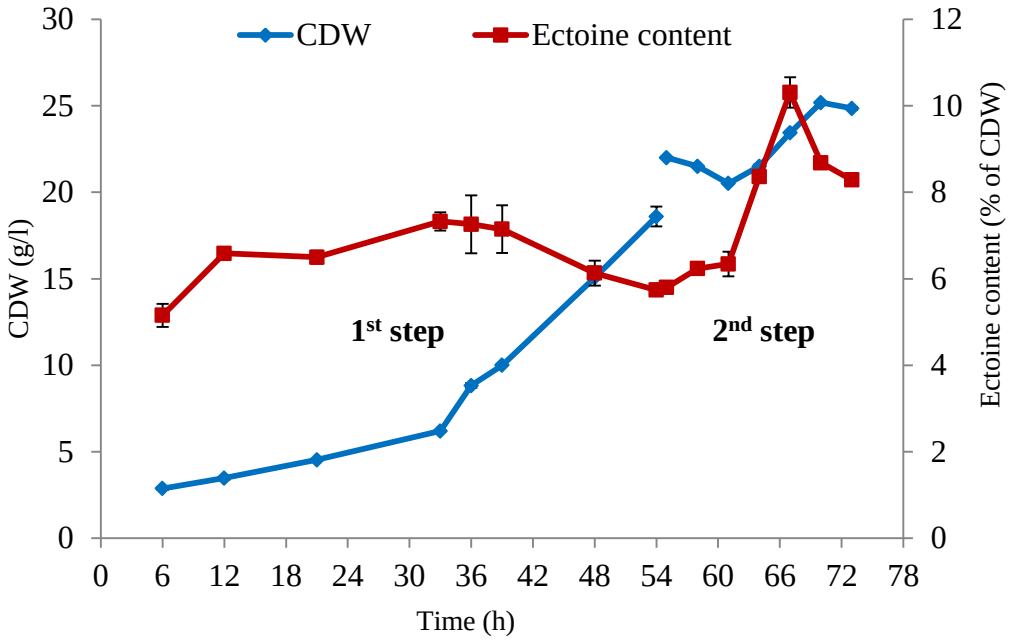

Figure 4. Two-step fed-batch fermentation for ectoine production by strain D209.

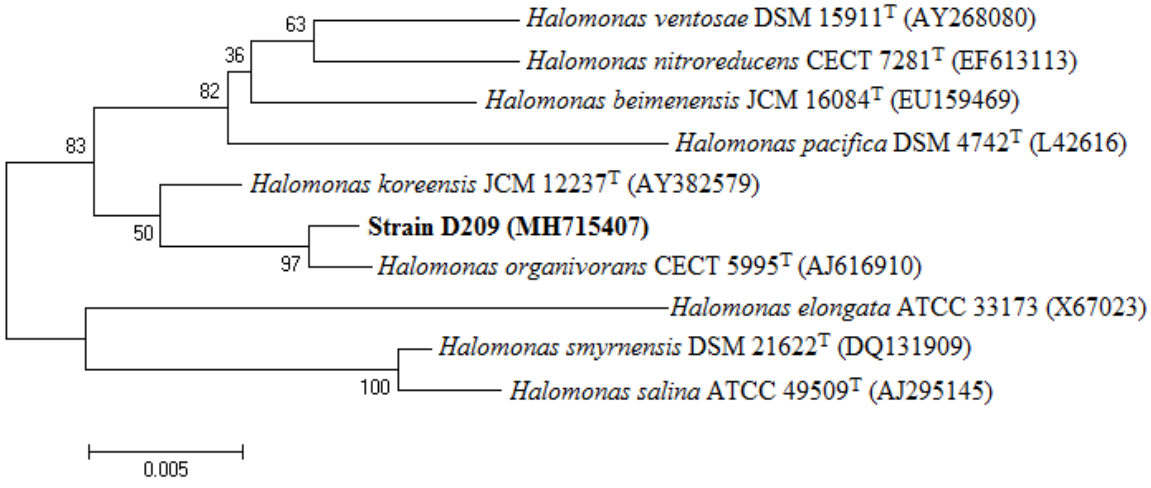

Figure 5. Phylogenetic tree constructed using 16S rRNA gene sequence of the strain D209 belonging to the genus *Halomonas*. Bar, five subtitutions per 1000 nucleotides.

4. CONCLUSION

The effect of some components of culture medium such as carbon and nitrogen sources, concentrations of NaCl on growth and ectoine accumulation by strain D209 was investigated. Sucrose and glucose were found to be good carbon sources, and monosodium glutamate was favorable nitrogen source for strain D209. Maximum biomass was obtained at NaCl concentration of between 4 to 6 %, whereas, high ectoine content was achieved at NaCl concentration of above 12 %. By using two-step fed-batch fermentation, high biomass of 25 g/l and ectoine content of 10.3 % were achieved. Strain D209 belonged to *Halomonas* genus and shared a closest relationship with *Halomonas organivorans* (99.5 %). The name of strain D209 will be *Halomonas* sp. D209.

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