

Selection Enhanced Nutrient Removal from Pig Farm Wastewater Using Co-Immobilized *Chlorella vulgaris* and *Azospirillum brasilense* Azo09 in Alginate Beads

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ABSTRACT

The livestock industry in Vietnam significantly contributes to agricultural production but also generates severe environmental pollution, particularly from untreated wastewater. Pig farm effluents contain high concentrations of nitrogen (N) and phosphorus (P), far exceeding regulatory limits. This study investigates an innovative wastewater treatment approach using co-immobilized *Chlorella vulgaris* microalgae and *Azospirillum brasilense* bacteria in alginate beads. The combination aims to enhance the removal of ammonium (N-NH_4^+), total nitrogen (TN), phosphate (P-PO_4^{3-}), and total phosphorus (TP), while also facilitating easier biomass recovery. Experimental wastewater, diluted tenfold, exhibited high pollutant concentrations: TN (540.7 mg/L), TP (185.7 mg/L), N-NH_4^+ (157.5 mg/L), P-PO_4^{3-} (19.3 mg/L), and BOD5 (189.4 mg/L). Over 12 days, different treatment setups were evaluated, with the most effective being the co-immobilized microalgae-bacteria system (VTVK treatment). Results showed significant reductions: N-NH_4^+ decreased by 93.8% (to 9.71 mg/L), TN by 85.9% (to 76.24 mg/L), P-PO_4^{3-} by 77.7%, and TP by 66%. These removal rates exceeded those of treatments using only *C. vulgaris* immobilized in alginate beads, confirming the beneficial role of *A. brasilense* in stimulating microalgal growth and nutrient assimilation. This bioremediation method effectively reduces nutrient loads in pig farm wastewater, meeting Vietnam's national discharge standards (QCVN 01-79:2011/BNNPTNT and QCVN 62-MT:2016/BTNMT). The study highlights the potential of microalgae-bacteria co-immobilization as a sustainable and scalable solution for livestock wastewater treatment, offering improved efficiency and simplified biomass recovery.

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1. INTRODUCTION

Livestock production contributes over 24.6% to Vietnam's agricultural sector. Despite its economic benefits, the livestock industry significantly contributes to environmental pollution, posing risks to human health and damaging ecosystems due to untreated or inadequately treated wastewater that fails to meet discharge standards. One of the most concerning sources of pollution is wastewater from pig farms, which contains high concentrations of solid waste, nutrients (mainly nitrogen and phosphorus),

and pathogenic microorganisms, often exceeding permitted standards (Nguyen, 2017).

Effectively treating the large volume of wastewater generated by the livestock industry is an urgent environmental priority. In recent years, microbial-based treatment methods have gained increasing attention due to their sustainability. These methods utilize microorganisms to metabolize and transform complex organic compounds into simpler, less harmful forms (Le & Luong, 2009). Among these, microalgae-based treatment is particularly promising due to its ability to photosynthesize, convert



solar energy into biomass, and absorb nutrients such as nitrogen and phosphorus for growth.

Among various microalgae species, *Chlorella vulgaris* (*C. vulgaris*) is widely studied for wastewater treatment. However, one limitation of algal-based treatment is the difficulty in recovering microalgal biomass, which can cause secondary pollution (Abdel-Raouf *et al.*, 2012). Several methods exist for harvesting micro-algal biomass, such as coagulation-flocculation and flotation, but these techniques often require high investment costs and specialized knowledge.

An innovative approach involves co-immobilizing *C. vulgaris* microalgae with *Azospirillum* sp. bacteria in alginate beads. This method enhances microalgal growth through phytohormones secreted by *Azospirillum* sp., improving nutrient removal efficiency (Bashan & Levanyov, 1990; Dao *et al.*, 2018; Han *et al.*, 2018; Peng *et al.*, 2020; Raffi & Charyulu, 2021; Zhang *et al.*, 2021). Furthermore, immobilizing both microorganisms in alginate beads facilitates easier biomass recovery, making the treatment process more practical and efficient.

2. MATERIALS AND METHODS

2.1. Subjects of Study

Alginate beads, *A. brasilense* Azo09 bacteria and *C. vulgaris* microalgae. Livestock wastewater collected from several pig farms in Hoa Bac, Hoa Vang, Da Nang, Vietnam.

2.2. Research Methodology

2.2.1. Determination of Ammonium, $N-NH_4^+$

Indophenol is an organic compound with the formula $OC_6H_4NC_6H_4OH$. It is deep blue dye that is the product of the Berthelot's reaction, a common test for ammonia. Indophenol is formed from phenol and hypochlorite in the presence of NH_4^+ ion and ammonia. The reaction of ammonium with hypochlorite forms blue amine monochloride in the presence of phenol catalyzed by nitroprusside ion. The absorbance of solution is measured at 606 nm.

2.2.2. Determination of $P-PO_4^{3-}$

Ammonium pentamolybdate and potassium antimony tartrate react with orthophosphate in an acidic medium to form an antimony phosphomolybdate complex. This complex is reduced by ascorbic acid to form a dark blue molybdenum complex. The intensity of the color is proportional to the concentration of phosphate in the sample. The absorbance is measured at 880 nm.

2.2.3. Determination of Total Nitrogen (TN)

Devarda alloy is used for the reduction of nitrogen compounds to ammonium. After evaporation, the nitrogen is converted to ammonium sulfate in the presence of concentrated sulfuric acid containing high concentrations of potassium sulfate to raise the boiling point of the mixture, and copper is present as a catalyst. The ammonia is liberated from the mixture by adding alkali and distilled into boric acid solution. The amount of ammonium in

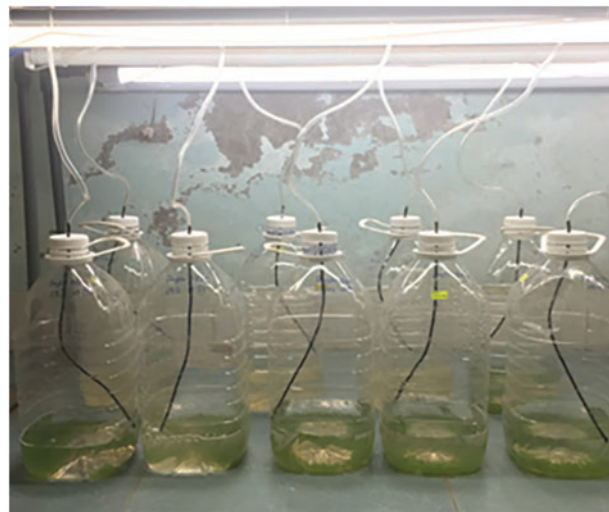


Fig. 1. Establishment of wastewater treatment model at the experimental house of the Faculty of Biology, Agriculture and Environmental Science, University of Science and Education, The University of Danang.

the distillate is determined by titration with acid or by spectrophotometry at 655 nm.

2.2.4. Determination of Total Phosphorus (TP)

Ammonium molybdate and potassium antimony tartrate react with PO_4^{3-} in a moderately acidic medium to form phosphomolybdic acid, which is dark blue in color as measured at 880 nm on the spectrophotometry.

2.2.5. Determination of BOD_5

Wastewater samples in the treatments were incubated in the dark at 20°C for 5 days, in a filled and sealed vessel. The DO content was determined before and after incubation. The BOD_5 consumed for 1 liter of sample was calculated.

2.2.6. Experimental Arrangement

Wastewater collected from pig farms was filtered, settled for 3–5 days, then the input content of $N-NH_4^+$, $P-PO_4^{3-}$, TN, TP and BOD_5 was determined. The efficiency of treating nitrogen and phosphorus compounds in unsterilized livestock wastewater by adding alginate beads to immobilize bacteria and microalgae in the following treatments (Fig. 1):

- Control treatment (K): 100% wastewater without alginate beads added.
- VT: adding *C. vulgaris* microalgae immobilized with alginate beads to wastewater at a ratio of 1:9 (v/v).
- VTK: adding *A. brasilense* Azo09 bacteria and *C. vulgaris* microalgae immobilized with alginate beads to wastewater at a ratio of 1:9 (v/v).

The treatments had a ratio of N:P were 10:1 that stabled by fixing phosphorus concentration and adjusting nitrogen concentration with NH_4Cl , pH = 6.5 and alginate bead diameter of 6 mm (Rhee & Gotham, 1980). The experiments conditions were light 1000 lux, 30°C $\pm$ 2°C, diurnal cycle is 16:8 and aeration. The output contents of BOD_5 , TN, TP, $N-NH_4^+$ and $P-PO_4^{3-}$ were analyzed after 12 days of treatment.

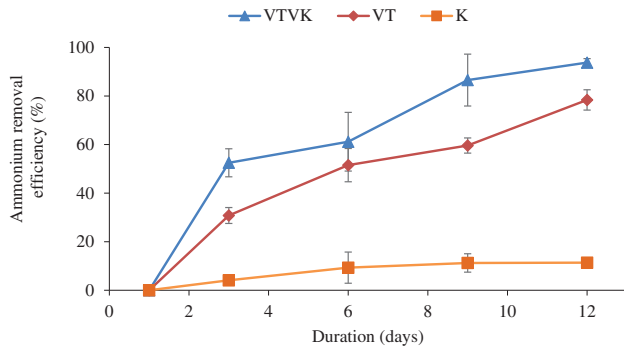


Fig. 2. Ammonium removal efficiency after 12 days of treatment. VTKV: *C. vulgaris* and *A. brasilense* Azo09 co-immobilized with alginate beads, VT: only *C. vulgaris* immobilized with alginate beads; K: only diluted pig wastewater.

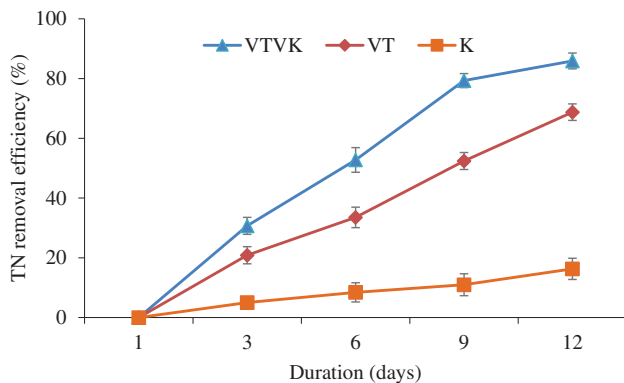


Fig. 3. Total nitrogen removal efficiency after 12 days of treatment. VTKV: *C. vulgaris* and *A. brasilense* Azo09 co-immobilized with alginate beads, VT: only *C. vulgaris* immobilized with alginate beads; K: only diluted pig wastewater.

2.3. Data Analysis

The collected data were analyzed by descriptive statistics using MS - Excel 2010. The differences between experimental groups were determined by ANOVA analysis of variance and Tukey's Test post hoc analysis.

2.4. Methods for Isolation and Preliminary Identification of *Bacillus* Bacteria

Put 10 ml of aquatic wastewater in a triangle jar, add 90 mL of physiological saline (NaCl 9‰), and heat in a thermostatic tank at 80°C for 10 minutes. Then, dilute the sample at 10^{-1} to 10^{-6} concentrations. Aspirate 100 μ L of sample in small diluted concentrations onto a jelly disc containing LB medium and incubated at 30°C. After two days, when colonies appear, colonies are selected that are loose and differ in color, shape, and size. Isolated *Bacillus* bacteria are preliminarily identified by physiological and biochemical characteristics of *Bacillus* bacteria.

3. RESULTS AND DISCUSSION

3.1. Characteristics of Diluted Pig Farm Wastewater

Pig farm wastewater was filtered to remove sediment and suspended solids before being diluted tenfold with distilled water. The analysis showed the following concentrations: total nitrogen (TN) 540.7 mg/L, total phosphorus (TP)

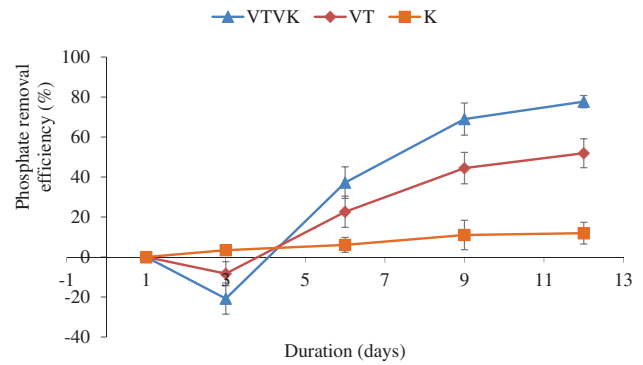


Fig. 4. Phosphate removal efficiency after 12 days of treatment. VTKV: *C. vulgaris* and *A. brasilense* Azo09 co-immobilized with alginate beads, VT: only *C. vulgaris* immobilized with alginate beads; K: only diluted pig wastewater.

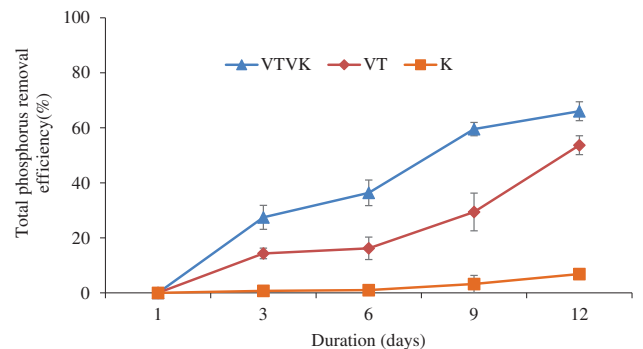


Fig. 5. Total phosphorus removal efficiency after 12 days of treatment. VTKV: *C. vulgaris* and *A. brasilense* Azo09 co-immobilized with alginate beads, VT: only *C. vulgaris* immobilized with alginate beads; K: only diluted pig wastewater.

185.7 mg/L, ammonium (N-NH_4^+) 157.5 mg/L, phosphate (P-PO_4^{3-}) 19.3 mg/L, and biological oxygen demand (BOD5) 189.4 mg/L.

Compared to the National Technical Regulation on Livestock Wastewater (QCVN 01-79:2011/BNNPTNT), the wastewater in this study was highly polluted, with N-NH_4^+ , TN, and TP levels exceeding permitted limits by 15.7, 18, and 30.9 times, respectively. Similarly, based on the National Technical Regulation on Livestock Wastewater (QCVN 62-MT:2016/BTNMT), TN levels exceeded the permissible limit by 3.6 times. Given its high nutrient content, this wastewater is suitable for treatment using the co-immobilization method with alginate beads.

3.2. Ammonium (N-NH_4^+) Removal Efficiency

Nitrogen in pig farm wastewater is primarily in the form of ammonium (N-NH_4^+). After 12 days of treatment, N-NH_4^+ levels decreased in all experimental conditions. The control treatment (K) achieved only an 11.1% removal efficiency, likely due to the natural activity of microorganisms present in the wastewater (Fig. 2).

The highest removal efficiency was observed in the VTKV treatment (wastewater supplemented with *C. vulgaris* microalgae and *A. brasilense* Azo09 bacteria co-immobilized in alginate beads), achieving a 93.8% reduction, reducing N-NH_4^+ content to 9.71 mg/L. In contrast, the VT treatment (only *C. vulgaris* immobilized in alginate beads) achieved a removal efficiency of 77.8%, leaving an N-NH_4^+ concentration of 34.1 mg/L. These

TABLE I: CHARACTERISTICS OF PIG WASTEWATER IN VTVK TREATMENT AFTER 12 DAYS

Parameters	Unit	Contents		QCVN 01-79:2011/BNNPTNT		QCVN 62-MT:2016/BTNMT	
		Input	Output	Column B	Column A	Column B	
N-NH ₄ ⁺	mg/L	157.5	9.84 ± 2.583	10	—	—	
TN	mg/L	540.7	76.24 ± 14.33	30	50	150	
TP	mg/L	185.7	58.57 ± 0.018	—	—	—	
P-PO ₄ ³⁻	mg/L	19.3	4.3 ± 0.585	6	—	—	
BOD ₅	mg/L	214.4	67.345 ± 3.78	50	40	100	

Note: QCVN 01-79:2011/BNNPTNT: Cattle and poultry farm—Hygiene inspection and evaluation procedure. QCVN 62-MT:2016/BTNMT: National technical regulation on the effluent of livestock.

results align with previous studies, such as [De-Bashan et al. \(2002\)](#), which reported that *C. vulgaris* combined with *A. brasilense* could remove 100% of NH₄⁺, while *C. vulgaris* alone achieved only 75% efficiency; [González Luz Estela et al. \(1997\)](#) reported that *C. vulgaris* microalgae has the ability to absorb 95% of NH₄⁺ in domestic wastewater ([González Luz Estela et al., 1997](#)).

3.3. Total Nitrogen (TN) Removal Efficiency

Pig farm wastewater contains substantial amounts of nitrogen, making removal by conventional methods challenging ([Nguyen, 2016](#)). After 12 days, the TN concentration in the control treatment (K) remained at 452.56 mg/L ± 19.18 mg/L, corresponding to a removal efficiency of only 16.3%. The VT treatment (wastewater supplemented with *C. vulgaris* immobilized in alginate beads) achieved a TN removal efficiency of 68.75%, 4.2 times higher than the control ([Fig. 3](#)).

The VTVK treatment exhibited the highest TN removal efficiency, reaching 85.9% and reducing TN levels to 76.24 mg/L ± 14.33 mg/L. The treated wastewater met the discharge standards specified in QCVN 62-MT:2016/BTNMT. These findings highlight the crucial role of *A. brasilense* Azo09 in stimulating *C. vulgaris* growth and facilitating organic nitrogen transformation into inorganic forms, making it more readily absorbed by microalgae.

[Fallowfield and Garrett \(1985\)](#) reported that TN content decreased by 54%–98% after five days of treating diluted sludge from pig farming with *C. vulgaris* microalgae ([Fallowfield & Garrett, 1985](#)); [Aziz et al. \(1992\)](#) showed that TN content decreased by 60%–70% after 15 days of treating waste and wastewater from industrial pig farms with *C. pyrenoidosa* microalgae ([Aziz et al., 1992](#)); [Vo Thi et al. \(2012\)](#) announced that TN content in diluted livestock wastewater decreased by 87.4%–90.18% after nine days of treatment with *C. vulgaris* microalgae ([Vo Thi et al., 2012](#)).

3.4. Phosphate (P-PO₄³⁻) Removal Efficiency

In the control treatment (K), phosphate (P-PO₄³⁻) levels decreased slightly, achieving an 11.9% removal efficiency. In contrast, the VT and VTVK treatments initially showed an increase in P-PO₄³⁻ concentrations within the first three days, followed by a significant reduction. The VTVK treatment demonstrated the highest efficiency, achieving a 77.7% removal rate after 12 days, while the VT treatment achieved only 51.9% ([Fig. 4](#)). This underscores the enhanced role of *A. brasilense* Azo09 in promoting *C. vulgaris* growth and increasing phosphorus uptake.

In a previous publication by [De-Bashan et al. \(2002\)](#) the removal efficiency of P-PO₄³⁻ reached 80% after 6 days in the treatment of artificial wastewater prepared in the laboratory using alginate beads co-immobilized with *A. brasilense* bacteria and *C. vulgaris* microalgae. However, the artificial wastewater in that experiment did not contain microorganisms and the phosphorus level was lower, so the treatment time was shorter ([De-Bashan et al., 2002](#)).

3.5. Total Phosphorus (TP) Removal Efficiency

TP removal efficiency improved across all treatments after 12 days, though the control (K) exhibited only a 6.86% reduction, bringing TP levels to 170.84 mg/L ± 4.52 mg/L. The VTVK treatment achieved the highest TP removal efficiency (66%), reducing TP levels to 61.57 mg/L ± 5.21 mg/L, surpassing the VT treatment ([Fig. 5](#)). The enhanced removal was attributed to *A. brasilense* Azo09, which aids in converting organic phosphorus into an inorganic form more easily absorbed by microalgae.

Previous studies, such as [Hernandez et al. \(2006\)](#), reported TP reductions of 42%–98% in pig farm wastewater treated with *C. vulgaris*, while [Malick and Rai \(1994\)](#) documented 50%–60% TP removal using *C. pyrenoidosa*. Our findings confirm that co-immobilizing *A. brasilense* and *C. vulgaris* in alginate beads enhances phosphorus removal efficiency compared to microalgae alone.

This finding was quite different from the results previously published by [Hernandez et al. \(2006\)](#), TP content in pig wastewater decreased by 42%–98% after four-five days when treated with *C. vulgaris* ([Hernandez et al., 2006](#)); [Malick and Rai \(1994\)](#) announced that TP content in wastewater from industrial pig farms decreased by 50%–60% after 15 days when treated with *C. pyrenoidosa* ([Malick & Rai, 1994](#)).

4. CONCLUSION

The results indicate that co-immobilizing *C. vulgaris* and *A. brasilense* in alginate beads significantly enhances nitrogen and phosphorus removal from pig farm wastewater. The treated wastewater from the VTVK treatment met Vietnam's national discharge standards (QCVN 01-79:2011/BNNPTNT and QCVN 62-MT:2016/BTNMT), confirming the effectiveness of this method ([Table I](#)). This study highlights the potential of microalgae-bacteria co-immobilization as a sustainable and scalable wastewater treatment solution, improving nutrient removal efficiency and simplifying biomass recovery.

CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest.

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