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AMMONIA-REMOVAL EFFICIENCY OF A NOVEL *ACINETOBACTER CALCOACETICUS* STRAIN ISOLATED FROM INDUSTRIAL WASTEWATER OF NINH BINH COAL NITROGENOUS FERTILIZER PLANT

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Abstract. Ammonium removal from wastewater has recently become a major concern of fertilizer manufacturers and industrial zones in Vietnam. Using aerobic ammonium remover may be an appreciate solution for reduction of treatment cost. This study describes the isolation and characterization of a novel bacterium for ammonium removal under aerobic conditions. Twelve ammonium remover strains were isolated from wastewater of a local fertilizer industry. The isolated strains were initially screened using solid media for their nitrifying activities. Among them two of the bacteria displayed the highest removal of ammonium without much accumulation of nitrite and nitrate. The isolates were identified as *Acinetobacter* based on biochemical characteristics and 16S rRNA sequence. One of these two isolates, *Acinetobacter calcoaceticus* HUST-C8 strain, showed 88 % ammonium removal from industrial fertilizer wastewater.

Keywords: Acinetobacter calcoaceticus, aerobic treatment, ammonium removal, fertilizer.

Classification numbers: 3.1.1, 3.3.1, 3.7.2.

1. INTRODUCTION

Ammonium ions are the primary form of widespread nitrogen pollution in the hydrosphere and cause an acute toxicity to fish and other forms of aquatic life at very low concentration, about 0.1 - 10 mg/L [1-6]. Furthermore, excess ammonium ions in receiving water can cause a remarkable increase of oxygen demand and biological eutrophication and a source of nitrite and nitrate ions in water. Thus, removing ammonium ions from municipal and industrial wastewater, especially from the ammonium-rich wastewater of nitrogenous fertilizer plants, prior to discharge is critical.

The most often employed method for removal of ammonium ions from wastewaters consists of nitrification carried out by autotrophic nitrifiers in an aerobic tank and denitrification

by heterotrophic denitrifier in another tank under anoxic conditions [1-6]. However, the slow nitrification makes this wastewater treatment more time-consuming and expensive [1-3].

Recently, bacteria capable of heterotrophic nitrification-aerobic denitrification simultaneously (such as *Paracoccus denitrifican, Alcaligenes faecalis, Bacillus* spp., *Pseudomonas* sp.) were isolated and characterized [1-7]. Under aerobic conditions, these hetetropes (which can faster grow than autotrophic nitrifiers) are able to convert ammonium to hydroxylamine, nitrite, or nitrate and immediately reduce these products to N_2O and/or N_2. As the results, removal of ammonium ions can be carried out in a single aeration phase without the need of either carrier or additional constructions. Therefore, the treatment time and construction cost are reduced. System operation is also simpler than the conventional treatment. Therefore, hetetotrophic nitrification-aerobic denitrification had drawn more and more attention. However, very limited researches have been done in this area in Vietnam while native microorganisms are diverse and hold considerable promise for effective ammonium removal.

In this work, a novel bacterium capable of ammonia removal likes aerobic hetetotrophic nitrifiers was newly isolated from wastewaters taken from Ninh Binh coal nitrogenous fertilizer plant and its ammonium removal characteristics were evaluated.

2. MATERIALS AND METHODS

2.1. Isolation and identification

Wastewater sample was obtained from waste water treatment plant of Ninh Binh coal nitrogenous fertilizer plant, Vietnam between December 2015 and March 2016. The characteristics of wastewater sample are listed in Table 1. Wastewater samples (1 mL) were incubated at 80°C for 30 minutes. 100 µL of diluted sample was spread on the blue bromthymol (BTB) medium plates under aseptic conditions and incubated at 37°C for one day. The BTB medium (pH 8.2) consists of the following components: 0.5 g of $(NH_4)_2SO_4$, 1 g of K_2HPO_4, 0.03 g of $FeSO_4.7H_2O$, 0.3 g of NaCl, 0.3 g of Mg SO_4 $.7H_2O$, 1.5 g of $CaCO_3$, 1 mL of blue bromthymol (1 % in ethanol) and 2 g of agar. Colonies forming yellow color/halo zones in the blue solid agar medium indicates the nitrification activities. Such colonies were further purified and separated on nutrient agar media and maintained as a single strain before subjecting them into further nitrification studies. Secondary screening for potential nitrifier screening based on ratio of yellow color halos and colony diameter. Colonies which showed higher D/d ratio were chosen for further screening in liquid culture. During the study, one separating colony of individual strains were inoculated into 20 mL of BTB liquid medium (without $CaCO_3$) in 100 mL Erlenmeyer flask and incubated one set under aerobic conditions. After 24 h of incubation, sample was withdrawn and centrifuged at 10.000 rpm for 5 min in 4°C to separate supernatant and residue of bacterial biomass. The strain with the highest nitrification ability was selected for further study.

Micrographs of the isolated strain were taken with an electron microscope (Nikon Elipse E100, Japan). The biochemical tests were performed according to the methods of Dong and Cai [8]. Genomic DNA of the isolated strain was extracted with DNA extraction kit (Qiagen, USA). The 16S rRNA gene was then amplified using forward primer 518f (5'-CCAGCAGCCGCGGTAATACG-30) and reverse primer 800r (5'-TACCAGGGTATCTAATCC-3'), and sequenced by First BASE Laboratories Sdn Bhd (Selangor, Malaysia).

2.2. Ammonium removal studies

A preculture of the isolated strain was inoculated into triplicate 500-ml glass jars containing 200 ml of diluted wastewater which was pre-adjusted to a constant ammonium and pH. The jars were incubated aerobically (with an aerator) at a constant temperature in range of 23 - 37 °C in a waterbath (Thermo Fisher Scientific). The appropriate medium without inoculation was used as the control. Samples were withdrawn periodically for the determination of ammonium. All experiments were carried out in triplicate.

2.2. Analytical methods

Ammonium, nitrate and nitrite were determined according to standard methods. Ammonium was determined by the method of Nessler's reagent spectrophotometry (TCVN 5988- 1995, ISO 5664-1984). Nitrite was determined by N-(1-naphthalene)-diaminnoethane photometry method (TCVN 6178: 1996, ISO 6777: 1984). Nitrate was measured by Spectrometric method using sulfosalicylic acid (TCVN 6180:1996). The growth of the isolate was measured by spectrophotometer at 600 nm. Antibiotic susceptibility testing was determined by single disk method [9].

Table 1. Characteristics of raw wastewater from Ninh Binh coal nitrogenous fertilizer plant.

Characteristics	pH	COD (mg/l)	Ammonium (mg/l)	Nitrite (mg/l)	Nitrate (mg/l)	BOD_5 (mg/l)	SS (mg/l)
Results	9.2	463	1180	0	0	246	< 100

3. RESULTS AND DISCUSSION

3.1. Strain isolation and identification

Twelve different colonies which formed yellow color and/or halos zones on BTB agar medium were isolated from waste water collected from the fertilizer plant. These potential ammonium remover strains were individually separated and employed in second screening based on ratio of yellow color halos and colony diameter. The results show that the strains coded with A7, A8, B8 and C8 had higher ratios than other isolates (Figrure 1-A). Therefore these four strains were further employed in liquid cultures for confirming the ammonium conversion. In liquid culture of the strains coded with A7 and A8 were found as ammonium remover. These isolates demonstrated excellent capability for ammonium removal. About 80% of ammonium was removed after 24 h of incubation (Figure 1-B). Accumulation of nitrate and nitrite was not observed during the process. Hence A8 and C8 isolates were selected for further studies.

The isolates A8 and C8 have similar morphological and biochemical characteristics. Their colonies in agar plate were rounded, whitish and opaque. They were gram-negative, and appeared as cocci or short rods. They could utilize sodium succinate as the sole carbon source. The strains gave positive results for carboxyl methyl cellulose hydrolysis. Positive tests were observed for catalase and protease. Both A8 and C8 were sensitive to almost normal antibiotics commerced in Vietnam (Fig. 2).

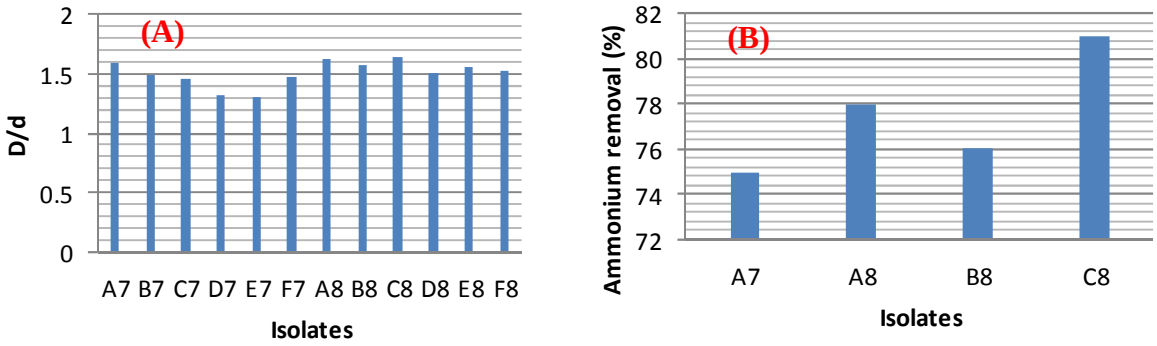

Figure 1. Ammonium removal capacity of isolates

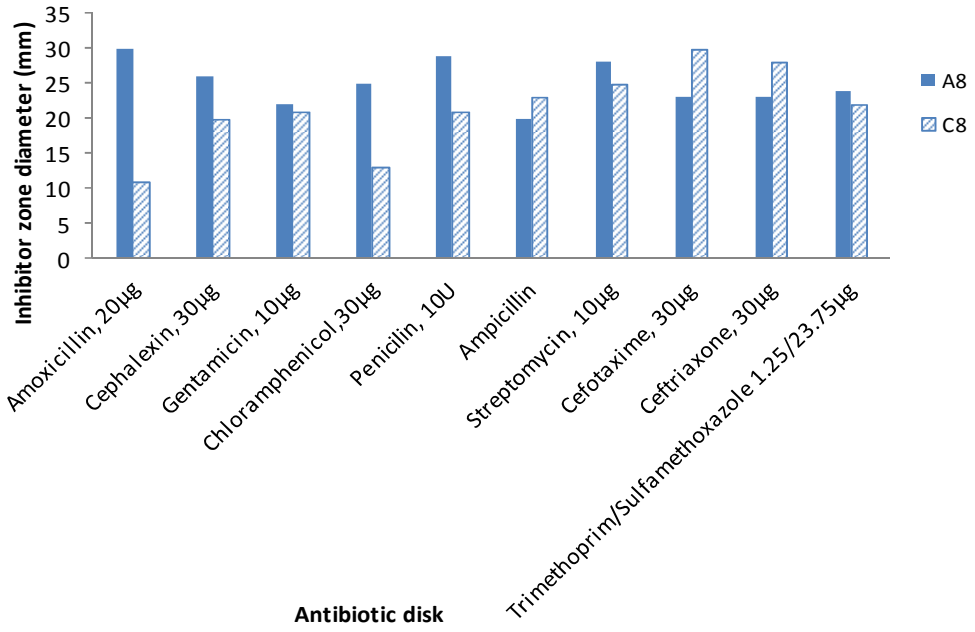

Figure 2. Antibiotic sensitivity of isolates A8 and C8.

Partial 16S rRNA gene of strain A8 (1378 bp) and strain C8 (1381 bp) were high similarity (99 - 100 %) to members of genus *Acinetobacter*. Phylogenetic tree constructed based on partial 16S rDNA sequence of A8 and some members of *Acinetobacter* showed that A8 was closely related to *A. calcoceticus* NCCB 22016 and *A. pitii* LMG 1035 (data not showed here). C8 was very closely related to *Acinetobacter calcoceticus* PHEA-2 which was a non-pathogen strain used to remove phenol from industrial wastewater [10] (Figure 3). This C8 strain was called *Acinetobacter calcoceticus* HUST-C8 and chosen for futher studies.

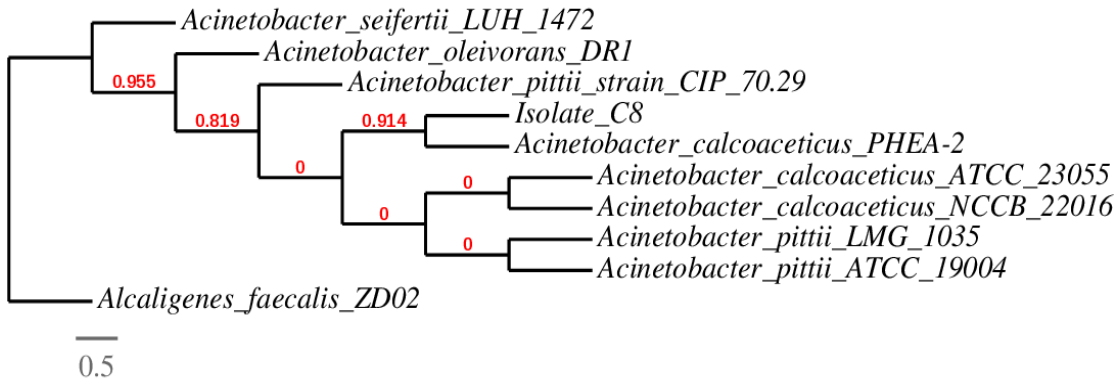

Figure 3. Phylogenetic tree based on the comparison of partial 16S rRNA gene sequences of strain C8 and other members of *Acinetobacter*. *Alcaligenes faecalis* ZD 02 was used as an out group. The tree was constructed using the "Click" mode with default settings in the Phylogeny.fr platform [11-16]. The number of bootstrap replicates is 100 [11]. The numbers above the branches are tree support values generated by PhyML using the approximate likelihood-ratio statistical test.

3.2. Effect of different factors on heterotrophic ammonium removal

Effect of initinal ammnonium and inoculation concentration

Through causing the osmotic pressure on the cell, solutes in the waste water greatly influenced on the growth of bacteria. Therefore, initinal ammonium concentration can affect the efficiency of ammonium conversion of the strain. Fig. 4A shows the lower the ammonium concentration (the more dilute the wastewater), the better ammonium removal. However, the ammonium consumption occurred at 100 mg/L of ammonium was not much higher than at 150 mg/L.

The effect of inoculation size on ammonium removal of the strain was shown in Fig. 4B showed that the highest ammonium removal was archived at inoculation size of 7 % v/v (number of cell to cultivate was 10^8 CFU/ml). When increasing inoculation size, the yield decreased.

Effect of incubation temperature and pH

Temperature is one of the important parameters which significantly affect the growth of the microorganism as well as its metabolic activities. To study the effect of temperature on ammonium removal of HUST-C8, the organism was inoculated into diluted sterilized wasterwater (with the initial ammonium concentration of 150 mg/L) then the jars were incubated at different temperature. The ammonium removal by HUST-C8 is shown in Fig. 4C. The organism may remove ammnium from wastewater in a wide temperature range from 23 to 44°C. This character of HUST-C8 is useful for applying in treatment in the plants in Vietnam where temperature significantly changes according to seasons. The optimal temperature for ammonium removal was 37 °C for strain HUST-C8.

The effect of pH on ammonium removal was studied by preparing the wastewater with initial pH 8.0, 8.5, 9.0, and 9.5. The pH of the medium was altered by using 1N HCl/NaOH solution. In the present study at a pH 8.5 a maximum ammonium removal by *Acinetobacter calcoaceticus* HUST-C8 was observed (Fig. 4D). At more alkaline pH, there is decrease in ammonium removal.

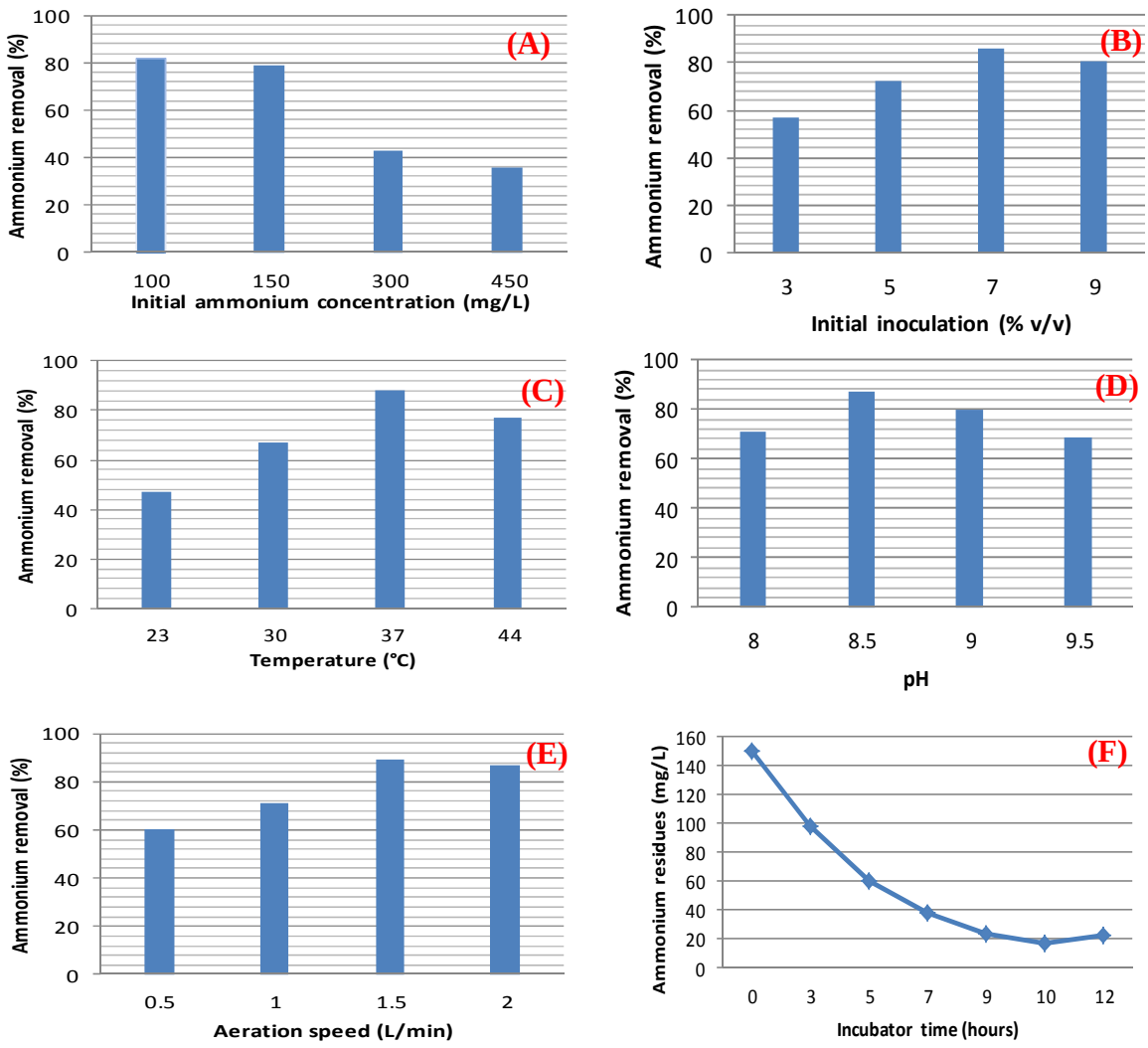

Figure 5. Effects of different factors on ammonium removal efficiency by *Acinetobacter calcoaceticus* HUST-C8. (A) Initial ammonium concentration; (B) Initial inoculation; (C) pH; (D) Temperature; (E) Aeration speed; (F) Incubator time.

Effect of aeration speed

The effect of aeration on ammonium removal was studied by varying the aeration speed of the jars containing 300 mL of the wastewater with initial ammonium of 150 mg/L. The jars were inoculated with the strain and incubated at 37 ºC at different aeration speeds 0.5 L/min, 1 L/min, 1.5 L/min and 2 L/min. The results (Fig. 4E) showed that, with increase in agitation speed, there was significant change in ammonium removal observed till 1.5 L/min. A maximum of 85 % ammonium elimation was achieved in the culture incubated at 1.5 L/min. It can be observed from the Fig. 4E that, at aeration speed of 2 l/min, the ammonium removal is less which may be due to rupture of the cells at higher agitation.

Effect of incubator time

Time-course data for the consumption of ammonium for the HUST-C8 strain is shown in Fig. 4F. The strain rapidly removed ammonium from diluted wastewater. Under aerobic conditions, after 10 h, the maximum ammonium removal was achieved 88 %, higher than the yield of 61.4 % at 20 °C (and 42 % at 35 °C) the isolate *Acinetobacter* sp. Y16 that Huang et al. reported in their study [3]. However, Fig 4F also show that ammonium still remained 18 mg/L. In addition, nitrate and nitrite appeared at 12 h in water at concentrations of 1.5 and 0.4 mg/L respectively. This event may be caused by the death of organisms in reactor because of an exhautation of substrates. The incubator time acts as the main control parameter for the efficience treatment of wastewater in bath reactor.

4. CONCLUSION

A novel *Acinetobacter calcoaceticus* HUST-C8 was isolated from local industrial wastewater. It exhibited efficient ammonium removal ability which yielded 88 % of initial 150 mg/L of ammonium after 10 hours at 37 °C, pH 8.5 with inoculator of 7 % and aeration speed of 4.5 v/v/min. The simple operation, efficiency and strong adaptability of strain HUST-C8 to raw wastewater of the industrial fertilizer plant made it a prospective candidate for aerobic nitrogen-rich wastewater treatment. However, further studies on carbon sources, continous feed of wastewater, aerobic metabolic pathway, sluge formation, and the role of the strain in microbial ecology should be done.

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