Application of salt-tolerant bacteria to treat wastewater from agar powder production

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ABSTRACT

The wastewater generated from the agar powder production process using seaweed as raw material is classified as saline wastewater, with NaCl concentrations ranging from 10 to 30 g/L and containing high starch content. Methods for treating saline wastewater using halophilic microorganisms are being studied. In this study, two saline bacterial strains, Bacillus sp. N9 and Enterococcus sp. N5, which have high starch degradation ability, were used to treat saline wastewater. Preliminary results showed that both strains were capable of starch degradation at 30 °C, pH 6, with the same initial concentration of 2%. Under these conditions, the combined use of two reactors and activated sludge to treat synthetic saline wastewater (initial COD: 960 mg/L; NH_4^+-N: 49.34 mg/L; PO_4^{3-}-P: 25.64 mg/L) achieved removal efficiencies of 90%, 80%, and 81% after 12 hours for COD, NH_4^+-N, and PO_4^{3-}-P, respectively. Using aeration to treat wastewater from an agar producer resulted in removal efficiencies of 71%, 75%, and 67% after 24 hours for COD, NH_4^+-N, and PO_4^{3-}-P, respectively.

Keywords: Agar; Starch; Salt-tolerant; Wastewater.

1. INTRODUCTION

Saline wastewater, or high-salinity wastewater, comprises various types such as domestic wastewater, livestock wastewater, and industrial effluents. These types of saline wastewater generally exhibit high concentrations of pollutants, including COD, total nitrogen (N), and total phosphorus (P), with NaCl concentrations reaching up to 10÷30 g/L [1]. In saline environments, common microorganisms tend to die due to osmotic pressure. Therefore, conventional biological treatment systems are often ineffective or have very low efficiency in removing organic pollutants from saline wastewater environments [1].

According to the report from the Vietnamese Agar Production Association, Vietnam's agar output reaches approximately 1,000 tons per year, with about 10 production facilities, most of which are located in Hai Phong. Wastewater generated from agar powder production plants, which use seaweed as a raw material, is classified as saline wastewater due to the marine origin of the seaweed. Field surveys reveal that although these facilities have installed wastewater treatment systems, the effluents from most plants still contain very high levels of COD (approximately 250÷300 mg/L), ammonium (around 30÷40 mg/L), and phosphate (about 30÷50 mg/L), which do not meet discharge standards according to QCVN 11-MT:2015 – National Technical Regulation on Aquatic Product Wastewater [2]. Consequently, this wastewater poses a significant environmental burden and threatens human health.

The primary cause of this issue lies in the high salinity of the industrial wastewater from agar powder production (NaCl concentration ≥ 10 g/L) affects microorganisms in activated sludge systems by increasing osmotic pressure, causing enzyme inactivation, and disrupting microbial growth and metabolism, which ultimately reduces pollutant removal efficiency, while the wastewater treatment systems currently employed by these facilities are still based on conventional biological processes [3]. In coastal seafood factories, where freshwater is limited, seawater is often used during processing, resulting in wastewater with salinity levels comparable to seawater (10÷30 g/L NaCl). Overall, wastewater salinity in various industries can range from as low as 0.2% to as high as 15% NaCl. For example, aquaculture wastewater typically ranges from 3.10% to 3.34%, pharmaceutical wastewater contains around 2.6% salt, and pickled vegetable processing can produce effluents with up to 15% salinity [4]. Additionally, domestic wastewater may also exhibit variable salinity depending on factors such as living standards, water usage habits, and the wastewater collection system. The results of wastewater composition analysis in this study show that: Chemical Oxygen Demand (COD): 768 mg/L; Ammonium (NH_4^+): 95,26 mg/L; Phosphate (PO_4^{3-}): 6,81 mg/L and Sodium Chloride (NaCl): 22000 mg/L (equivalent to 2,2%).

The study by Le Thanh Hien et al. successfully demonstrated the use of lightweight gravel as a carrier material for immobilizing halophilic microorganisms with biological activity in the treatment of shrimp-farming wastewater in Do Son (Hai Phong). The results indicated significant pollutant reduction, with COD decreasing by 1.3 times, BOD by approximately 3 times, and NH_4^+ by about 3.4 times compared with initial concentrations [5].

At present, various halotolerant microbial products have also been introduced in the Vietnamese market for the treatment of high-salinity wastewater. This product contains specially selected microbial strains possessing active enzyme systems and surface-active compounds, thereby enhancing treatment efficiency. It has been reported to effectively reduce organic pollutants (BOD, COD), mitigate odor problems, suppress algal biomass growth, and address other operational challenges commonly encountered in saline water environments with salinity ranging from 1–3%.

2. MATERIALS AND METHODS

2.1. Materials

Microbial strains: Two halotolerant strains, *Bacillus* sp. N9 and *Enterococcus* sp. N5, capable of degrading starch and cellulose, were obtained from the microbial collection of the Institute of Materials, Biology, and Environment. These strains were isolated from the Truong Sa Islands, identified, and registered in GenBank with accession numbers PQ219502 and PQ219504, respectively. The strains were preserved in 20% glycerol at - 20 °C. Before use, they were inoculated onto slant agar and activated by overnight cultivation in liquid medium to serve as an inoculum for subsequent studies.

Activated sludge samples were collected from the area of Lang Station, Hanoi (X: 21º00'26.1''N, Y:105º48'54.7''E).

Synthetic wastewater: Prepared to simulate the pollution levels (COD, ammonium, and phosphate) of actual wastewater from an agar powder processing plant. The synthetic wastewater contained 3% NaCl, COD = 960 mg/L, total nitrogen (N) = 49.34 mg/L, and total phosphorus (P) = 25.64 mg/L. The actual wastewater at Viet Xo Vegetables and Fruits Joint Stock Company, located in An Trang Industrial Zone, Hai Phong City, contains 2.2% NaCl, with COD = 768 mg/L, Ammonium (NH_4^+) = 95.26 mg/L, and Phosphate (PO_4^{3-}) = 6.81 mg/L

2.2. Methods

2.2.1. Selection of culture conditions affecting starch degradation by the two strains

- Effect of temperature: Cultures were incubated at different temperatures (25, 30, 37, and 42 °C) in mineral medium containing 1% starch and 0.5% peptone at pH 7 for 48 h. After incubation, cultures were centrifuged to collect the supernatant for residual starch analysis.

- Effect of pH: Cultures were inoculated at 2% (v/v) in mineral medium containing 1% starch and 0.5% peptone, adjusted to initial pH values (4, 5, 6, 7, 8, and 9), and incubated at 30 °C for 48 h. After incubation, the supernatant was collected for residual starch analysis.

- Effect of inoculum size: Cultures were inoculated at different inoculum sizes (0.3, 0.5, 1.0, 1.2, 1.5, 2.0, and 2.5% v/v) in mineral medium containing 1% starch and 0.5% peptone at pH 7 and incubated at 30 °C for 48 h. The supernatant was collected for residual starch analysis.

- Starch degradation analysis: by the phenol–sulfuric acid (phenol–H_2SO_4) colorimetric method, following the protocol as described by Nguyen Van Mui [7]. In this method, starch in the reaction mixture is hydrolyzed under acidic conditions to yield monosaccharides, which react with phenol and concentrated sulfuric acid to produce a yellow-orange chromogen. The intensity of the resulting color is measured spectrophotometrically, typically at 490 nm and is proportional to the carbohydrate (starch) concentration remaining in the sample [7].

2.2.2. Evaluation of pollutant degradation in synthetic wastewater

2.2.2.1. Pollutant degradation with activated sludge and without salt-tolerant bacterial addition

Raw sludge was pretreated by sieving and settling, then mixed activated sludge 10% (50 mL) with 500 mL of synthetic saline wastewater as above and cultivated in a 1-L aerated reactor under aerobic conditions (DO > 2 mg/L, 28 °C, pH 6.5÷7.5) for 7 days. Removal efficiencies of COD, NH_4^+, and PO_4^{3-} were evaluated at 4, 8, and 12 hours.

2.2.2.2. Pollutant degradation with activated sludge with single bacterial inoculation

To assess the performance of each strain, 10% activated sludge in 500 mL of synthetic wastewater was combined with individual bacterial strains at 2% (v/v). Samples were aerated for 8 hours, and key pollutant parameters were measured post-treatment.

2.2.2.3. Pollutant degradation with activated sludge with combined bacterial inoculation

For dual-strain trials, 50 mL of activated sludge was added to 500 mL of synthetic saline wastewater, with bacterial inoculum concentrations ranging from 2% (v/v). Samples were aerated continuously, and pollutant concentrations (COD, NH_4^+, PO_4^{3-}) were monitored at 4, 8, and 12-hour intervals. This setup aimed to evaluate the synergistic effect of both strains under saline conditions.

2.2.3. Analytical methods

COD content was analyzed according to SMEWW 5220C: 2023, using COD VELP Heater Sample Digester. Model: ECO 8 Manufactured in Italy. NH_4^+ content was analyzed on UV-2401PC Spectrophotometer Manufactured in Japan, are analyzed by Colorimetric comparison using Nessler reagent. PO_4^{3-} content are analyzed by TCVN 6202:2008 using UV-2401PC Spectrophotometer Manufactured in Japan.

3. RESULTS AND DISCUSSION

3.1. Affecting factors in culturing two salt-tolerant bacteria strains

3.1.1. Incubation temperature

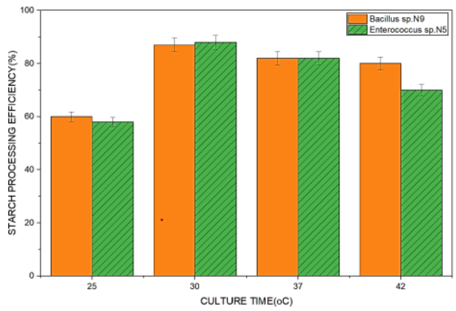

Figure 1. *Starch processing efficiency at different temperatures.*

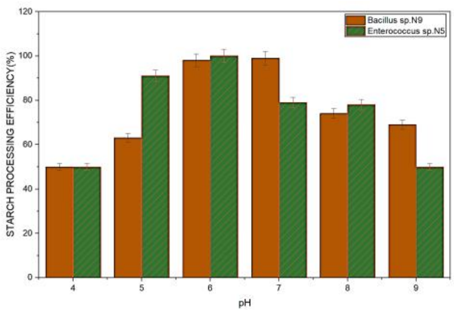

Figure 2. *Starch processing efficiency from the initial to the pH concentration.*

A 1% (v/v) inoculum of *Bacillus* sp. N9 and *Enterococcus* sp. N5 was cultured in liquid medium at 25, 30, 37, and 42 °C for 48 h, with gentle shaking after 24 h. Starch removal efficiencies for *Bacillus* sp. N9 were 60%, 87%, 82%, and 80%, and for *Enterococcus* sp. N5 were 58%, 88%, 82%, and 72%, respectively (figure 1). Both strains exhibited optimal performance at 30÷37 °C (82÷88%), reflecting this range's suitability for microbial growth and amylase activity. Lower temperatures slowed metabolism, while higher temperatures risked enzyme denaturation, particularly in *Enterococcus* sp. N5.

3.1.2. Effect of initial pH

A 1% (v/v) inoculum of *Bacillus* sp. N9 and *Enterococcus* sp. N5 was introduced into liquid media adjusted to pH 4 ÷ 9 and incubated at 30 °C for 48 h. After 24 h, cultures were gently shaken to enhance oxygen exchange. Starch removal efficiencies for *Bacillus* sp. N9 were 50%, 63%, 93%, 98%, 74%, and 69% at pH 4÷9, respectively, while *Enterococcus* sp. N5 achieved 50%, 91%, 97%, 79%, 78%, and 50%. Optimal pH ranges were 6 ÷ 7 for *Bacillus* sp. N9 and 5÷6 for *Enterococcus* sp. N5 (figure 2). These differences reflect physiological adaptations: *Bacillus* sp. N9 grows best and secretes stable amylase in neutral to mildly alkaline conditions, whereas *Enterococcus* sp. N5, a lactic acid bacterium, maintains high enzyme activity under mildly acidic conditions but loses activity when pH exceeds its tolerance range.

3.1.3. Bacterial concentration

The bacterial suspensions of *Bacillus* sp. N9 and *Enterococcus* sp. N5 were prepared according to the volumes specified, under conditions of pH 6 and a constant incubation temperature of 30°C. The cultivation process was carried out over a period of 2 days; after 24 hours, the culture media were shaken for 5 minutes to ensure appropriate aerodynamic conditions.

The effect of bacterial concentration on starch degradation efficiency is illustrated in Figure 3. The results indicate a significant difference in degradation performance between the two bacterial strains at varying concentrations. Specifically, the average starch removal efficiency of both *Bacillus* sp. N9 and *Enterococcus* sp. N5 increased progressively with increasing bacterial concentration, reaching the highest values at 2% inoculation.

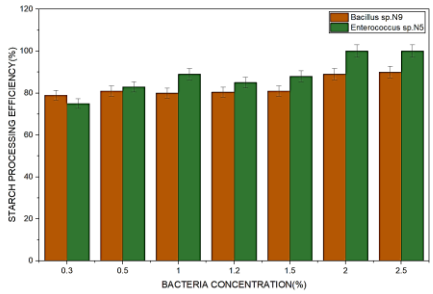

Figure 3. *Starch treatment efficiency as a percentage of bacterial concentration.*

3.2. Treatment efficiencies of synthesized wastewater

3.2.1. Sludge without bacteria added

Following the aeration of activated sludge (figure 4), the obtained sludge showed limited treatment efficiency without the addition of halotolerant bacteria. Removal rates for COD, NH_4^+, and PO_4^{3-} were only 6%, 9%, and 4%, respectively. This slight decrease suggests residual microbial activity, possibly from native aerobic bacteria partially adapting to salinity, along with pollutant adsorption onto sludge flocs. However, the high salinity and organic load quickly inhibited non-halotolerant microbes, resulting in poor overall performance. These results highlight the need to introduce halotolerant bacterial strains to improve saline wastewater treatment.

3.2.2. Sludge with each bacteria added

Treatment efficiency of synthetic wastewater by *Enterococcus* sp. N5: *Enterococcus* sp. N5 was evaluated under the same treatment conditions: 500 mL of synthetic wastewater with 3% salinity, aerated continuously for 8 hours. The results were as follows: COD, NH_4^+ and PO_4^{3-} decreased 61%, 39% and: 67% respectively.

Treatment efficiency of synthetic wastewater by *Bacillus* sp. N9: The experiment was conducted using 500 mL of synthetic wastewater with 3% salinity, employing *Bacillus* sp. N9 as the biological agent. Continuous aeration was applied for 8 hours to maintain aerobic conditions. The post-treatment analysis revealed the following removal efficiencies for key pollutants: COD: 65% and NH_4^+: 80%, PO_4^{3-}: 33% (figure 4).

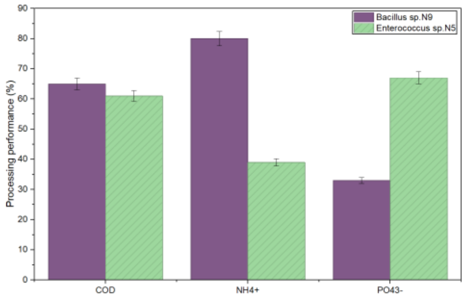

Figure 4. *COD, NH_4^+ and PO_4^{3-} removal efficiency of two bacterial strains.*

3.2.3. Sludge with two-strain bacteria adding

a. COD parameter

The initial chemical oxygen demand (COD) concentration in the synthetic wastewater was set at 960 mg/L to simulate the typical level of organic pollution in starch-rich effluents. The COD removal results under various tested conditions are illustrated in figure 5, covering different bacterial concentrations and aeration durations of 4, 8, and 12 hours. The COD removal efficiency increased progressively with bacterial concentration, especially between 0.3% and 1.5%. At a concentration of 1.5%, optimal removal efficiency was achieved, ranging from approximately 70% after 4 hours of aeration to nearly 90% after 12 hours. This indicates that an appropriate bacterial density combined with extended aeration time facilitates the biological oxidation of organic compounds in the wastewater. At 2%, COD removal remained high, suggesting the stability of the microbial system under these conditions.

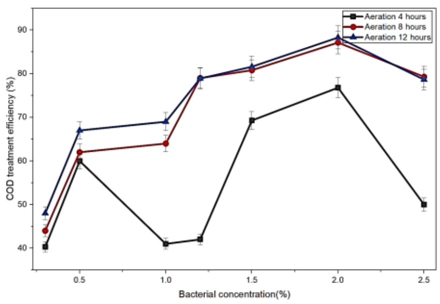

Figure 5. *Average COD treatment efficiency in 4, 8, 12 hours.*

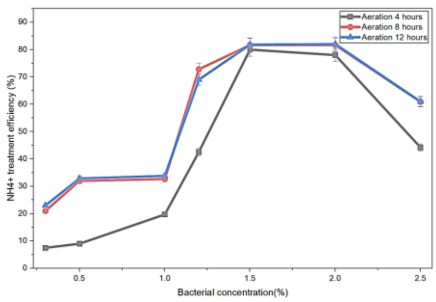

Figure 6. *Average NH_4^+ treatment efficiency in 4, 8, 12 hours.*

b. NH_4^+ parameter

The initial concentration of ammonium ions (NH_4^+) in the synthetic wastewater was 49,34 mg/L. The efficiency of NH_4^+ removal through aerobic treatment using halotolerant bacteria at varying bacterial concentrations is presented in figure 6. The results indicate that NH_4^+ removal efficiency ranged from 7.5% to 81.83%, depending on the bacterial concentration and the duration of aeration. As the bacterial concentration increased from 0.3% to 2%, NH_4^+ removal efficiency showed a marked improvement. At concentrations of 1.5% and 2%, combined with 8-hour and 12-hour aeration, the removal efficiency reached optimal levels, exceeding 80%. This suggests that ammonium oxidation occurred effectively under aerobic conditions, supported by sufficient cell density and prolonged contact time.

c. PO_4^{3-} parameter

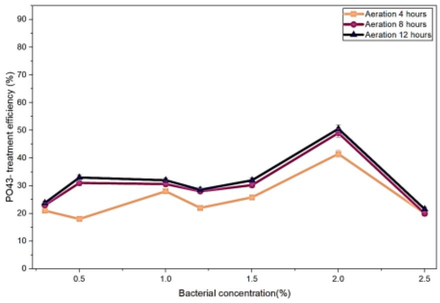

Figure 7. *Average PO_4^{3-} treatment efficiency in 4, 8, 12 hours.*

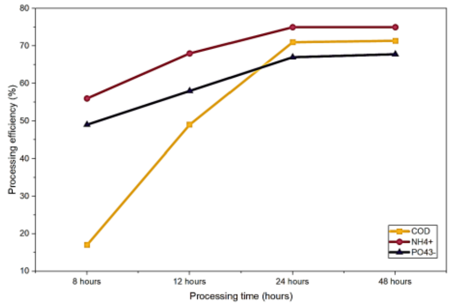

Figure 8. *Wastewater treatment efficiency of the Agar powder factory.*

With an initial concentration of phosphate ions (PO_4^{3-}) at 25.64 mg/L in the synthetic wastewater, the efficiency of phosphate removal via aerobic treatment using halotolerant bacteria is presented in figure 7. The results show that the PO_4^{3-} removal efficiency ranged from 7.5% to 81.83%, depending on the bacterial concentration and the aeration duration.

The data indicate that increasing the bacterial concentration from 0.3% to 1.5% led to a progressive improvement in phosphate removal, particularly notable at 8-hour and 12-hour aeration intervals. The combination of 2% bacterial concentration with 12 hours of aeration yielded the highest removal efficiency, exceeding 81%, suggesting that this represents the optimal condition within the tested range.

3.3. Effectiveness of wastewater treatment for Agar powder production

The wastewater treatment efficiency of the Agar powder factory with a salt concentration of 2.2% is shown in figure 8. At the 8-hour treatment mark, COD removal efficiency was only 17%, while NH_4^+ and PO_4^{3-} achieved 56% and 49%, respectively. Although microbial activity had initiated, the short contact time and high salinity conditions likely hindered the complete biological oxidation of organic compounds, resulting in relatively low COD removal. When the treatment time was extended to 12 hours, removal efficiency improved significantly: COD reached 49%, NH_4^+ reached 68%, and PO_4^{3-} reached 58%. These findings suggest that the microbial community had gradually adapted to the saline environment, and the halotolerant strains began to effectively degrade organic matter and nitrogen- and phosphorus-containing compounds.

At 24 hours, the removal efficiency peaked across all three parameters: COD reached 71%, NH_4^+ achieved 75% and PO_4^{3-} achieved 67%. This marks the phase where the microbial system was most active, and aerobic biological processes were stable and efficient. In addition, extracellular enzymes responsible for the degradation of high-molecular-weight organic compounds were likely highly active, contributing to enhanced treatment performance. During the experiments with simulated wastewater, the results indicated that a treatment time of 12 hours achieved the highest pollutant removal efficiency. However, for actual wastewater from agar powder production plants, due to the influence of more complex factors in terms of composition and characteristics, the required treatment time tends to be longer. In comparison, the study by Motoki Kubo (2000) investigated two halotolerant bacterial strains, *Staphylococcus* sp. and *Bacillus cereus,* isolated from soil, for treating high-salinity wastewater from pickled plum production [8]. Both strains exhibited good growth at NaCl concentrations from 0 to 15%. Under aerobic cultivation for 72 hours, COD removal efficiency reached 70% in laboratory settings and up to 90% at pilot-scale (1 m³ reactor volume) [8]. Similarly, Xiang Wu et al. (2013) applied *Bacillus* sp. SCUN, a halotolerant strain capable of biodegrading organic pollutants in saline wastewater [7]. This strain thrived within a pH range of 6.0–8.0 and at an optimal temperature of 30 °C, tolerating up to 4% NaCl [9]. It successfully treated wastewater with an initial COD of 22,800 mg/L, achieving a COD removal efficiency of 58.3%.

4. CONCLUSIONS

This study successfully employed two halotolerant strains, *Bacillus* sp. N9 and *Enterococcus* sp. N5, for treating saline wastewater from agar production. Optimal growth and starch degradation occurred at 30 °C, pH 6, and 3% salinity. *Bacillus* sp. N9 achieved 65% COD and 80% ammonium removal within 8 hours, linked to strong amylase activity. *Enterococcus* sp. N5 showed 67% phosphate and 61% COD removal, consistent with phosphorus assimilation by lactic acid bacteria. Co-cultivation at 3% salinity and 8-hour aeration yielded ~80% COD, 81.6% ammonium, and 51% phosphate removal. In real wastewater (2.2% NaCl), 24-hour aeration achieved 71–75% COD and ammonium removal, and ~67% phosphate removal.

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TÓM TẮT

Ứng dụng vi khuẩn chịu mặn để xử lý nước thải sản xuất bột agar

Nước thải phát sinh từ quá trình sản xuất bột agar sử dụng rong biển làm nguyên liệu được phân loại là nước thải nhiễm mặn, với nồng độ NaCl dao động từ 10 đến 30 g/L và chứa hàm lượng tinh bột cao. Các phương pháp xử lý nước thải nhiễm mặn sử dụng các vi sinh vật chịu mặn đang được quan tâm nghiên cứu. Trong nghiên cứu này, hai chủng vi khuẩn chịu mặn là Bacillus sp. N9 và Enterococcus sp. N5, có khả năng phân hủy tinh bột cao, được sử dụng để xử lý nước thải nhiễm mặn. Kết quả sơ bộ cho thấy cả hai chủng đều có khả năng phân huỷ tinh bột ở 30 °C, pH 6 với nồng độ giống ban đầu 2%. Trong điều kiện này, việc sử dụng kết hợp 2 chủng và bùn hoạt tính để xử lý nước thải nhiễm mặn tổng hợp (COD ban đầu: 960 mg/L; NH_4^+-N: 49,34 mg/L; PO_4^{3-}-P: 25,64 mg/L) đạt hiệu suất loại bỏ lần lượt là 90%, 80% và 81% sau 12 giờ đối với COD, NH_4^+-N và PO_4^{3-}-P tương ứng. Sử dụng hỗn hợp trên để xử lý nước thải từ một nhà máy sản xuất agar cho thấy hiệu suất loại bỏ lần lượt là 71%, 75% và 67% tương ứng với COD, NH_4^+-N và PO_4^{3-}-P sau 24 giờ.

Từ khoá: Agar; Tinh bột; Chịu mặn; Nước thải.