J. Microbiol. Biotechnol. (2010), **20**(8), 1251–1258
doi: 10.4014/jmb.1003.03002
First published online 6 July 2010

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Ammonium Production During the Nitrogen-Fixing Process by Wild *Paenibacillus* Strains and Cell-Free Extract Adsorbed on Nano TiO_2 Particles

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Received: March 1, 2010 / Revised: May 11, 2010 / Accepted: May 13, 2010

During the nitrogen-fixing process, ammonia (NH_3) is incorporated into glutamate to yield glutamine and is generally not secreted. However, in this study, NH_3-excreting strains of nitrogen-fixing *Paenibacillus* were isolated from soil. The ammonium production by the *Paenibacillus* strains was assayed in different experiments (dry biomass, wet biomass, cell-free extract, and cell-free extract adsorbed on nano TiO_2 particles) inside an innovative bioreactor containing capsules of N_2 and H_2. In addition, the effects of different N_2 and H_2 treatments on the formation of NH_3 were assayed. The results showed that the dry biomass of the strains produced the most NH_3. The dry biomass of the *Paenibacillus* strain E produced the most NH_3 at 1.50, 0.34, and 0.27 µM NH_3/mg biomass/h in the presence of N_2 + H_2, N_2, and H_2, respectively, indicating that a combined effluent of N_2 and H_2 was vital for NH_3 production. Notwithstanding, a cell-free extract (CFE) adsorbed on nano TiO_2 particles produced the most NH_3 and preserved the enzyme activities for a longer period of time, where the NH_3 production was 2.45 µM/mg CFE/h over 17 h. Therefore, the present study provides a new, simple, and inexpensive method of NH_3 production.

Keywords: Ammonium, *Paenibacillus*, nano TiO_2 particles, nitrogenase, nitrogen-fixing process

Nitrogen-fixing bacteria convert N_2 to NH_3 using a nitrogenase enzyme. This enzyme is biosynthesized by 15 to 20 different nitrogen fixation (*nif*) gene products [3, 9]. The product of nitrogen fixation, ammonia, is assimilated by a glutamine synthetase (GS) enzyme, where the ammonia is incorporated into glutamate to yield glutamine, while glutamate is regenerated by the activity of glutamate synthase (GOGAT). Thus, the activities of GS and GOGAT supply cells with the key intermediates of nitrogen metabolism, glutamate, and glutamine. Glutamate is involved in 90% of all nitrogen-containing metabolite synthesis, whereas glutamine provides about 10% of all N-containing metabolites in cells [3, 8, 9]. In GOGAT-deficient mutants, glutamate can also be formed by the amination of ketoglutarate (KT) *via* glutamate dehydrogenase (GDH) or from the breakdown of other amino acids [2, 3]. Therefore, ammonium, the product of nitrogen fixation, is assimilated and not released. The first report of a nitrogen-fixing bacterium excreting NH_3 was in the case of a mutant strain of *Klebsiella pneumonia*, where deficient ammonium assimilation led to an extracellular concentration of NH_3 up to 5 mM in the culture medium [3]. There have also been several other reports that nitrogen fixation may lead to ammonium secretion [2, 8, 13, 17, 19]. In the case of bacteria with GOGAT, GS, or GDH mutants, when this mutation has an impact on the system assimilating the NH_3 produced by the nitrogenase enzyme, NH_3 is released into the medium.

H_2 is a by-product of biological nitrogen fixation and inhibits nitrogenase activity, as a minimum of 25% of the electron flux through purified nitrogenase is allocated to H_2 production [14]. However, Allen *et al.* [1] suggest that the H_2 uptake system in *Azorhizobium* functions as a scavenger of O_2 that is harmful to the nitrogenase enzyme. H_2-dependent ATP formation has already been reported in blue-green algae, *Azotobacter chroococcum*, and a particulate preparation of *Rhizobium leguminosarum* bacteroids [4, 7, 19–21]. In addition, H_2 utilization *via* the hydrogenase system in blue-green algae may provide a reductant for nitrogenase. Emerich *et al.* [4] reported that the H_2-oxidizing hydrogenase in the *R. japonicum* strain USDA 122 DES bacteroids provides a mechanism for the respiratory protection of nitrogenase, increases the ATP supply, and supports nitrogenase activity.

The sol-gel process is an efficient method for making identical porous films by incorporating an organic polymer

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into the precursor solution. Recently, nanoporous materials with a very high surface area and controllable pore size, like TiO_2 and SiO_2, have attracted attention as a host for biomolecules, such as enzymes [6, 11, 12].

Accordingly, this study analyzed the biotechnological potential of the free ammonium production by different nitrogen-fixing bacteria, and the effects of different H_2 and N_2 treatments on the NH_3 formation. The adsorption of a cell-free extract containing a nitrogenase enzyme on a nano solid support, TiO_2 particles, was also investigated for NH_3 production.

Materials and Methods

Organisms and Growth Conditions

Different *Paenibacillus* strains from the soil and rhizosphere of rice (*Oryza sativa*), wheat (*Triticum aestivum*), and alfalfa (*Medicago sativa*) were isolated by heat-shock and incubation in an anaerobic jar. Meanwhile, different *Azotobacter* strains from the rhizosphere of rice, wheat, and alfalfa were isolated using different sugars as the sole carbon source (glucose, mannitol, fructose, mannose, sucrose, and starch purchased from Merck Co.). To isolate the *Paenibacillus* and *Azotobacter* strains, a nitrogen-free mannitol agar medium was used containing mannitol 20; NaCl 4; K_2HPO_4 0.50; $Na_2MoO_4{\cdot}2H_2O$ 0.02; KH_2PO_4 0.25; $CaCO_3$ 0.3; $MgSO_4{\cdot}7H_2O$ 0.4; and $FeSO_4$ 0.4; in grams per liter, pH=7.2 (to isolate the different strains, mannitol was replaced by different sugars). The identification of the isolates was initially carried out on the basis of their morphological, culture, and biochemical characteristics using standard methods; in *Bergey's Manual of Determinative Bacteriology* [5], and then confirmed by the CinnaGene Company (Tehran, Iran) according to the nifH and 16S rRNA, where PCRs using DNA extracts were performed with the universal primers RW01 (5'-AAC TGG AGG AAG GTG GGG AT-3') and DG74 (5'-AGG AGG TGA TCC AAC CGC A-3') that flank a ~370-bp fragment found in the 16S rRNA gene of all bacterial species.

Screening of Isolates for Ammonium Production

Stocks of all the strains were prepared with an optical density of 0.8 (OD=0.8) in a TSB (tryptone soya broth) medium. Approx. 10 ml of each stock was inoculated into 1 l of the TSB medium for biomass production and incubated at 30°C for 48 h. The cells were then centrifuged at 5,000 rpm for 30 min, washed with distilled water three times, and resuspended in 1 l of a nitrogen-free medium with two different sugars (mannitol and sucrose) as the sole carbon sources at 30°C for one week. Thereafter, the biomass was harvested by centrifugation at 5,000 rpm for 30 min, the supernatant liquid mixed with the Nessler reagent (purchased from Merck Co.), and the ammonium concentration determined immediately using spectroscopic absorbance measurements at a wavelength of 410 nm according to a standard curve.

Ammonium Production in U-Tube System

To enable the NH_3 to be collected separately from the container holding the NH_3-producing bacteria, a simple U-tube system was designed. As shown in Fig. 1, a U-tube is used to connect tube 1, containing the bacteria, with tube 2 that contains distilled water for collecting the ammonium. As such, when the ammonium gas produced by the bacteria in tube 1 enters tube 2, it is solved and accumulated in the distilled water. The U-tube system was used in three different experiments to assay the NH_3 production by three *Paenibacillus* strains (E, H, and SH), the best producers of ammonium, from an initial stock with an optical density of 0.8 (OD=0.8) in a TSB medium.

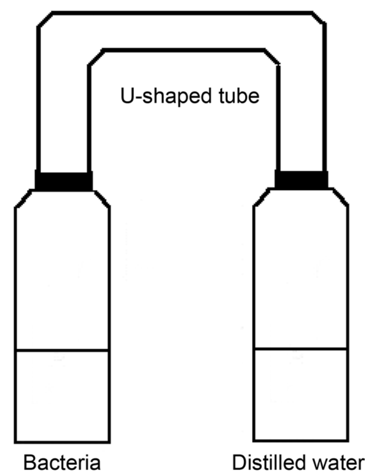

Fig. 1. Simple U-tube system designed for ammonium production: connected by a U-tube, tube 1 contains the bacteria, and the ammonium is collected in tube 2 that contains distilled water.

NH_3 production assay with nitrogen-free broth medium. 10 ml of the bacterial stocks were inoculated into one liter of the TSB medium for biomass production, and incubated at 30°C for 48 h. The cells were then centrifuged at 5,000 rpm for 30 min, washed with distilled water three times, suspended in one liter of a nitrogen-free medium to induce nitrogenase production, and placed in tube 1. The NH_3 gas dissolved in the distilled water in tube 2, i.e. the NH_3 gas production was assayed colorimetrically until the production approached zero (the distilled water in tube 2 was analyzed and replaced with fresh distilled water every week).

NH_3 production assay with wet biomass. Whereas the biomass production and cell induction for the nitrogenase production were the same as described above, a total wet biomass was obtained by centrifugation, washing with distilled water three times, and placing in tube 1. The NH_3 gas production was then assayed colorimetrically until the production approached zero.

NH_3 production assay with dry biomass. After following the same biomass production, cell induction for nitrogenase production, and total wet biomass production as described above, the wet biomass was washed three times with distilled water, dried at 30ºC using an evaporator to obtain a dry biomass, and placed in tube 1. The NH_3 gas production was then assayed colorimetrically until the production approached zero.

Each experiment was performed in triplicate with each of the three *Paenibacillus* strains, and the averages were used as the results.

Ammonium Production by Ammonificators

Non-nitrogen-fixing bacteria, like *Listeria monocytogenes, Streptococcus faecalis*, and *Staphylococcus aureus*, were also used as controls to compare the ammonium production from ammonification and nitrogen fixation during seven weeks when using the U-tube system.

Here, 10 ml of bacterial stocks with an optical density of 0.8 (OD=0.8) was individually inoculated into 1 l of a TSB medium for 48 h. Three similar experiments (with a broth medium, wet biomass, and dry biomass) were then conducted using the U-tube system, and the NH_3 production was examined colorimetrically until production approached zero. Each experiment was performed in triplicate and the averages were used as the results.

Composite of TiO_2 Nanostructure

A composite TiO_2 nanostructure was prepared according to the method of Habibi *et al*. [6] with a sol-gel technique using nanopowder titanium dioxide filler (purchased from Aldrich Co.) mixed with a sol, spin coating, and heat treatment. The structural properties were then characterized by scanning electron microscopy (SEM).

Ammonium Production by Innovative Bioreactor System Containing N_2 and H_2

Next an innovative bioreactor designed for ammonium production was tested. Using a three-way connector, capsules of H_2 and N_2 are linked to the bacteria and then to a tube with distilled water to collect the ammonium produced (Fig. 2). Then 10 ml of a stock of the *Paenibacillus* strain that produced the most ammonium in the U-tube system (strain E) was inoculated into 1 l of a TSB medium for biomass production and incubated at 30°C for 48h. After inducing nitrogenase activity in an N-free medium, the cells were harvested, as in the U-tube experiments, and their ammonium production in the bioreactor was then examined colorimetrically in different experiments.

Control stage. First, the ammonium production in the presence of 0.01 atmosphere of the two gases (H_2 and N_2) without any bacteria was assayed for one week.

NH_3 production assay with wet biomass. A 10-ml stock of strain E was inoculated into 1 l of a TSB medium and incubated at 30°C for 48 h. The cell biomass was then harvested by centrifugation at 5,000 rpm for 30 min, washed with distilled water three times, and resuspended in 1 l of a nitrogen-free medium to induce nitrogenase production. The total wet biomass of the induced cells was harvested by centrifugation, washed with distilled water three times, and placed in the bioreactor. The ammonium production in the tube with distilled water was then examined with 0.01 atmosphere of a dry stream of the two gases (H_2 and N_2) until the production approached zero.

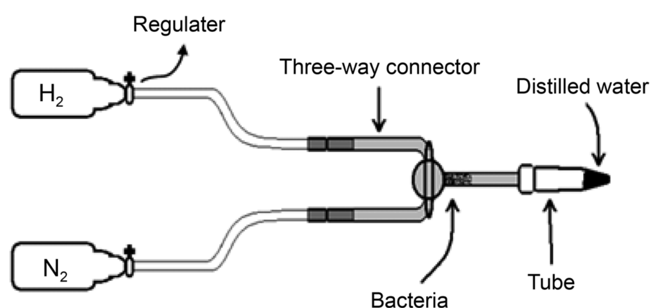

Fig. 2. Structure of an innovative bioreactor designed for ammonium production.

A three-way connector is used to link capsules of H_2 and N_2 to the bacteria, and a tube containing distilled water to accumulate the ammonium produced.

NH_3 production assay with dry biomass. After following the same procedures as described above, the total wet biomass was dried and placed in the bioreactor, and the ammonium production examined with 0.01 atmosphere of a dry stream of the two gases (H_2 and N_2) until the production approached zero.

NH_3 production assay with cell-free extract. After following the same procedures for biomass production and induction of nitrogenase production, the cells were centrifuged at 5,000 rpm for 30 min, washed three times in a 10 mM potassium phosphate buffer (pH=7.5), and resuspended in 0.1 M potassium phosphate (pH=7.5). The cell suspension was then disrupted using ultrasonication (VP 2004, Dr. Hielscher Gmb4) for 5 min and centrifuged at 6,000 rpm for 30 min at 5°C. The resulting supernatant solutions containing a cell-free extract were dried, placed in the bioreactor, and the ammonium production was assayed colorimetrically.

NH_3 production assay with cell-free extract adsorbed on TiO_2 particles. After obtaining the cell-free extract, as described above, it was adsorbed on a 2% powder of TiO_2 particles by shaking for 2 h at 4°C, dried, placed in the bioreactor, and the NH_3 production was assayed colorimetrically.

To determine the effects of the N_2 and H_2 gases on the ammonium formation, the production of NH_3 by the dry biomass without the H_2 and N_2 gases was assayed first. Thereafter, the ammonium production by the dry biomass in the presence of N_2 and then in the presence of H_2 was examined.

Each experiment was performed in triplicate and the averages were used as the results.

Statistical Analyses

The data were statistically analyzed using a one-way analysis of variance (ANOVA) (Tukey HSD and Duncan) and SPSS software 15.

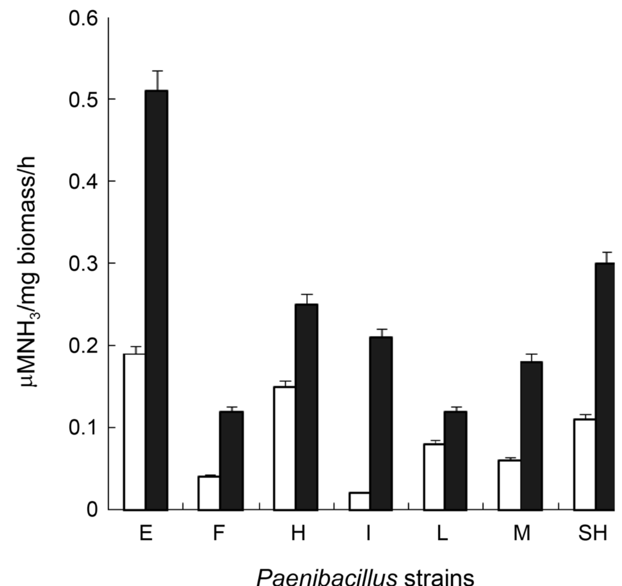

Fig. 3. Ammonium production by *Paenibacillus* strains in nitrogen-free medium with two different carbon sources [mannitol (□) and sucrose (■)] during 7 days of incubation at 30°C.

The strains produced more NH_3 in the nitrogen-free medium with sucrose as the sole carbon source than with mannitol; strains E, H, and SH were the best producers of NH_3.

Results

Isolation of Strains and Ammonium Production Assay

Fourteen strains of *Paenibacillus* spp. and 12 strains of *Azotobacter* spp. were isolated from different plant root rhizospheres and soils. The strains were then identified based on biochemical tests and their sugar fermentation behavior, as described in the *Bergey's Manual of Determinative Bacteriology* (data not shown), and the identification was finally confirmed by the CinnaGene Company according to the nifH and 16S rRNA. When screening the ability of the strains to produce ammonium during the nitrogen-fixing process, only *Paenibacillus* strains (seven strains) exhibited this ability. The results revealed that the NH_3 production in an N-free medium with sucrose as the sole carbon source was higher than the production with mannitol as the sole carbon source. Moreover, this difference due to the carbon source was statistically significant ($P<0.05$). As shown in Fig. 3, *Paenibacillus* strain E produced the most ammonium at 0.19 µM NH_3/mg biomass/h in the N-free medium with mannitol as the sole carbon source, and much more when sucrose was used as the sole carbon source (0.52 µM/mg biomass/h). Strains H and SH also exhibited a high NH_3 production at 0.24 and 0.32 µM/mg biomass/h, respectively,

Table 1. Biochemical tests for identification of *Paenibacillus* strains (E, H, and SH).

Tests	Strain E	Strain H	Strain SH
Gram reaction	+	+	+
Spore	+	+	+
Growth on N_2-free medium	+	+	+
Pigment	-	-	-
Cell morphology	Bacillus-spore	Bacillus-spore	Bacillus-spore
Oxidase	-	-	-
Catalase	+	+	+
Thioglycolate	+	+	+
Starch	-	+	+
Citrate	-	-	-
Glucose (A)	+	+	+
Sucrose	+	+	+
Xylose	+	+	+
Sorbitol	+	+	+
Adonitol	-	-	+
Trehalose	-	-	+
Lactose	-	+	+
Arabinose	-	-	-
Mannose	+	+	+
MR	-	-	+
VP	+	+	-
Licethinase	-	-	+
BHI	+	+	+
SIM	-/-/+	-/-/+	-/-/+
NO_3 reduction	+	+	+

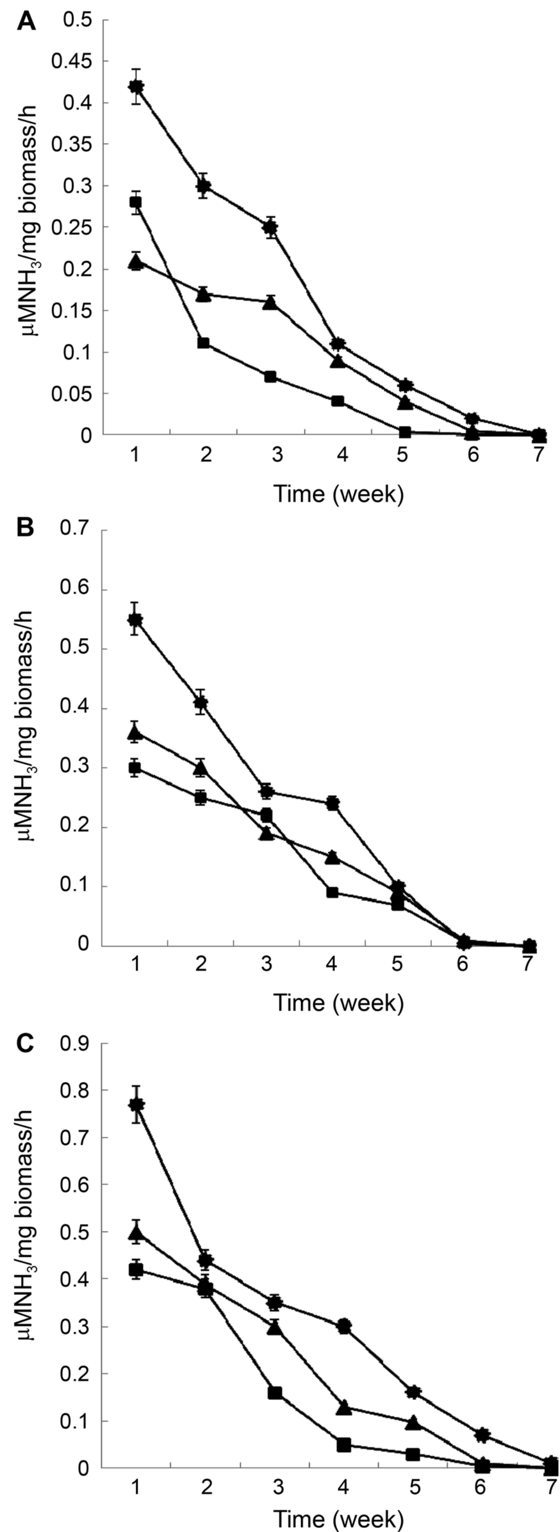

Fig. 4. Ammonium production with N-free broth medium (**A**), wet biomass (**B**), and dry biomass (**C**) of *Paenibacillus* strains (E, H, SH) incubated at 30°C for 7 weeks using the U-tube system. Among the three strains, strain E produced the most ammonium when grown in the N-free media and when using its biomass. In the case of all three *Paenibacillus* strains, the dry biomass produced more NH_3 than the wet biomass or broth medium. Strains: E (●), H (■), and SH (▲).

in the medium with sucrose as the sole carbon source. Thus, in the following experiments, sucrose was used as the sole carbon source in the N-free medium. Using biochemical tests according to *Bergey's Manual of Determinative Bacteriology* (as shown in Table 1), the best ammonium producers were characterized as *Paenibacillus* strains (E, H, and SH), which are Gram-positive, oxidase-negative, spore-forming nitrogen-fixing bacteria that grow on nitrogen-free media. These results also were confirmed by the CinnaGene Company according to the 16S rRNA.

Ammonium Production in U-Tube System

The results of the ammonium production by the *Paenibacillus* strains (E, H, and SH) in the U-tube system are shown in Fig. 4A, 4B, and 4C, where measurements were taken for 7 weeks until the production approached zero. Among the three strains tested, *Paenibacillus* strain E produced the most ammonium at 0.42, 0.55, and 0.77 µM NH_3/mg biomass/h when using the N-free broth medium, wet biomass, and dry biomass, respectively (Fig. 4A, 4B, and 4C). In addition, all three *Paenibacillus* strains produced more NH_3 when using the dry biomass, over the wet biomass and broth medium. The NH_3 production with the U-tube system reached a maximum after one week, and then decreased slowly until the production approached zero after seven weeks.

Ammonium Production by Ammonificators

The ammonium production from the ammonification process by the three non-nitrogen-fixing bacteria (*Listeria monocytogenes*, *Streptococcus faecalis*, and *Staphylococcus aureus)* is shown in Fig. 5, where the maximum NH_3 production was 0.20 µM/mg biomass/h by *Staphylococcus areus*.

Ammonium Production by Bioreactor System Containing N_2 and H_2 Gases

The best ammonium producer, strain E, was used to examine the production of ammonium with different gas treatments in a specially designed bioreactor. As expected, the control with no bacteria produced no ammonium after 7 days in the presence of the two gases (H_2 and N_2). Meanwhile, the highest amount of ammonium produced by the wet biomass and dry biomass of strain E was 0.68 and 1.50 µM/mg biomass/h, respectively, in 0.01 atmosphere of H_2 and N_2 (Fig. 6), which also emphasized the results of the U-tube system, where the dry biomass produced more NH_3 than the wet biomass. Here, the NH_3 production reached a maximum after 17 h, and then decreased slowly until the production approached zero after 165 h. Thus, more NH_3 was produced by the dry biomass and wet biomass when using the bioreactor than with the U-tube system, and the difference was statistically significant ($P<0.05$).

The NH_3 production results when using different gas treatments and the dry biomass of *Paenibacillus* strain E are shown in Fig. 7. Whereas 1 mg of the cell biomass of strain E without the H_2 and N_2 gases produced only 0.08 µM ammonium per hour (0.08 µM/mg biomass/h), the highest amounts of NH_3 were 1.50, 0.34, and 0.27 µM

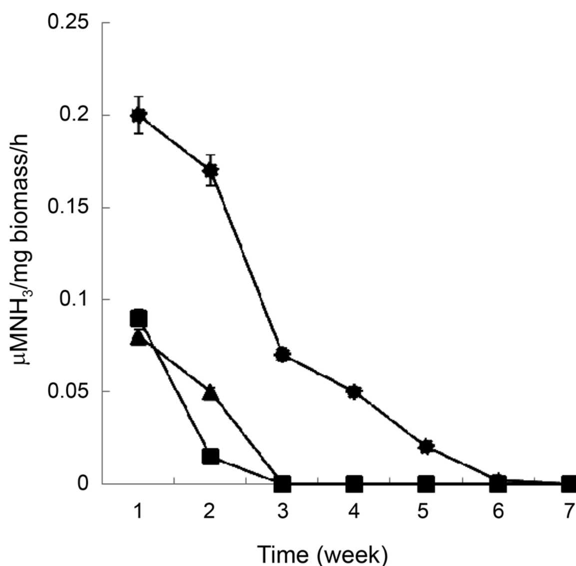

Fig. 5. Ammonium production by dry biomass of non-nitrogen-fixing bacteria incubated at 30°C for 7 weeks.

The amount of ammonium produced by these bacteria was lower than that produced by the *Paenibacillus* strains. *Listeria monocytogenes* (▲), *Streptococcus faecalis* (■), and *Staphylococcus aureus* (●).

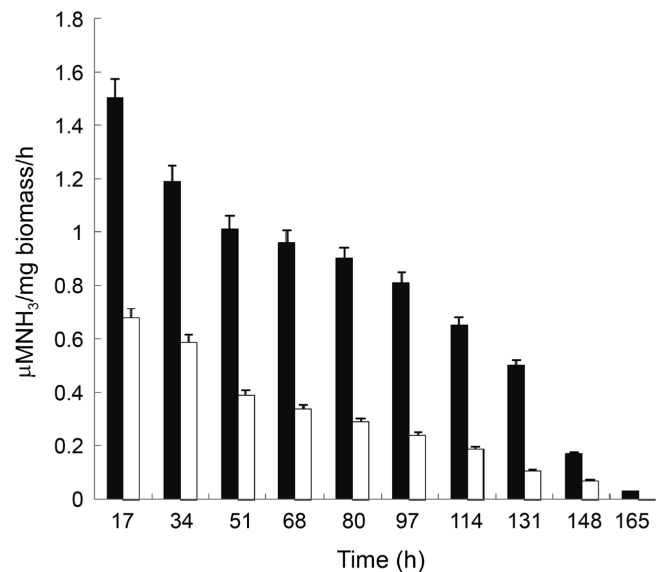

Fig. 6. Comparison of ammonium production by dry (■) and wet (□) biomass with 0.01 atmospheres of H_2 and N_2 during 165 h.

The dry biomass produced more NH_3 than the wet biomass.

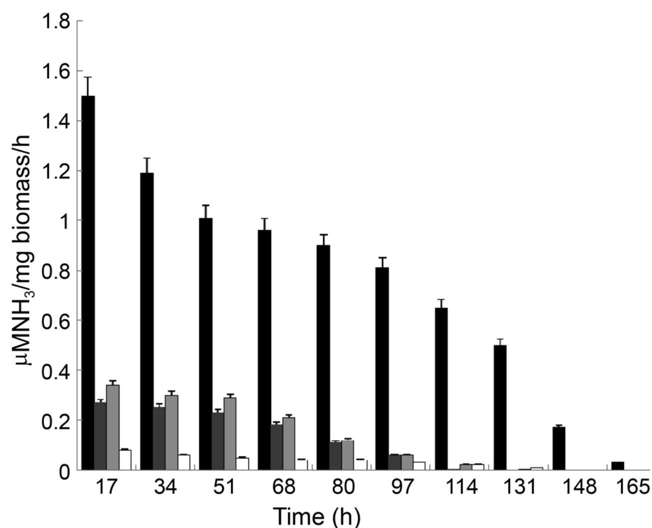

Fig. 7. Production of ammonium by dry biomass with different gas treatments.

The highest production of NH_3 was observed with 0.01 atmosphere of both N_2 and H_2. NH_3 production with $N_2 + H_2$ (■), N_2 (■), H_2 (■), and without gas (■) was tested.

NH_3/mg biomass/h in the presence of $N_2 + H_2$, N_2, and H_2, respectively. Therefore, a combined effluent of N_2 and H_2 was vital for the NH_3 production, which was significantly reduced when N_2 or H_2 was omitted.

A SEM image of the surface of the TiO_2 nanoparticles (Fig. 8) demonstrates the nanostructure of this composite. In the next experiment, a cell-free extract of *Paenibacillus* strain E adsorbed on the nanostructure of TiO_2 was used for NH_3 production, and the results were compared with those for the non-adsorbed form, as shown in Fig. 9. The highest NH_3 production by the cell-free extract (CFE) was 2.7 µM/mg CFE/h after 0.5 h, which then decreased until it approached zero after 2.5 h. Meanwhile, the cell-free extract adsorbed on TiO_2 produced 2.4 µM/mg CFE/h, yet the NH_3 production only approached zero after 17 h.

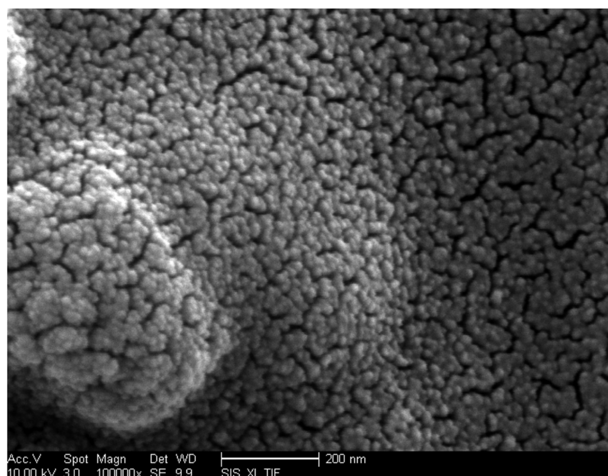

Fig. 8. SEM image of the composite nanostructure of TiO_2.

Discussion

In this study, NH_3-excreting strains of N-fixing *Paenibacillus* were isolated from soil. Colnaghi *et al*. [3] previously showed that ammonium assimilation by glutamine synthetase (GS) can be diminished or prevented by treating the bacteria with chemicals that inhibit GS activity, and in some diazotrophs, such treatment can result in the excretion of up to 15 mM ammonium into the liquid growth medium. Shanmugam and Valentine [16] created mutants that excrete fixed N_2 as NH_4^+ by genetic manipulation of nitrogenase and key glutamate-forming enzymes. A derepressed N_2 fixation mutant (SK-24) was also isolated that excreted up to 20.2 µmol of fixed N_2 as NH_4^+ per milligram of cell protein in 24 h at room temperature. Furthermore, Islam *et al*. [10] reported on ammonium production by certain free-living diazotrophic bacteria in peptone water.

The results of this study showed that seven strains of *Paenibacillus* were able to produce ammonium in the nitrogen-fixing process. All the strains were wild bacteria, freshly isolated with no genetic manipulation, and did not necessarily have natural enzymes that were involved in the assimilation of ammonium. Therefore, this study developed a new biotechnological method for ammonium production during the nitrogen-fixing process of *Paenibacillus* using a

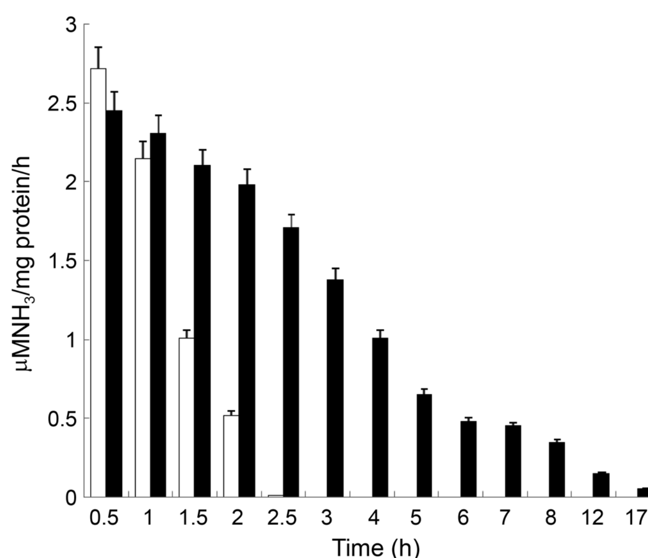

Fig. 9. Ammonium production by cell-free extract (□) and cell-free extract adsorbed on nanostructure of TiO_2 (■) with 0.01 atmosphere of H_2 and N_2.

The adsorption of the cell-free extract on the nanostructure of TiO_2 preserved the enzyme activities for a longer period of time (17 h).

simple U-tube system and bioreactor containing H_2 and N_2. The results showed that a dry biomass of the strains always produced more NH_3. Indeed, the high solubility of NH_3 in water caused a reduction in the NH_3 production by the wet biomass and broth medium when compared with that by the dry biomass. The *Paenibacillus* strains produced more NH_3 when using the bioreactor than with the U-tube system, and the highest production of NH_3 was observed in the presence of N_2 and H_2 together. Thus, both gases were essential for the maximum production of NH_3 under the different conditions, and the omission of either one caused a significant reduction in the NH_3 production. Rasche and Arp [14] showed that H_2 is a by-product of biological nitrogen fixation and inhibits the reduction of N_2 by nitrogenase. However, Allen *et al*. [1] suggested that H_2 functions as a scavenger of O_2, which is harmful to the nitrogenase enzyme and inhibits its inactivation. Emerich *et al*. [4] reported that the H_2-oxidizing hydrogenase in *R. japonicum* strain USDA 122 DES bacteroids increases the ATP supply, and supports nitrogenase activity. Here, it was shown that H_2 was needed for the maximum production of ammonium, and its omission caused a significant reduction in the NH_3 production. Recently, nanoporous materials with an extremely high surface area and controllable pore size have been used as a support host for biomolecules, such as enzymes [11, 12]. In the present study, to stabilize the enzyme function, a cell-free extract of NH_3-excreting *Paenibacillus* was successfully adsorbed on TiO_2 particles prepared by a sol-gel process. The TiO_2 particles were prepared with low-cost materials and had a high surface area. Although, the surface active site of TiO_2 and other nanostructure materials decreases with immobilization (coating on a substrate) or adsorption, by nanopowder TiO_2 with a high surface area, this reduction is modified [6]. Thus, the NH_3 production by the cell-free extract in the bioreactor was the highest in a short time, whereas the adsorption of the cell-free extract on the nanostructure of TiO_2 preserved the enzyme activities for a longer period of time.

In summary, this study has provided a simple method for producing NH_3 that facilitates product recovery (NH_3 is accumulated in a separate container that only contains distilled water, allowing easy recovery). Ammonia is currently made using the Haber-Bosch process invented in 1910, where pure nitrogen and hydrogen are reacted at a high temperature (~500^oC) and high pressure (~351 kPa). Thus, preparing these conditions is expensive and energy-intensive, requiring large amounts of natural gas, coal, or petroleum that are nonrenewable energy sources. In addition, the process produces CO_2, a gas implicated in the greenhouse effect [18]. In contrast, NH_3 production by *Paenibacillus* is possible at room temperature without considerable pressure, thereby reducing costs. Future studies will focus on optimizing the conditions for product enhancement and the industrial production of ammonium using this new method.

Acknowledgments

This study was supported by the University of Isfahan and "Standard and industrial research of Isfahan state". The authors would also like to thank Afsaneh Shokri, Dr. Arman Rostamzad, Dr. M. Khosravi, Dr. M. Habibi, and Amir Morshedi for their valuable assistance.

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