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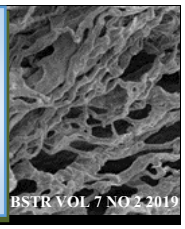

Bacillus sp. UPM-AAG1 for The Bioremediation of Ammonia in Aquaculture Wastewater

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ABSTRACT

The widespread activity of the aquaculture industry has led to environmental pollution resulting from the uncontrollable production of ammonia from aquaculture activities. The presence of ammonia in the environment is a major threat due to its toxicity that can bring harm to organism especially aquatic organism. Bioremediation is an efficient technique for the remedy of ammonia pollution as it provides complete assimilation of the ammonia. In this work, a bacterial isolate identified as *Bacillus* strain UPM-AAG1 shows the best performance out of the four isolates screened for remediating ammonia. The characterisation of *Bacillus* strain UPM-AAG1 was conducted to identify the optimum conditions for ammonia utilization by *Bacillus* strain UPM-AAG1. The optimum pH for ammonia remediation by *Bacillus* strain UPM-AAG1 was determined to be acidic at pH 6.0. While the optimum condition for ammonia concentration was determined to be at 10 mg/mL. For the optimum temperature for ammonia remediation by *Bacillus* strain UPM-AAG1 was determined to be at 30 °C.

INTRODUCTION

Aquaculture has developed as monoculture from past decades, from the easiest retention of fish in ponds to the more technologically advanced fish farms that use food, hormones and often antibiotics with a known environmental effect. A number of studies have shown the impacts of aquaculture that contribute to pollution in the environment. The rapid global expansion of the aquaculture industry has caused many environmental problems, such as water pollution, ecosystem degradation, and disease outbreaks, suggesting that this expansion may not be sustainable [1]. This is now considering the aquaculture of the aquatic environment as a potential polluter.

The aquaculture industry is heavily struck by the occurrence of diseases due to high stock densities that meet the high demand for fish [2]. Ammonia is mainly produced from the breakdown of feed from fish that will accumulate in the aquaculture wastewater. The uncontrolled production of ammonia can cause the outbreak of the disease in aquatic organisms and water pollution. Because of this, the need to understand ammonia degradation is vital as it can lower the potential of ammonia as a pollutant that contaminates in the environment [3–7].

The removal of ammonia from polluted areas has been widely applied using different physical, chemical and biological methods. Bioremediation of ammonia to treat pollutant and contaminant using bacteria have been widely accepted as is proven to be effective, cost-effective and reliable [7–14].

MATERIALS AND METHODS

Sample collection of ammonia-utilizing bacteria

Four different bacterial samples were collected from previously isolated bacteria from the Bioremediation and Biomonitoring laboratory sample collection. They are labelled as *Bacillus sp.* strain UPM-AAG1*, Bacillus 6*, *Bacillus 14* and *Bacillus 16*.

Microorganism and culture condition

The four selected bacterial strains, *Bacillus* strain UPM-AAG1, B.6, B.14 and B.16 were thereafter tested for the ammonia removal efficiency in a basal media under aerobic condition. 1

mL active bacteria culture of *Bacillus* strain UPM-AAG1, *B.6, B.14* and *B.16* grown in Luria Bertani (LB) medium overnight was inoculated into 100 mL synthetic basal media (SBM) for incubation. The composition of SBM (g/L) consisting of 1 g of $(NH_2)_4SO_4$, 10 g of glucose, 0.10.4 g of NaCl, 2.15 g of $Na_2HPO_4.12H_20$, 0.09 g of KH_2PO_4 and 3 mL of trace elements solution. The trace elements solution contained 0.3 g of $MgSO_4.7H_20$, 0.1 g of $MnSO_4$, 0.112 g of H_3BO_3, 0.03 g of $FeSO_4.7H_2O$, 0.06 g of $CaCl_2$ and 0.042 g $Na_2MoO_4.H_2O$ (per litre) [15].

The prepared media had a pH of 7.3. The concentrations of $(NH_4)_2SO_4$ as the sole nitrogen source and glucose as carbon source were adjusted maintaining at a C: N ratio (w/w) of 10, incubated under aerobic conditions at 30°C and 150 rpm. After 24 h of incubation, the optical density (OD) of the bacteria culture was measured at 600 nm to check the concentration of bacteria. The samples were collected at 4 h interval to detect the growth of the bacteria. The bacteria strain that had highest OD was selected for further tested for characteristic conditions.

Selection of conditions for pre-optimization

Preliminary study on the characteristics of ammonia-utilizing bacteria such as pH, ammonia concentration and temperature is important to maximize the growth of ammonia- utilizing bacteria. The result from this study is crucial in designing effective bioremediation strategies.

Estimation of pH range

The aim of this parameter study was to determine the optimum pH for the growth of ammonia-utilizing bacteria. 100 mL of a sterile synthetic basal medium (SBM) were prepared at different initial pHs, which were 6.0, 6.5, 7.0, 7.5 and 8.0 in 250 mL conical flasks. The medium was prepared according to the method described in section 1.2.2. About 1 mL of bacterial sample was pipetted out into the medium and was incubated at room temperature for 24 h on a 150 rpm rotary shaker. The experiments were carried out in triplicates. After 24 h incubation, the optical density, OD_{600} was measured to check the growth of bacterial culture.

Selection of ammonia concentration

The influence of the ammonium concentration on the ammonia-utilizing bacteria capacity was assessed in SBM with pH 6.0 (optimum pH). The medium was prepared according to the method described in section 1.2.2. Initial NH_4^+N concentration were adjusted to 5, 10, 15, 20, 25 and 30 mg/mL NH_4^+N using $(NH_4)_2SO_4$. About 1 mL of bacterial sample were pipetted out into the medium and was incubated at room temperature for 24 h on a 150 rpm rotary shaker. After 24 h incubation, the optical density (OD) was measured at 600 nm to check the growth of bacterial culture. The experiments were carried out in triplicates.

Estimation of temperature range

The optimum temperature for the growth of the ammonia-utilizing bacterium was determined by growing the sample in 100 mL of synthetic basal salt medium with an initial pH 6.0 (optimum pH) and 10 mg/mL of $(NH_4)_2$ SO_4 (optimum concentration) at 25, 30, 35, 40 and 45 °C. The medium was prepared according to the method described above. The temperature range was chosen based on the mesophilic temperature range as the bacteria were isolated from the tropical environment [6,8,10,11]. 1 mL of bacterial sample was pipetted out into the medium and incubated at respective temperature for 24 H incubation, the optical density, OD_{600} was measured to check the concentration of bacterial culture.

RESULTS AND DISCUSSION

Four isolates which were *Bacillus* strain UPM-AAG1, *Bacillus* isolate 6, *Bacillus* isolate 14 and *Bacillus* isolate 16 were sub-cultured from glycerol stock collection from Bioremediation, Biomonitoring and Ecotoxicity laboratory which were then sub-cultured and proceed for one-factor-at-time (OFAT). Luria Bertani (LB) broth was used to grow the bacteria within 24 h (**Fig. 1**). LB is a broadly utilized bacterial culture medium today yet it has its roots in the field of bacteriophage genetics [16]. After 24 h incubation, the broth turned from clear to cloudy that showed the present of bacteria.

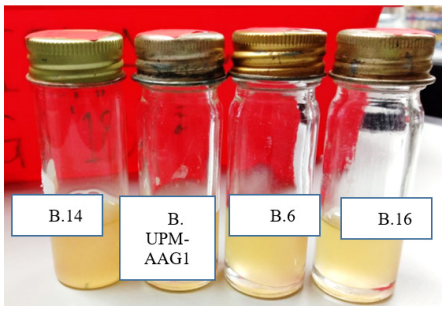

Fig. 1. The growth of various *Bacillus* spp. strain in LB broth. The solutions of the broth turn cloudy after 24 h incubation.

Screening of ammonia-utilizing bacteria

Four isolates of ammonia-utilizing bacteria; *Bacillus* strain UPM-AAG1, *Bacillus* 6, *Bacillus* 14 and *Bacillus* 16 were screened in synthetic basal medium (SBM) medium for 24 h with samples were collected at 4 h interval to detect the growth of the bacteria. The absorbance value was then measured at 600 nm using UV spectrophotometer to monitor bacterial growth. The bacterial growth curves for all *Bacillus* spp. bacteria screened on ammonia as the nitrogen source shows no lag period (**Fig. 2**). *Bacillus* isolate 16 shows the lowest growth based on ANOVA analysis at hour 24 whilst *Bacillus* strain UPM-AAG1 was the best.

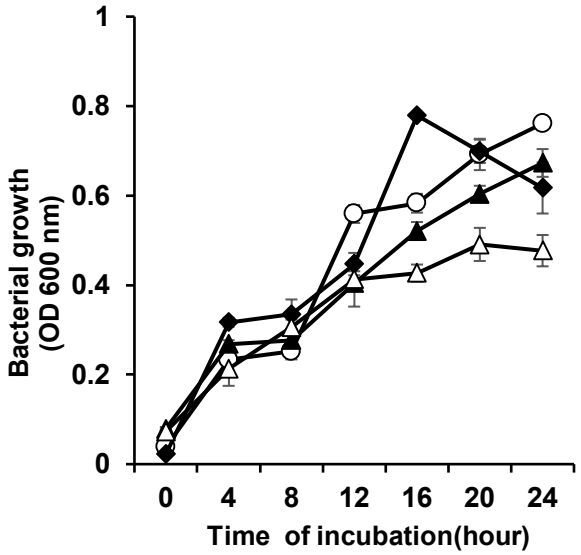

Fig 2. Growth of *Bacillus* spp. (*Bacillus* strain UPM-AAG1 (○), isolate 6 (◆), isolate 14 (▲) and isolate 16 (△)) on ammonia as the nitrogen source. The error bars represent the mean ± standard deviation of three replicates.

Effect of pH on growth of bacteria

The study of the effects of pH on the growth of *Bacillus* strain UPM-AAG1 was carried out at room temperature by altering the pH of the SBM media. The purpose of this experiment is to find the best pH for optimal bacterial growth. The choice of pH was verified by previous studies [17,18] Based on **Fig. 3**, it shows that this bacterium is able to grow at relatively wide pH range, from 6 to 8 with optimal growth at pH 6. As the pH increase, the growth of the bacteria gradually decreases. From the result, alkaline pH seems to slow down the growth of bacteria. From this, we can conclude that *Bacillus* strain UPM-AAG1 grow at acidic pH rather than an alkaline pH. Other *Bacillus* spp grow best at alkaline pH [18] while most of *Bacillus* spp grow best at neutral pH values [19–25]. Moreover, pH lower than 6.0 is not explored because it is beyond PO_4 buffer pKa and lower pH requires organic acids (citric or acetic- both can disrupt the effect of C sources).

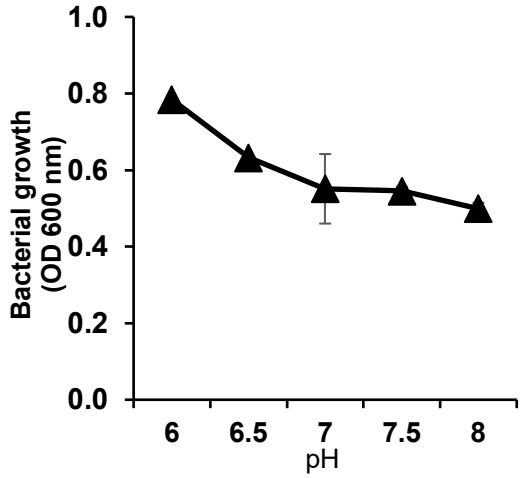

Fig. 3. Effects of pH on the growth of *Bacillus* strain UPM-AAG1 in SBM. The error bars represent the mean ± standard deviation of three replicates.

Effect of ammonia concentration on growth

The ammonia concentration analysed were 5, 10, 15, 20, 25 and 30% with optimum pH (6.0). Based on **Fig. 4**, the optimum concentration of ammonia is 10 mg/mL or about 1% (w/v) as *Bacillus* strain UPM-AAG1 was able to grow highest at that concentration. While as the concentration of ammonia increase, a dramatic decrease in the growth of bacteria. In previous studies [26] it has been shown that ammonia is the main cause of *Bacillus* species growth inhibition, not ammonium ion. Most of the *Bacillus* spp. ammonia-utilizing bacteria to date prefer between 0.5 to 5 mg/mL concentration of ammonia for optimal growth [26–35].

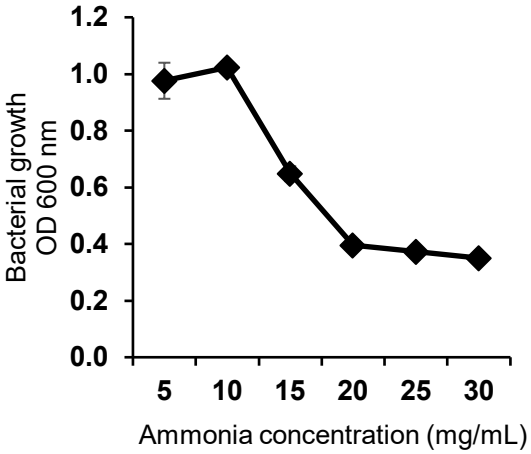

Fig. 4. Effects of ammonia concentration on the growth of *Bacillus* strain UPM-AAG1 in SBM. The error bars represent the mean ± standard deviation of three replicates.

Effect of incubation temperature on growth

The study on the effects of incubation temperature on the growth of *Bacillus* strain UPM-AAG1 was also carried out by setting the initial pH at 6.0 (optimum pH), the ammonia concentration at 10 mg/mL and the media was incubated at different temperatures. Based on **Fig. 5**, the optimum growth of *Bacillus* sp. strain UPM-AAG1 occurred at between 30 to 35 ºC. As the temperature increase, the growth was declined. Temperature plays an important role in determining the capability of bacteria in ammonia utilizing. The increase in rates of enzymatic reaction as the temperatures are increased is limited to the effect of high temperature on the denaturation of enzymes leading to cellular death. Most of the *Bacillus* spp ammonia-utilizing bacteria to date prefer between 25 to 35 ºC for optimal growth [26–35] and the bacterium in this study falls within this range.

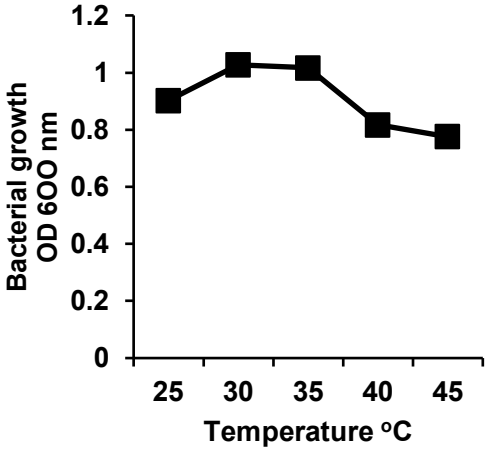

Fig. 5. Effects of temperature on the growth of *Bacillus sp.* strain UPM-AAG1 in SBM. The error bars represent the mean ± standard deviation of three replicates.

CONCLUSION

Four isolates (*Bacillus* strain UPM-AAG1*, Bacillus 6, Bacillus 14* and *Bacillus 16*) collected from the bacterial collection of the bioremediation and biomonitoring laboratory were tested on synthetic basal medium to see which isolate utilize ammonia the best. Among the four isolates, *Bacillus* strain UPM-AAG1 showed the best growth pattern indicating its capability to utilize ammonia. The characterisation of *Bacillus* strain UPM-AAG1 was conducted to identify the optimum conditions for ammonia utilization by *Bacillus* strain UPM-AAG1. The optimum pH for ammonia remediation by *Bacillus* strain UPM-AAG1 were determined to be acidic at pH 6.0. While the optimum condition for ammonia concentration was determined to be at 10 mg/mL. For the optimum temperature for ammonia remediation by *Bacillus* strain UPM-AAG1 was determined to be at 30ºC.

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