



Consortium of heterotrophic nitrification bacteria *Bacillus* sp. and its application on urea fertilizer industrial wastewater treatment

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ABSTRACT

Aims: Industrial wastewater can be processed by physical, chemical and biological treatment. Most biological treatment have no negative impact on environment and can minimize more cost. The aim of this study was to reduce ammonia in urea fertilizer industrial wastewater by *Bacillus* bacterial consortium. This strategy tried to develop biological processing of ammonia in wastewater with nitrification process.

Methodology and results: Bacterial consortium consisted of three isolates: isolate W1.6 identified as *Bacillus* sp. A16ZZ, isolate W2.5 identified as *Bacillus* sp. BNPK-13, and isolate S2.6 identified as *B. cereus* strain CP1. Consortium culture was made based on shortest generation time on each isolate in order to be in exponential phase when it was used. Bacterial consortium was able to decrease ammonia concentration in wastewater seven days after incubation.

Conclusion, significance and impact of study: Consortium of heterotrophic nitrification works optimally with concentration of 350 mg/L ammonia and decrease 96.28%. This consortium has potency to be developed as alternative biological agent in reducing ammonia compounds in high-concentrated urea fertilizer industrial wastewater.

Keywords: Ammonia, *Bacillus* sp., consortium, nitrification

INTRODUCTION

Industrialization and technological changes can lead to the environmental degradation and ecosystem imbalance. This situation is mainly caused by a load of pollution from industrial wastewater containing many toxic chemical compounds. One of the wastewater generated from the process of urea fertilizer industry is ammonia.

Urea fertilizer industry produces wastewater containing ammonia. In water solution, ammonia exists in two forms, ionized (NH_4^+) and unionized ammonia (NH_3) which the balancing of them depends on water pH (Princic *et al.*, 1998). It is toxic for aquatic and terrestrial organisms in higher level of pH, starting from mild disturbance to serious ecological threat (Abeliovich, 1992).

Effort to reduce ammonia become an important part in the industrial wastewater treatment system. One of the technology used is the biological method. Wastewater treatment with this method is cheaper and easy to operate (Sheela *et al.*, 2014).

Utilization of nitrogen compounds as ammonia, not only carried out by autotrophic bacteria such as *Nitrosomonas* and *Nitrobacter*, but also by heterotrophic nitrification bacteria. Most autotrophic nitrification bacteria cannot tolerate high concentration of ammonia and organic materials (Kim *et al.*, 2005). Long generation period makes these bacteria unprofitable compared with heterotrophic bacteria. Heterotrophic nitrification bacteria uses an external carbon source to remove ammonia in wastewater. *Bacillus* is known to be involved in oxidizing ammonia heterotrophically (Mevel and Prieur, 2000), but its ability to remove ammonia in wastewater treatment industry remains unknown.

Heterotrophic nitrification occurs in a series of reactions facilitated by enzymes in the internal membrane system. Oxidation of ammonia to hydroxylamine is catalyzed by ammonia monooxygenase enzyme, then hydroxylamine is oxidized by hydroxylamine oxydoreductase to be nitrite and nitrite is oxidized to be

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nitrate by nitrite oxydoreductase (Wen and Wei, 2011).

Nitrification bacteria found in large quantities in wastewater and sludge, such as industrial wastewater and drainage (Su *et al.*, 2006). Many species of heterotrophic nitrification bacteria have been isolated and characterized for the wastewater treatment system (Joo *et al.*, 2005). Several recent studies have reported capability of *B. subtilis* A1 and *B. cereus* strains GS5 on nitrification under aerobic conditions by utilizing organic carbon sources, termed heterotrophic nitrification bacteria (Yang *et al.*, 2011; Rout *et al.*, 2014).

This study aimed to get nitrification bacterial consortium that able to decrease ammonia concentration in urea fertilizer industrial wastewater. The consortium divided into three isolates of *Bacillus* species isolated from wastewater and sludge in urea fertilizer industry wastewater treatment plant. It is expected to be utilized as a biological agent in ammonia wastewater treatment in a wider scale.

MATERIALS AND METHODS

Identification of nitrification bacteria

Isolates W1.6, W2.5 and S2.6 were successfully isolated from wastewater and sludge of aerobic pond of urea fertilizer industry wastewater treatment plant. Morphological and biochemical properties of all isolates were characterized including test of colony and cell shape, Gram nature, and endospores staining. Identification was carried out by following the Bergey's Manual of Determinative Bacteriology as a reference. A 24 h old culture isolates were sent to the Indonesian Center for Biodiversity and Biotechnology, Bogor, West Java, Indonesia to analyze the 16S-rRNA sequence. The 16S rRNA gene was amplified using PCR machine. Sequencing was performed by PCR amplification using bacterial forward and reverse universal primers 27F such as (5'-AGAGTTTGTATCMTGGCTCAG-3') and 1492R (TACGGYTACCTTGTACGACTT 5'-3') (Zhang *et al.*, 2012). The PCR conditions used, namely pre-denaturation (95 °C, 5 min), denaturation (95 °C, 15 sec), annealing (53 °C, 15 sec), elongation (72 °C, 15 sec), and post-elongation (72 °C, 5 min). The PCR process was done for 35 cycles. Furthermore, sequence data was aligned with GenBank using BLAST from NCBI website to determine the species similarity of isolates tested. Phylogeny tree was prepared by Mega 6.0 program (Kumar *et al.*, 2001).

Making growth curve

Density of bacterial cells culture (cells per milliliter) was created using a standard curve of bacterial cell density. Standard curve was made based on the procedure proposed by Cappuccino and Sherman (2005). Optical density was measured at the wavelength of 640 nm. The linear regression equation of bacterial cell density standard curve had been obtained by plotting the relationship between the density of bacterial cells per milliliter and the optical density.

Ten percent of each 48 h old culture isolates with a density of 10^{-7} cells/mL and 0.13 mg/L NH_3 were added to 50 mL of medium treatment. Isolates were incubated on shaker with 120 rpm at room temperature for 24 h. Bacterial cell density was monitored every 3 h until the amount was decrease and then the growth curve of each bacteria was made. The shortest generation time (g) for each bacteria growth curve was determined in the exponential growth phase (Madigan *et al.*, 2010). Shortest g of each isolate was calculated using the formula:

$$g = \frac{\log 2 (\tau)}{(\log X_t - \log X_0)}$$

Preparation of consortium culture

Consortium culture was prepared by following the shortest g of each isolate. Starter for culture consortium was created by the shortest g of each isolate in a single culture. Ten percent of each single culture isolates starter was put in a liquid selective medium and incubated on shaker with 120 rpm at room temperature. After all starter achieved the shortest g, the culture was mixed and incubated for 48 h in order to make the culture homogeneous and stable, at the same to increase the cell number.

Preparation of treatment medium

The 2,000 mL wastewater was treated in a tank with aerator, and concentrations of ammonia were 350, 400, 450, 500 and 550 mg/L, respectively. The source of carbon was added in the form of glucose ($\text{C}_6\text{H}_{12}\text{O}_6$) with a value of C/N ratio was 10 (Avnimelech, 1999), then sterilized at 121 °C for 15 min (Cappuccino and Sherman, 2005). The number of C in glucose added toward the N of the ammonia concentration contained in the wastewater was calculated to increase the C/N ratio up to 10. In ammonia concentration of 350 mg/L of wastewater, for example, the addition of glucose was 2.87 g/L. Medium was cooled and values of initial pH, nitrite, nitrate, TSS and COD as treatment parameters then were measured. Ten percent of consortium culture was used for each tank. Parameter measurements carried out on the seventh day incubation period.

Method of analysis

Observed variables in this study were measured by the following methods: $\text{NH}_3\text{-N}$ by Nessler method, TSS by gravimetry method, COD by bichromate potassium oxidizer ($\text{K}_2\text{Cr}_2\text{O}_7$), nitrite by N (1-naphthalene)-ethylene with 543 nm wavelength, and phenol disulphonic acid method by 420 nm wavelength for nitrates. All variables were measured based on the standard method procedures for water and wastewater testing (APHA, 2005). Value of pH was measured by digital pH meter (Hach type Sension 4, USA). Oxidized ammonia (OA) calculated from the formula:

$$[\text{Oxidized ammonia}] = \frac{[\text{Ammonia initial} - \text{Ammonia final}]}{[\text{Ammonia initial}]} \times 100\%$$

Statistical analysis

The entire experiment was repeated in triplicate. One-way ANOVA followed by Duncan Multiple Range Test was performed to evaluate significant differences among different treatments, with $p < 0.05$ indicating statistical significance. All statistical analyses were performed by SPSS for windows 17.00 version.

RESULTS AND DISCUSSION

Identification

Three nitrification bacteria isolates were successfully isolated from wastewater and sludge of aerobic pond of urea fertilizer industry wastewater treatment plant. Two isolates, W1.6 and W2.5 were isolated from wastewater and S2.6 from sludge. Early identification of isolates was performed by morphological and biochemical tests using Bergey's Manual of Determinative Bacteriology as a reference. The colony shape and colour of the three isolates were different. W1.6, W2.5 and S2.6 isolates were identified as *Bacillus* sp. (Table 1). According to Buchanan and Gibbons (1974), *Bacillus* is rod-shape bacterium with endospores which are different from its vegetative cells. It is usually gram positive and active with lateral flagella. This isolate has also high resistance of heat and is able to grow at 25 °C up to more than 75 °C. *Bacillus* is also either obligate aerobes or facultative anaerobes, and can produce catalase.

Table 1: Morphological and biochemical properties of isolates.

Test	Isolate W1.6	Isolate W2.5	Isolate S2.6
Gram's staining	Positive	Positive	Positive
Endospora	Positive	Positive	Positive
Colony colour	Yellowish	White	Whitish
Colony margin	Erose	Lobate	Circular smooth
Motility	Positive	Positive	Positive
Methyl Red	Positive	Positive	Positive
Voges Proskauer	Positive	Positive	Negative
Citrate	Negative	Negative	Negative
Urease	Negative	Negative	Negative
Starch hydrolysis	Positive	Positive	Positive
Gelatin liquefaction	Positive	Positive	Positive
Glucose fermentation	Positive	Positive	Positive

Amplified 16S rRNA of W1.6, W2.5, and S2.6 isolates using primers 27F and 1492R produced approximately 1500 bp length band (Figure 1).

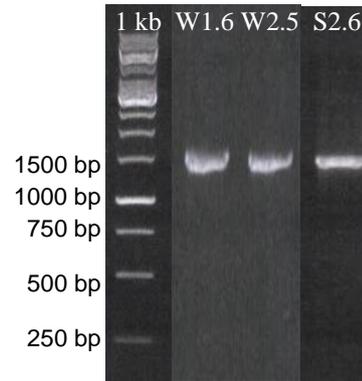


Figure 1: Amplification of 16S rRNA W1.6, W2.5 and S2.6 isolates genes.

BLAST results showed that isolates W1.6 was 96% similar with *Bacillus* sp. A16ZZ, W2.5 isolates was 98% similar with *Bacillus* sp. BNPK-13, and isolates S2.6 was 98% similar with *Bacillus cereus* strain CP1. Phylogenetic tree was obtained by Neighbour Joining (NJ) method with 1000x bootstrap (Figure 2).

Growth curve of isolates

Growth curve is required to determine the bacteria growth phase. Each isolate showed different growth pattern (Figure 3a). All isolates were in lag phase at 0th hour until 3rd hour and then were in exponential phase till 15th hour (W1.6) and 18th hour (W2.5 and S2.6). Stationary phase of S2.6 was at 18th hour while other isolates were not in stationary phase, but directly were in death phase at 15th hour (W1.6) and 18th hour (W2.5). It is because stationary phase occurred fast and was not detected during the three hours interval of observation time.

Exponential phase is set as incubation period for the availability of starter inoculum for biodegradation of ammonia in wastewater. Growth activity of isolate was most active at exponential phase or called as fast proliferation. In this phase, isolate would be in maximum growth rate and metabolism with intensive proliferation and increase in the number of cell. The exponential phase is used in determining the optimal time of treatment of urea fertilizer industrial wastewater to enhance biodegradation process of ammonia.

This isolate, which had reached its exponential phase, was further used in consortium culture preparation that was required in the processing and biodegradation of ammonia in wastewater. Each bacteria was mixed when it reached its exponential phase and maximum rate. This situation was aimed to create optimal condition for bacteria to degrade ammonia in wastewater.

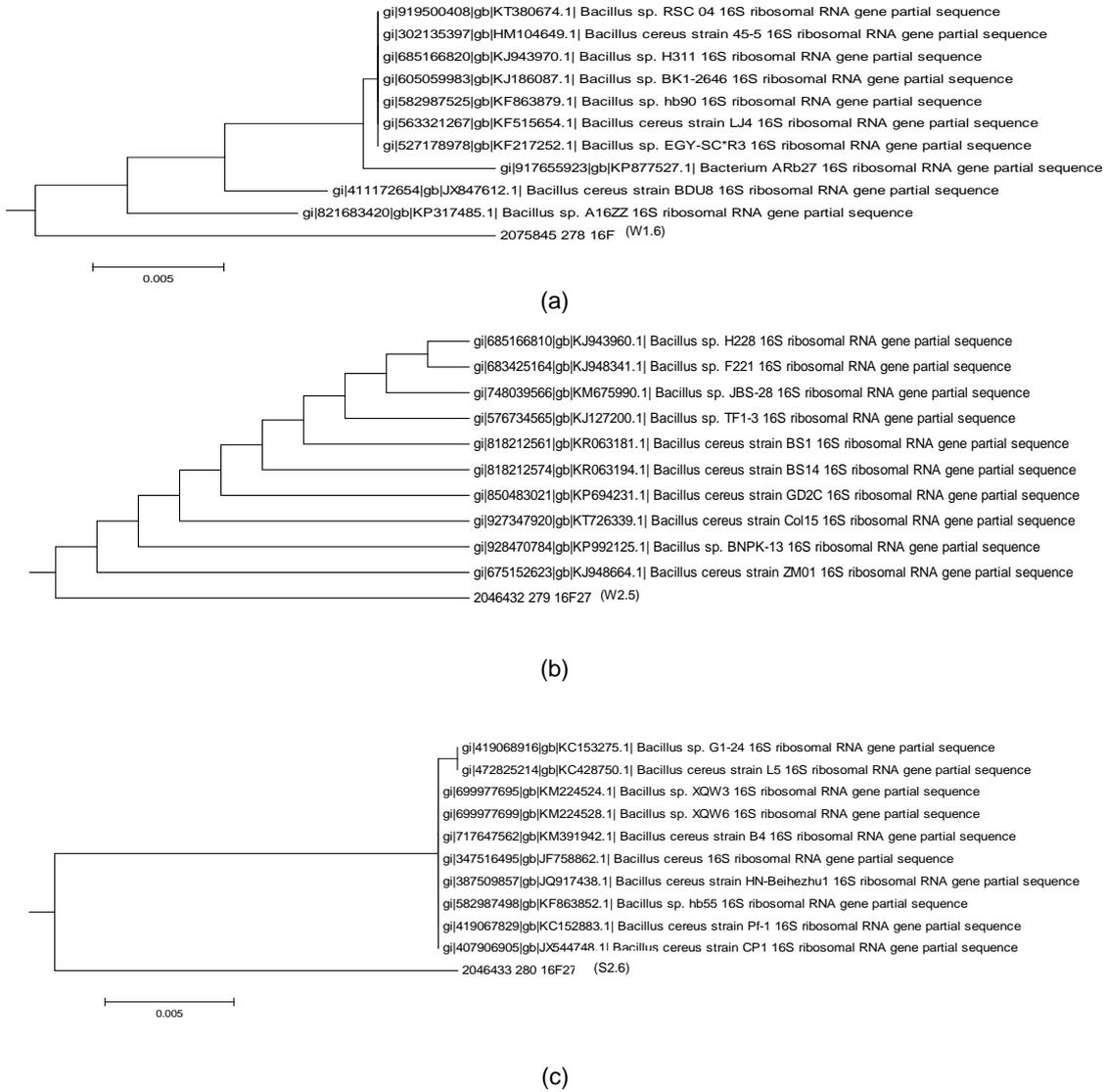


Figure 2: Phylogenetic tree of (a) W1.6; (b) W2.5 and (c) S2.6.

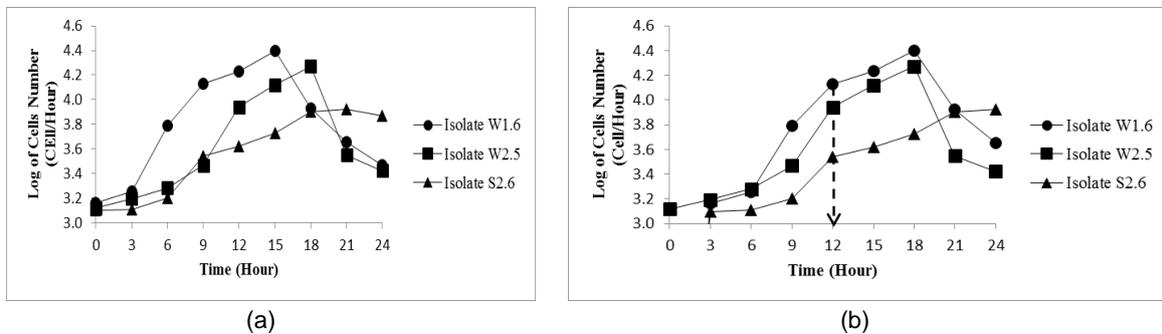


Figure 3: Growth curve (a) time of making single culture and (b) incubation period.

During the exponential phase, isolates as nitrification bacteria consortium would utilize ammonia in the wastewater as source of nitrogen for growth, either in free-ammonia (NH₃) or ammonium ions (NH₄⁺). It is the most important phase in biodegradation of wastewater ammonia because in this phase, degradation runs faster and ammonia loss is greater. As their nutrition requirements were fulfilled optimally, they could growth fast and respond well on various external factors. After exponential phase, isolates growth would reach stationary phase where the increase number of cell equalled to the number of cell death because nutrients in medium decreased. As a consequence, isolate number would be constant in a long period before it reached its death phase. Death phase occurred when ammonia in medium functioned as nutrient source for isolates growth had been utilized completely.

Preparation of culture consortium

Consortium cultures of isolates that had reached the exponential phase were required the process of wastewater biodegradation. In making consortium, isolates that had reached the exponential phase and reached its maximum speed was mixed. It aimed to make culture of the consortium were in optimal conditions when it degraded ammonia in wastewater. Inoculation time strategy is needed in making culture in order to set the consortium can work optimally and relieve the detrimental competition of degradation performance.

Based on log of cell number of each isolate, W1.6 and S2.6 had a similar shortest generation time, which was 9 h, while W2.5 was 12 h (Table 2). The shortest g was reached at initial exponential phase that was the peak of growth. As a consequence of different time of generation, single culture from each isolate based on shortest g was required. For that purpose, strategy of inoculation time was carried out that isolates could work together optimally in degrading ammonia in wastewater and no disadvantageous competition on degradation occurred.

Preparation of the consortium initiated first by making a single culture W2.5. After 3 h, two single culture of W1.6 and S2.6 were made and incubated for 9 h. The exponential growth of these single culture bacteria occurred on the 12th hour, which were ready to use as a consortium culture (Figure 3b). Bacterial consortium created is expected to provide optimal results of process during ammonia biodegradation in urea fertilizer industrial wastewater.

Industry wastewater treatment

Wastewater treatment for seven days resulted a change in the value of NH₃-N, NO₃, TSS, COD and pH (Figure 4). The consortium was able to decrease concentration of ammonia in all treatments. Heterotrophic nitrification bacteria consortium was able to oxidize ammonia in wastewater due to their ammonia monooxygenase (AMO) enzyme activity and the addition of organic carbon in the wastewater which were used as an energy source for

growth. The highest decrease of NH₃-N (96,28%) occurred at a concentration of 350 mg/L compared to the treatment of other concentration, respectively 93.75%, 92,9%, 91.4% and 88.36% (Figure 4a).

Table 2: Linear equation and shortest generation time (g).

No	Isolate	Time	Abs	Log of cells number (cell/h)	Linear equation	Generation time (h per generation)
1	W1.6	9	1.341	4.128	Y = 2.896 + 0.919X	2.800
2	W2.5	12	1.284	3.942	Y = 3.061 + 0.686X	4.388
3	S2.6	9	0.721	3.539	Y = 3.036 + 0.697X	6.170

Substrate concentration is one of the most significant environmental factors in nitrification process. Bacterial growth is limited by the balance of nutrients in the water. Therefore, the dynamics of bacterial population is strongly associated with the availability of substrate functioning as nutrient (Jones and Hood, 1980). Cell population significantly increased in liquid medium with ammonia as the primary nitrogen source (Wen and Wei, 2011). Low substrate concentration leads to higher amount of nitrification bacteria compared with the high quantity of substrate (Princic *et al.*, 1998). Moreover, this relationship occurs only when the ammonia concentration do not exceed the limit of bacterial tolerance. Tolerance of nitrification bacteria on ammonia in wastewater depends on wastewater pH. Higher pH will form unionized ammonia (NH₃) in water which is toxic for nitrification bacteria and affects the oxidation of ammonia. High ammonia concentrations in wastewater caused bacterial tolerance to be reduced then nitrification activity was also reduced. At neutral pH most of ammonia is in the form of ammonium ions which is not toxic to bacteria. According to Zhang *et al.* (2012), maximum ability of nitrification bacteria to oxidize ammonia in wastewater is in a pH range of 7-8.5. pH wastewater in this study increased along with concentration. Thus, bacterial tolerance was reduced.

Ammonia concentration in wastewater significantly influenced the increase of nitrate after seven days of incubation (Figure 4c). Nitrate accumulation occurred due to oxidation of ammonia to nitrates through nitrification. At concentration of 350 mg/L, there was more accumulation of nitrate (76.51%). Accumulation of nitrate occurred because consortium consisted of *Bacillus* species which were heterotrophic nitrification bacteria and nitrates contributor. Nitrification process cause nitrate accumulation without the addition of nitrite, and it occur in this study (Figure 4b). It was because nitrite produced from oxidation of ammonia was converted directly to nitrate. This phenomenon has also been reported by other researchers (Su *et al.*, 2006). The nitrite concentration did not increase in wastewater and this indicated that nitrite oxidoreductase which oxidize nitrite to nitrate works

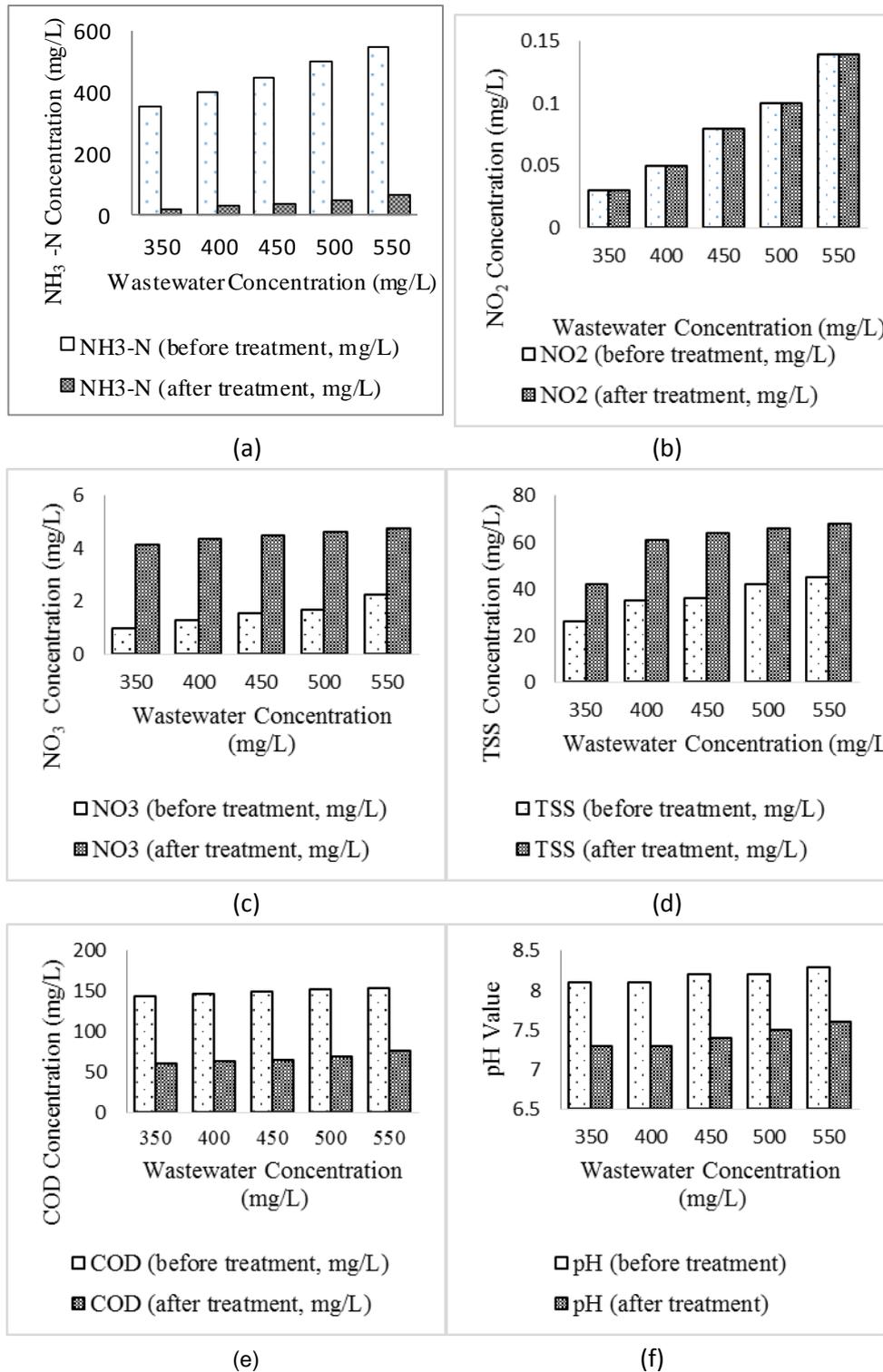


Figure 4: Wastewater parameter (a) NH₃-N; (b) NO₂; (c) NO₃; d, TSS; (e) COD; (f) pH.

continuously.

Nitrification is a process where ammonia is converted to nitrite and nitrate by using oxygen. Consortium consisted of isolate which converted ammonia to nitrite and nitrite to nitrate mediated its enzymes. Nitrite that was oxidized to nitrate caused nitrate as nitrification product would be increased, although it was not in equal amount. According to Rout *et al.* (2014), this was due to the two stage of nitrification process which includes oxidation of ammonia to nitrite and nitrite to nitrate, were not simultaneously occurred, yet sequentially.

Concentration of ammonia significantly influenced the increase of TSS. The initial value of TSS in each treatment concentration was different. The 61.54% of suspended solid was formed at a concentration of 350 mg/L from the initial TSS concentration of 26 mg/L. Both of growth of inoculated bacterial consortium and degradation of nutrients in the growth medium increased TSS levels. At a concentration of 550 mg/L, increase of TSS level occurred at 51.11% (Figure 4d). Most of the ammonia oxidized by *Bacillus* sp. as a source of energy is not aerobically removed from the medium, but assimilated into the cell. As a consequence, it will produce a number of biomass (sludge).

Ammonia oxidized was utilized by *Bacillus* sp. as energy source for its cells growth. In consequence, cell number increased, either the newly divided cell or dead cell. Biomass produced was measured as TSS. However, biomass was not affected by nitrification process but depended on the type and the shortest generation time (g) of each nitrification bacteria. Nitrification bacteria *Bacillus* sp. produced 300 mg/L of biomass in bioremediation of ammonia which was considered to be low in heterotrophic nitrification process (Sheela *et al.*, 2014). This result then became the reason in choosing *Bacillus* to be used in wastewater treatment system. Low biomass produced means that number of suspended solid is less, thus it will not create pollution if wastewater produced by this treatment is discharged to water. Furthermore, *Bacillus* can be selected for industrial wastewater treatment system in a wider scale.

The substrate added to the treatment medium has a C/N ratio of 10 which according to Kim *et al.* (2005), it contains a high carbon source. The treatment of high ammonia concentrations would require an additional carbon, consequently, the higher the concentration treatment the COD value will be higher. The COD of wastewater after treatment decreases significantly along with the increase of the treatment concentration (Figure 4e). the COD declined due to the organic materials and inorganic wastewater is largely used by bacteria in the consortium for metabolism and growth. The COD reduction occurred at a concentration of 350 mg/L is 57.64%.

The range of pH value before treatment is 8.1 - 8.3, which is the optimal pH range for bacteria consortium growth. The pH range for the growth of nitrifying bacteria culture is 5.8-8.5, below 5.8 the nitrification process is inhibited (Princic *et al.*, 1998). At pH 5.0-5.5 the nitrification process will be stalled (Titiresmi and Sopi

2006). The higher pH value is along with the increase of the concentration treatments. The pH value of wastewater after treatment decreases significantly with the increase of the treatment concentration (Figure 4f). This is due to the formation of hydrogen ions during the processing, which is derived from respiration and the nitrification process of the bacteria consortium. The mechanism of nitrifying bacteria in influencing the pH can be explained by the reaction (Khin and Annachhatre 2004) below:



The decrease of pH value after processing is within the tolerance range for the growth of nitrifying bacteria. the pH decreases to 7.3 at a concentration of 350 mg/L and 7.6 at 550 mg/L.

CONCLUSION

Consortium of heterotrophic nitrification bacteria *Bacillus* species are able to decrease the concentration of ammonia in urea fertilizer industrial wastewater. The decreasing was along with the decreasing of pH and COD, and increasing of TSS and nitrate while concentration of nitrite remained constant. The consortium significantly works best on the ammonia concentration of 350 mg/L although they can reduce ammonia at higher concentrations, up to 550 mg/L. Culture was prepared when all isolates in consortium were in the exponential phase. Thus increasing of ammonia concentration in wastewater occurred faster with high decreasing percentage.

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