SYNTHESIS OF AMMONIUM SOAP AND ITS *IN VITRO* ANTIBACTERIAL ACTIVITY AGAINST *XANTHOMONAS ORYZAE* PV.*ORYZAE* WHEN COMBINED WITH COPPER NANOPARTICLES/CHITOSAN

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ABSTRACT:

This paper presented a method for synthesizing ammonium soap (AS) from palm oil and NH_3 gas. The obtained AS was viscous, opaque white, and pH ~8.0. *In vitro* antibacterial efficiency (AE) against *Xanthomonas oryzae* pv.*oryzae* (*Xoo*) of AS reached 86.70% at a concentration of 5000 mg/L and that of Cu nanoparticles/chitosan (CuNPs/CS) reached 100% at a concentration of 30 mg/L Cu. When using CuNPs/CS at a concentration of 20 mg/L Cu supplemented with 500 mg/L AS, AE reached 100%. Thus, the combination of AS and CuNPs/CS is considered an effective solution for resisting *Xoo* at low concentrations.

Keywords: ammonium soap, copper nanoparticles/chitosan, *Xanthomonas oryzae* pv. *Oryzae*.

1. Introduction

In agriculture, surfactants are commonly used to enhance the adhesion and dispersion of pesticides [1, 2]. Soap is made by hydrolyzing fats (mainly triglycerides) with an alkaline solution, producing carboxylate salts of fatty acids [3]. The bases commonly used for soap production are NaOH (solid soap) and KOH or NH_4OH (liquid soap) [4, 5]. Soaps made from plant oils or animal fats are safe because they do not produce harmful waste [6].

Ammonium soaps (AS) can be prepared by reacting plant oil with excess NH_4OH solution at high concentration (25%) [7], aerating NH_3 gas into the plant oil, or adding $(NH_4)_2CO_3$ to the plant oil and heating it to decompose the carbonate salt into NH_3 [8]. Ammonium soap has been confirmed to have antibacterial, insect, and pest repellent properties [9, 10, 11]. The mechanism of action of AS on insects is to penetrate the cell membrane, causing cell damage and disrupting the respiratory system [10]. Ammonium soaps release NH_3 slowly, thus repelling larger animals such as deer and rabbits [9]. The disadvantage of using AS in plant disease control is that it must be applied repeatedly at high concentrations of 0.5–1% [12]. Therefore, AS is often used in low-concentration supplements to increase the effectiveness of pesticides [13].

Copper-based nanomaterials (CuO, Cu_2O, Cu) have potential applications in agriculture due to their anti microbial activity at low concentrations

[14, 15]. In addition, Cu is also a plant nutrient, so CuNPs is considered a safe plant protection agent because it is consumed by plants [16]. Various plant-damaging microorganisms such as *Xanthomonas axonopodis pv. punicae, Fusarium oxysporum, Alternaria alternata, Curvularia lunata,* and *Phoma destructive* have been shown to be inhibited by Cu nanomaterials [17, 18]. The effect of CuNPs on microorganisms is due to the interaction of Cu with carboxyl groups on the surface of microorganisms [19]. Moreover, CuNPs also stimulate the production of reactive oxygen species, causing damage and changing the permeability of cell membranes, leading to enzyme inactivation and protein dysfunction in microorganisms and cell death [20]. The antibacterial activity of CuNPs/polymer depends on the surface coating, chitosan (CS) biopolymer coating has been shown to have a synergistic effect in the fields of resistance to harmful microorganisms [21].

For the above reason, this study presented a method for preparing AS from palm oil by a heterogeneous reaction when aerating NH3 gas in a heated palm oil solution to produce a biodegradable surfactant, providing nitrogen nutrition for plants when decomposing and reducing its negative impact on the environment compared to traditional alkaline metal soaps. In addition, the *in vitro* antibacterial activity of AS and CuNPs/CS against *Xoo* causing rice leaf blight disease were also investigated in this study with the aim of reducing the concentration of active ingredients and ensuring environmental safety.

2. Methodology

2.1. Materials

Chemicals used in the experiments were of analytical grade, including palm oil (Palma Tech Product Sdn.Bhd., Malaysia), ammonia gas (NH_3) 99.8% (PetroVietnam Fertilizer and Chemicals, Vietnam); standard KOH-ethanol (Merck, Germany); zeolite4A (Guangdong Xintao Technology Co., Ltd., China); HCl; C_2H_5OH 99.7%; phenolphthalein (Xilong, China); CuNPs/CS including 5,000 mg/L Cu and 5% (w/v) CS (Institute of Applied Matterials Science, Vietnam); the *Xoo* bacterial (Institute of Agriculture Science for Southern Viet Nam, Vietnam). The deionized water was used throughout the experiment.

2.2. Methods

Determine the saponification value of palm oil [22]: 2 g of palm oil was placed in a 100 ml conical flask, 25 ml of 0.5 M standard KOH-ethanol solution, and 0.2 g of zeolite4A was added, and the flask was connected to a reflux condenser. The mixture was boiled for 60 min, and the mixture was titrated with HCl 0.5 M using phenolphthalein as an indicator. The same procedure was performed with a blank sample and the volume of HCl solution used for titration was recorded until the pink color disappeared. The experiment was repeated three times and the results were presented as mean ± standard error (SE). The saponification value (SN) was calculated according to formula (1).

$$SN\ (mg/g) = \frac{(V_0 - V_1) \times c \times 56.1}{m} \qquad (1)$$

Where SN (mg/g, calculated as KOH) is the saponification value, V_0 and V_1 (ml) are the volumes of standard HCl solution used for titration in the blank and sample, respectively, c (mol/L) is the concentration of the standard HCl solution, and m (g) is the mass of the sample.

Preparation of AS: 250 g of palm oil was added to a 500 ml glass beaker and heated on a magnetic stirrer to ~90°C. NH_3 gas was added from a pressure vessel with a flow meter to bubble into the beaker containing palm oil, adjusting the NH_3 gas flow to ~20.5 L/h for 60 mins, the amount of NH3 was calculated by the number of moles of KOH to saponify palm oil. Continue aerating NH_3 gas for another 5 min to increase the saponification efficiency.

Determination of the efficiency of saponification reaction (SH) [8]: 5 g of AS was added in a conical flask with a stopper, 10 ml of ester was added, closed the lid, and shaken the mixture on a shaker. Then, the mixture was transferred to an extraction flask, left for 120 mins and the AS at the bottom of the extraction flask was collected. The remaining AS mass was weighed and determined. The experiment was repeated 03 times and the results

were presented as mean ± SE, SH was calculated according to formula (2):

$$SN\,(\%) = \frac{m}{m_0} \times 100 \qquad (2)$$

Where m (g) is the mass of the initial AS and m0 (g) is the mass of the remaining AS.

Determination of pH of AS [22]: Dissolved 1 g of AS in 50 mL of deionized water and stirred well, the pH was measured with a pH meter.

Determination of surface tension: Add 0.05% AS to deionized water and determine the surface tension on a digital surface tensiometer DST-60 (SEO, Korea) at 25°C.

Determined the characteristic properties of CuNPs/CS: The size of CuNPs was calculated from transmission electron microscopy (TEM) images measured on a JEM 1010 (JEOL, Japan) and presented as mean ± standard deviation (SD). The crystalline properties of the material were determined by X-ray diffraction (XRD) spectroscopy on an XRD D8 Advance X-ray diffractometer (Bruker, Germany), using Cu kα (λ = 1.5406Å). The powdered CuNPs/CS sample for XRD measurement was prepared by precipitating in 99.7% C_2H_5OH with a volume ratio of C_2H_5OH:sample solution of 3:1. The precipitate was filtered through filter paper, washed three times with C_2H_5OH, and then dried at 60°C.

In vitro antibacterial efficacy (AE, %) [23, 24]: Inject 100 µl of bacterial enrichment solution into a test tube containing 15 ml of Luria Broth medium. The experiment included treatments supplemented with AS at concentrations of 500, 2000, 3000, 4000, and 5000 mg/L; treatments supplemented with CuNPs/CS at concentrations of 20, 25, and 30 mg/L Cu; treatments supplemented with CuNPs/CS at concentrations of 20 mg/L Cu and 500 mg/L AS; control treatment using distilled water. Incubation was conducted under the same conditions (28-30°C), the treatments were repeated 03 times. Determine the colony density after 24 hours of cultivation by measuring the optical density at a wavelength of 660 nm on a SmartReader 96 (Benchmark - USA) and calculate AE according to formula (3):

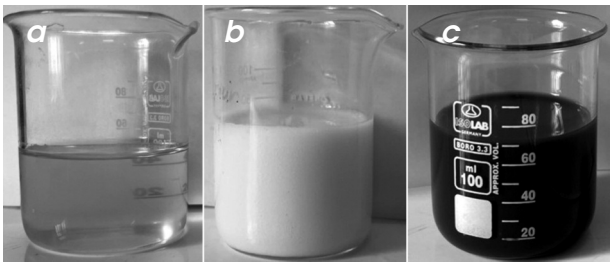
$$AE\,(\%) = 100 \times \frac{(C_0 - C)}{C_0} \qquad (3)$$

Where C_0 and C (cfu/mL) are the colony densities of the control and treated treatments, respectively. The colony densities were calculated from the standard curve with the empirical regression equation (1) for *Xoo:* y = 13.661x – 0.2431 (R^2 = 0.9904), x is the optical density, y is the colony density (cfu/mL) in the range 0.00–5.12 × 10^8 cfu/mL.

3. Results and discussion

After NH_3 gas reacted with palm oil (Figure 1a) at 90°C, AS was obtained in the form of a thick, opaque white liquid (Figure 1b). The mixture did not separate after many days of storage.

Figure 1. Photographs of palm oil (a), AS (b), and CuNPs/CS (c)



Source: Analysis by authors

The SN of palm oil was determined to be 196.73 mg KOH/g, this result is similar to the research of some previous authors [25]. According to our calculations, 250 g of palm oil for saponification needs to react with 14.93 g of NH_3 gas equivalent to 0.0205 m^3. The saponification reaction efficiency was determined to be 93.4% ± 0.5%, this result is similar to the study of Reiling (1962) when using the agent $(NH_4)_2CO_3$ to saponify plant oil (reaction efficiency ~92%) [8]. When the reaction was completed, the pH of AS was determined to be 8.0, which was lower than that obtained using the alkaline agent NaOH [22], and suitable for many applications. The obtained AS had a calculated solid content of ~83.5% (the rest was water and glycerol). The surface tension of water was determined to be 6.17 mN/m when 500 mg/L AS (~417.5 mg of solid soap) was added, which was lower than that of deionized water at ~72

mN/m.This result was similar to the study of Mckinney et al. (1962) when studying saponification from tung oil and ammonia [7].

CuNPs/CS from the Institute of Applied Materials Science had a reddish-brown color as shown in Figure 1c. Through the TEM image in Figure 2a, it could be seen that CuNPs have a spherical shape with an average size of 21.61 ± 2.18 nm. The XRD pattern of CuNPs/CS (Figure 2b) showed two diffraction peaks at 2θ ~10.14° and 20.10°, which are characteristic of the crystallization of CS [26]. In addition, the diffraction peaks at 2θ ~43.29°, 50.54°, and 74.25° correspond to the diffraction planes (111), (200), and (220) assigned to the crystal structure of elemental copper according to JCPDS No.003-1018 [27].

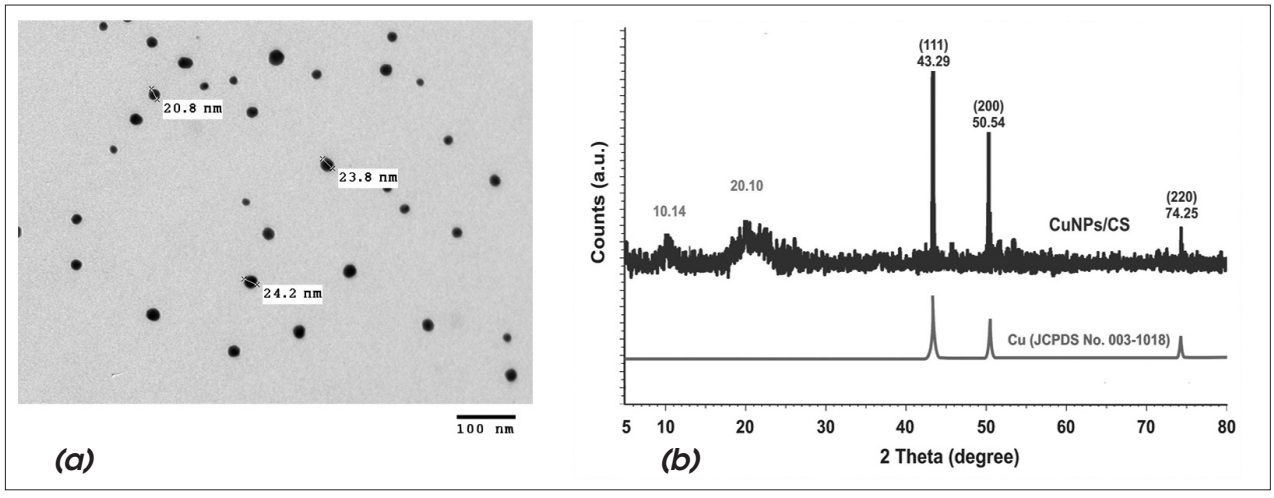
The AE against *Xoo* of AS and CuNPs/CS after 24 hours of incubation is shown in Table 1. The results showed that AS had a low AE (17.45%) when using a concentration of 500 mg/L, while at a concentration of 5000 mg/L, the AE reached > 80%. The half maximal inhibitory concentration (IC_{50}) of AS was 2909 mg/L calculated from the regression equation: y = 0.0163x + 2.5754 (R^2 = 0.9841), where y is AE (%) and x is AS concentration (mg/L). The antibacterial mechanism of AS was explained by Wang et al. (2023) as being due to the cationic surfactants being able to act on negatively charged bacterial membranes, causing cell membrane destruction [28]. Although AS was recommended by the EPA for use in agriculture, farmers often use it empirically, and to date, there have been very few studies on the antimicrobial potential of AS [29]. Quaternary ammonium surfactants have recently been studied and demonstrated to have antimicrobial activity against several medical bacteria such as *Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, Pseudomonas aeruginosa, Acinetobacter baumannii,* and *Staphylococcus aureus* with minimum inhibitory concentrations (MIC) ranging from 0.25–4.00 mg/mL [30]. Thus, AS is a material that is effective against Xoo at high concentrations of 5000 mg/L.

The results in Table 1 showed that CuNPs/CS exhibited antibacterial activity against Xoo at low concentrations of 20–30, AE reached 63.71% at 20 mg/L Cu concentration and increased to 100% at 30 mg/L Cu concentration. Notably, in the treatment supplemented with 500 mg/L AS, the AE of CuNPs/CS at 20 mg/L Cu concentration increased from 63.71% to 100%. Thus, the combination of AS and CuNPs/CS created a synergistic antibacterial activity that reduced the concentration of Cu metal but still achieved high antibacterial efficiency.

4. Conclusion

In this study, AS was synthesized by aerating

Figure 2. TEM image (a) and XRD pattern (b) of CuNPs/CS



Source: Analysis by authors

Table 1. In vitro antimicrobial activity of AS and CuNPs/CS against *Xoo*

Treatments	Colony density (cfu/mL)	AE (%)
Control	$3.61 \pm 0.21^{a} \times 10^{8}$	0.00
AS (500 mg/L)	$2.98 \pm 0.17^{a} \times 10^{8}$	17.45
AS (2000 mg/L)	$2.47 \pm 0.16^{b} \times 10^{8}$	31.58
AS (3000 mg/L)	$1.87 \pm 0.09^{b} \times 10^{8}$	48.20
AS (4000 mg/L)	$1.14 \pm 0.12^{b} \times 10^{8}$	68.42
AS (5000 mg/L)	$4.80 \pm 0.28^{c} \times 10^{7}$	86.70
CuNPs (20 mg/L Cu)	$1.31 \pm 0.15^{b} \times 10^{8}$	63.71
CuNPs (25 mg/L Cu)	$0.79 \pm 0.06^{b} \times 10^{8}$	78.12
CuNPs (30 mg/L Cu)	0.00^{c}	100
CuNPs (20 mg/L Cu) + AS (500 mg/L)	0.00^{c}	100

Note: Different letters in the same row indicate significant differences at $P < 0.05$
Source: Analysis by authors

NH_3 gas through heated palm oil with a reaction efficiency of > 93%. The obtained AS had a low pH and exhibited the properties of a surfactant, which is to reduce the surface tension of the solution. At a concentration of 5000 mg/L, AS was effective against *Xoo* at > 86%. Besides, CuNPs/CS also showed effective antibacterial ability at low concentrations. The use of 500 mg/L AS to supplement CuNPs/CS significantly increased the antibacterial effect of the material. The results of this study show that AS and CuNPs/CS are promising materials for safely inhibiting plant pathogens ■

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TỔNG HỢP XÀ PHÒNG AMONI VÀ HOẠT TÍNH KHÁNG KHUẨN *IN VITRO* CỦA NÓ ĐỐI VỚI VI KHUẨN *XANTHOMONAS ORYZAE* PV.*ORYZAE* KHI KẾT HỢP VỚI NANO ĐỒNG/CHITOSAN

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TÓM TẮT:

Bài viết này trình bày phương pháp tổng hợp xà phòng amoni (AS) từ dầu cọ và khí NH_3. AS thu được có dạng sệt, màu trắng đục và pH ~8.0. Hiệu quả kháng khuẩn (AE) trong phòng thí nghiệm đối với vi khuẩn *Xanthomonas oryzae* pv. *oryzae (Xoo)* của AS đạt 86,70% ở nồng độ 5000 mg/L và của nano Cu/chitosan (CuNPs/CS) đạt 100% ở nồng độ 30 mg/L Cu. Khi sử dụng CuNPs/CS ở nồng độ 20 mg/L Cu bổ sung 500 mg/L AS, AE đạt 100%. Như vậy, sự kết hợp giữa AS và CuNPs/CS được coi là giải pháp hiệu quả trong việc kháng lại vi khuẩn *Xoo* ở nồng độ thấp.

Từ khóa: xà phòng amoni, nano đồng/chitosan, vi khuẩn *Xanthomonas oryzae* pv. *Oryzae.*