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The correlation of carbon source and ammonium accumulation in culture broth by nitrogen-fixing bacterial isolates

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Abstract

Several nitrogen-fixing bacteria were isolated from the agricultural soil samples of Kyaukse District, Mandalay Division, Myanmar. Among the isolated strains K-3 and T-7 were identified as *Azotobacter beijerinckii* and *Azotobacter vinellandii* and and M4 was identified as *Stenotrophomonas maltophilia*.. Although other studies have reported that wild types of the nitrogen-fixing bacteria cannot produce excess ammonia, our experiments indicated that these three strains accumulate relatively high amounts of ammonia in medium with a low concentration of sugar. *Azotobacter beijerinckii* accumulated approximately 0.48 mM of ammonia after incubation for 96 hour in 0.5% nitrogen free glucose mineral medium (NFGMM), while *A. vinelandii* accumulated approximately 0.36 mM in 0.25% nitrogen free fructose mineral medium (NFFMM) after 96 hour. *Stenotrophomonas maltophilia* accumulated highest amounts of ammonia in glucose supplemented medium approximately 0.52mM after 96h incubation. *A. beijerinckii* also accumulated ammonia in fructose, galactose, and sucrose media. *Azotobacter vinelandii* accumulated ammonia mainly in fructose and *Stenotrophomonas maltophilia* accumulated ammonia in glucose, sucrose and mannitol. In this research work, it was noted that ammonia began to accumulate when no sugar remained in the medium, therefore we suggested that carbon source plays a key role in ammonia accumulation.

Keywords: Agricultural soil samples, Ammonia accumulation, *Azotobacter beijerinkii*, *Azotobacter vineladii*, *Stenotrophomonas maltophilia*, Nitrogen-fixing bacteria.

INTRODUCTION

Myanmar is an agricultural country, and the agriculture sector is the backbone of its economy. One major economic objective in Myanmar is "Development of agriculture as a base and all-round development of other sectors of the economy as well." Therefore, it is important to increase agricultural production and the improvement of soil is one of the most common strategies. The currently used agricultural inputs are mostly chemical. The poor farm management technique and improper use of agrochemical has a result in both soil quality and environmental degradation. In order to avoid these problems, application of biofertilizer is considered today to limit the use of mineral fertilizer and supports an effective tool for desert development under less polluted environment, decreasing agricultural costs, maximizing crop yield due to providing them with an available nutritive elements and growth promoting substances [1] . Biofertilizers are living microbial inoculants that are added to the soil to improve the plant growth and can be used as an alternative source of chemical fertilizer [2]. Use of soil microorganisms which can either fix atmosphere nitrogen, solubilizing phosphate, synthesis of growth promoting substances, will be environmentally begin approach for nutrient management and ecosystem function [3]. Establishing environmentally friendly agricultural practices that do not emit carbon dioxide from the use of fossil fuels is imperative. Therefore, one must understand the natural ecosystems that utilize nitrogen-fixing bacteria to produce fertilizers such as ammonia that can be applied to agriculture production. Free-living nitrogen-fixing micro-organisms, such as *Azotobacter* and other bacteria that are widely distributed in the soil and rhizosphere, are targeted for these types of studies [4,5]. Nitrogen-fixing bacteria are being investigated because of their potential to replace the ammonia fertilizer produced by industrial processes. Although there have been many reports for nitrogen fixation of *Azotobacter* and *Rhizobium* species, there may have many other unknown strains that can fix atmospheric N_2. Reinhardt *et al*., 2008 found that new strain for nitrogen fixation, *Stenotrophomonas* strain, can fix atmospheric

nitrogen. They also said that in accordance with Liba *et al*., 2006, nitrogen fixation ability was a new character not previously reported for *Stenotrophomonas*, and only one publisher refers to a *Stenotrophomonas*-like strain able to fix atmospheric nitrogen [6]. *S. maltophilia* is a potential plant growth promoting bacterium. And Berg, 2006, said that *Stenotrophomonas* can be used for biological control of several plant pathogens (*Rhizoctonia solani*, *Verticillium dahliae*, *Pythium ultimum*). For bioremediation, *Stenotrophomonas* can use Xenobiotics, RDX and cocaine as carbon source. Suckstorff and Berg, 2003, reported that negative effects of *S. maltophilia* are on stem length and positive effects are on root growth and hair development. So, they reported that *S. maltophilia* from clinical source can produce 1.5µg IAA ml^{-1} and from environmental source can produce 3.4 µg IAA ml^{-1} [7].

We screened nitrogen-fixing bacteria using glucose as a carbon source from soils and found that three soil isolates of typical nitrogen-fixing bacteria (i.e., *A. Beijerinckii*, *A.vinelandii*, *S. maltophilia*) grown in nitrogen-free mineral medium (NFMM) can accumulate ammonia. Here, we report the accumulation of ammonia in wild-type nitrogen-fixing bacteria, the correlation with the carbon source in the medium, the time course, and ammonium accumulation by both wild-type cultures *Azotobacter* strains and *Stenotrophomonas* strain to demonstrate the generality of ammonia excretion by free-living nitrogen-fixing bacteria.

MATERIALS AND METHODS

Materials

The Visocolor Alpha Ammonia Detection Kit was obtained from Macherey-Nagel (Duren, Germany). All other chemicals used in this research were of the highest quality available from Kanto Chemical (Tokyo, Japan), Nacalai Tesque (Kyoto, Japan), and Wako Pure Chemical Industries (Osaka, Japan).

Media

Nitrogen-free mineral medium (NFMM) was used, and the composition of the screening medium for nitrogen-fixing bacteria was as follows (g/L): 1.0 K_2HPO_4, 1.0 $CaCl_2$, 0.5 NaCl, 0.25 $MgSO_4 \cdot 7H_2O$, 0.01 $FeSO_4 \cdot 7H_2O$, 0.01 $Na_2MoO_4 \cdot 2H_2O$, 0.01 $MnSO_4 \cdot 5H_2O$, 0.025 Bromo-Thymol Blue (BTB; if needed), and a carbon source that was either glucose (5 g/L) or sucrose (5 g/L). The pH of the medium was adjusted to 7.0. To study the relationship between the carbon source and ammonia accumulation, the medium used was the same as the screening medium, but carbon source was used with glucose, fructose, sucrose, galactose, mannose, citrate, mannitol or succinate at the same concentration. Solid medium was produced by adding 1.5% agar.

Isolation and screening of nitrogen fixing bacteria

20 soil samples were collected from the agricultural sites of Kyaukse District, Mandalay Division, Myanmar. To screen for nitrogen-fixing bacteria, 1 g of soil was suspended in 10 mL of sterilized dH_2O in a test tube that was left to stand until the soil solution settled. A 1-mL aliquot of supernatant was then added to 200 mL of NFGMM liquid medium and incubated for 1 week on a rotary shaker at 120 rpm and 35°C. Subculture was carried out twice by adding 2 mL of liquid culture to 200 mL of new NFGMM medium and incubated as before. Single-colony isolation was performed on NFGMM agar medium. Nitrogen-fixing activity was tested by growing the strains on NFGMM plates supplemented with BTB. Colonies that changed colour green to blue were stored in 80% glycerol at –80°C.

Identification of isolated bacteria by*16S rDNA* sequencing method

DNA extraction was performed using the Miniprep DNA Purification Kit (TaKaRa, Tokyo, Japan). Bacterial 16S rDNA was amplified using universal primers and 35 PCR cycles. Each cycle consisted of denaturation for 1 min at 94°C, annealing for 30 s at 60°C, and extension for 4 min at 72°C. DNA purification was done by using the DNA Extraction Kit (Roche Diagnostics GmbH, Mannheim, Germany). Nucleotide sequences were analysed using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and BLAST on the NCBI BLASTN.

Detection of Ammonia accumulation in culture broth

Ammonia was detected using Nessler's reagent. The bacterial culture solution was centrifuged at 13,000 rpm for 10 min at room temperature, and 0.5 mL of the supernatant was transferred into a new test tube. One drop of Nessler's reagent was added to the supernatant and mixed well. A yellow color is produced in the presence of ammonia; at higher ammonia concentrations, a brown precipitate forms.

The concentration of ammonia was estimated using the Visocolor Alpha Ammonia Detection Kit. After centrifuging the sample at 13,000 rpm for 10 min at room temperature, 1 mL of supernatant was transferred into a test tube. Two drops of NH_4-1 were added to the sample and mixed well, after which one-fifth spoon of NH_4-2 was added. After mixing, the sample was left at room temperature for 5 min. One drop of NH_4-3 was then added, mixed well, and the sample was left at room temperature for 5 min. The ammonium concentration was estimated from the color of the sample: yellow indicated 0 mg/L, whereas green indicated 3 mg/L of ammonium.

The concentration of ammonia was also detected using ion chromatography. After centrifugation at 13,000 rpm for 10 min at room temperature, the supernatant was filtered through a 0.2-µm filter before determining the concentration of ammonium using 861 Advanced Compact Ion Chromatography (Metrohm, Herisau, Switzerland). The cation eluent used was 4 mM H_3PO_4 with 5 mM 18-crown 6-ether. The separation column was an IC YK-421 (Shodex, Tokyo, Japan) and the guard column was an IC-YK-G (Shodex). A standard ammonium solution was prepared from $(NH_4)_2SO_4$; the concentration was adjusted to 1000 parts per million (ppm) and diluted to appropriate concentrations to obtain a standard curve. All experiments were replicated three times.

Selection of the best carbon source for three isolates to study the growth and ammonia accumulation

Three strains were cultured on 0.5% NFGMM plates with BTB at 35°C for 2–3 days before they were inoculated in a test tube in nitrogen-free liquid medium having different carbon sources. The concentration of the carbon sources was 0.5%. Bacteria were cultured by shaking at 35°C for approximately 2 days. Samples were taken at days 3 to determine the OD_{600}, pH, and concentration of ammonia in the medium. Each experiment was replicated three times.

Study on the effect of carbon concentration on ammonia accumulation by *Azotobacter* and *Stenotrophomonas* isolates

Azotobacter beijerinckii and *Stenotrophomonas maltophilia* were grown on 0.5% nitrogen free glucose mineral medium (NFGMM) plates for 2 days and then inoculated into 6 mL of NFGMM with various concentrations of glucose. The two strains were then incubated for 2 days. The optical density (OD), pH, and ammonium concentration of

the culture solution were measured to examine the relationship between the carbon source concentration and ammonia accumulation. *Azotobacter vinelandii* was also grown in nitrogen free fructose mineral medium (NFFMM) with various concentrations of fructose and incubated the same as for NFGMM. Three replicates of each experiment were performed.

Study on the growth and ammonia accumulation by *Azotobacter and Stenotrophomonas isolates* with the best carbon sources

The best carbon source concentration was chosen to examine the correlations among incubation time, ammonia accumulation, and carbon uptake. *A. beijerinckii*, *A. vinelandii* and *S. maltophilia* were pre-cultured in 6 mL of NFGMM and NFFMM, respectively, for 2 days, and 2 mL of liquid culture was added to 200 mL of fresh medium in 500-mL baffle flasks. Culture samples were taken at various times to measure the OD, pH, ammonium ion concentration, and remaining sugar. All incubations were carried out aerobically at 35°C on a rotary shaker at 200 rpm. Three replicates of each experiment were performed.

Estimation of remaining sugar in the culture medium

Remaining glucose in the medium was tested using a Glucose C_2 Kit purchased from Wako (Osaka, Japan) using the Mutarotase-GOD method and following procedural protocol. The same procedure was used to prepare glucose standard curves. Each experiment was replicated three times.

RESULTS

Isolation and screening of nitrogen fixing bacteria and 16S rDNA identification

From the 20 soil samples collected, three strains that showed colour changes to blue in the medium containing BTB were obtained, suggesting the excretion of ammonia into the agar medium. The nucleotide sequences of K-3 and T-7 showed high similarity (99%) to *A. beijerinckii and A.vinelandii and* M4 showed high similarity to *Stenotrophomonas maltophilia* (99%). Therefore an experiment was subsequently performed to determine the generality of ammonia accumulation by *Azotobacter and Stenotrophomonas* in which the typical culture strains used were *A. beijerinckii and A. vinelandii and Stenotrophomonas maltophilia*.

Selection of the best carbon source for three isolates to study the growth and ammonium accumulation

Azotobacter beijerinckii grew well in glucose, fructose, galactose, and sucrose media, but weak growth was observed in mannose and citrate and succinate media. The bacteria accumulated ammonium in almost all media. The highest quantity of ammonium was accumulated in glucose medium. Nearly the same amount of ammonium was accumulated in medium containing galactose, fructose and sucrose.

The highest growth of *A. vinelandii* was in fructose medium, followed closely by sucrose, whereas small amount of ammonia accumulated in glucose, galactose, and mannose media. For *A. vinelandii*, we were unable to detect ammonia in citrate and succinate media.

Stenotrophomonas maltophilia grew well in glucose and sucrose media, medium growth in mannose and citrate medium but weak growth in fructose, succinate and galactose media. The highest amount of ammonium was accumulated in glucose medium and was slightly higher than the amount accumulated in the sucrose medium. Small amount of ammonium was accumulated in fructose, mannose and galactose media. No ammonium was detected in succinate and citrate media. All the results are shown in Table 1.

Table 1: OD and Ammonium accumulation of bacterial isolates in culture broth with various carbon sources after two days incubation

Carbon source	K-3 (*Azotobacter beijerinckii*)		T-7 (*Azotobacter vinelandii*)		M-4 (*Stenotrophomonas maltophilia*)	
	OD_{600}	NH_4^+ (mM)	OD_{600}	NH_4^+ (mM)	OD_{600}	NH_4^+ (mM)
Glucose	0.928	0.256	0.542	0.028	0.701	0.298
Fructose	0.512	0.221	0.703	0.193	0.238	0.025
Mannose	0.256	0.052	0.562	0.017	0.466	0.040
Sucrose	0.682	0.228	0.655	0.179	0.693	0.254
Galactose	0.571	0.201	0.573	0.025	0.219	0.012
Succinate	0.236	0.048	0.361	ND	0.116	ND
Citrate	0.114	0.020	0.223	ND	0.397	ND

Study on the effect of carbon concentration on ammonia accumulation by *Azotobacter* and *Stenotrophomonas* isolates

For the isolated strains, growth increased with higher concentrations of the carbon source in the medium. Ammonia accumulation by *A. beijerinckii* and *S. maltophilia* cultured in NFGMM with various concentrations of glucose (0.1, 0.25, 0.5, 0.7, 1.0, and 2.0%) increased as the concentration of glucose increased from 0.1 to 0.5%, but decreased in 0.7%, 1.0% and 2.0% NFGMM (Table 2).

Table 2: OD and Ammonium accumulation of bacteria isolates in culture broth with various concentration of glucose after two days incubation

Glucose Concentration	*Azotobacter beijerinckii*		*Stenotrophomonas maltophilia*	
	OD600	NH4+(mM)	OD600	NH4+(mM)
0.10%	0.145	0.062	0.428	0.086
0.25%	0.486	0.117	0.574	0.288
0.50%	1.109	0.202	0.728	0.403
0.70%	1.406	0.080	1.611	0.226
1.00%	1.698	0.026	1.721	0.130
2.00%	1.522	ND	1.528	0.056

Azotobacter vinelandii had increased growth in medium with higher concentrations of fructose. Ammonium accumulation increased gradually as the fructose concentration increased from 0.10 to 0.25%, but decreased in 0.5% NFFMM. No ammonia was detected in 1.0 and 2.0% NFFMM. Thus, the optimum fructose concentration for ammonia accumulation by *A. vinelandii* was 0.25% (Table 3).

Table 3: OD and Ammonium accumulation of bacteria isolate in culture broth with various concentration of fructose after two days incubation

Fructose Concentration	*Azotobacter vinelandii*	
	OD_{600}	NH_4^+
0.10%	0.389	0.091
0.25%	0.578	0.206
0.50%	0.750	0.175
0.70%	1.391	0.046
1.00%	1.710	ND
2.00%	1.948	ND

Study on the growth, ammonia accumulation and remaining sugar by *A. beijerinckii* and *S.maltophilia* in glucose medium

For *A. beijerinckii and S.maltophilia,* the pH of the medium slowly decreased as the population grew due to the production of acidic substances from glycolysis. A sharp decrease in pH to around 6 was observed after 16 h of incubation. The pH of the medium started to increase at the end of the log phase of population growth, or early in the stationary phase due to the production of ammonium around 30 h after the start of incubation. Almost no glucose remained in the medium after 30 h of incubation. The pH of the medium was maintained at approximately 7.1–7.2 from the middle of the stationary phase, whereas the amount of ammonium gradually increased to 0.48 mM for *A. beijerinckii* and 0.52mM for *S.maltophilia* after 72 h of incubation.

Study on the growth, ammonia accumulation and remaining sugar by *A. vinelandii* in fructose medium

As with *A. beijerinckii* and *S. maltophilia*, the bacterial growth of *A. vinelandii* and the pH of the medium slowly decreased due to the production of acidic substances from glycolysis. The pH sharply decreased to 6.4 after 16 h of incubation, and the pH of the medium started to increase at the end of the log phase, or early in the stationary phase, due to the production of ammonium at approximately 16 h after the start of incubation. Medium pH remained neutral between 7.1 and 7.2 beginning at the middle of the stationary phase, whereas ammonium gradually increased and reached 0.36 mM after 72 h of incubation.

Thus, for three bacterial isolates, ammonia began to accumulate at the end of the log phase or in the early stationary phase. No carbon source was detectable in the medium at these times. Higher ammonia accumulation was observed in medium after longer incubation times, suggesting that the mechanism of nitrogen fixation might be influenced by sugars in the medium.

DISSCUSSION

In the case of *A. beijerinckii* and *S.maltophilia*, glucose is required for bacterial growth until the late logarithmic phase. The fixation of nitrogen during this time probably supports bacterial growth. Ammonium starts to accumulate when no more glucose is in the medium; this was confirmed by glucose determination using the glucose detection kit after 30 h of incubation. This suggests that the *nifL* and *nifA* genes might not function in the absence of carbon. Normally, with excess ammonium or ammonia, *nifL* is expressed and inactivates *nifA* so that ammonia is no longer produced [8]. We assume that a lack of glucose in the medium prevents the *nifL* system from functioning, so that *nifA* continues to produce ammonium outside of the cells, resulting in the ammonium accumulation in the medium.

For *A. vinelandii*, almost no ammonium accumulation was detected in the culture broth with glucose as the carbon source. However, ammonium accumulation was detected using fructose as the carbon source. Similar to the pattern for *A. beijerinckii*, ammonium accumulation started 16 h after incubation. At this time, the fructose remaining in the medium had decreased, and no fructose was detected using the Somogyi–Nelson method after 30 h of incubation. Similar to the case with *A. beijerinckii*, ammonium accumulation is thought to occur when no more carbon exists in the medium, indicating that no ammonia accumulation is detected if sufficient carbon is in the medium. In this case, however, ammonia may be detected with longer incubation times.

Our results suggest that a novel mechanism is involved in ammonia production by nitrogen-fixing bacteria in the absence of carbon in the medium. When the medium contains sufficient carbon, the bacterial cells retain the ammonia they produce within the cell, and no ammonia accumulates in the medium. When the medium contains no carbon, the bacterial cells lose their capacity to store ammonia, and the ammonia is excreted outside of the cell. Indirectly, the nitrogenase function is also influenced by the presence of carbon.

This research was highly relevant to Iwata,K, *et.al* (2010) which paper said ammonium clearly began to accumulate in the medium when all of the carbon had been used [9].

CONCLUSION

For three bacterial isolates in this study, ammonia began to accumulate at the end of the log phase or in the early stationary phase. No carbon source was detectable in the medium at these times. Higher ammonia accumulation was observed in medium after longer incubation times, suggesting that the mechanism of nitrogen fixation might be influenced by sugars in the medium.

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REFERENCES

1. El-Ghany Abd, Bouthaina F. 2010. Effect of some soil microorganisms on soil properties and wheat production under North Sinai Conditions. Journal of Applied sciences Research, 4(5):559-579.
2. Ivanova Evelina. 2005. Alginate based micro-capsule as inoculants carriers for production of nitrogen fixing biofertilizer. Department of Food Process Engineering, National High School of food Technology, France.
3. Wu, S.C, and ZH Cao, zg Li, K, C Cheung and M.H Wag. 2005. Effect of biofertilizer containing N-fixer, P and K solubilizers and AM fungi on maize growth: A green house trail. Geoderma, 125: 155-166.
4. Martinez-Toledo, M. V., Gonzalez-Lopez, J., and Ramos-Cormenzana, A. (1985) Isolation and characterization of Azotobacter chroococcum from the roots of Zea mays. FEMS Microbiol Ecol 31, 197-203.
5. Kennedy, I. R., and Tchan, Y. T. (1992) Biological nitrogen fixation in non-leguminous field crops: recent advances. Plant Soil 141, 93-118.
6. Elo and Haahtela, (1999). Nitrogen fixing bacteria isolated from forest soils in Finland. In: Current Plant Science and Biotechnology in agriculture (Pedrosc, F. O., Hungria, M., Yates, M. G., Newton, W. E., eds.), Kluwer Academic Publishers, Dordrecht 38, p.190.

7. Berg, (2006). Genotypic and Phenotypic Relationships between Clinical and Environmental Isolates of Stenotrophomonas maltophilia. J. Microbiology, November 1999, p.3594-3600,Vol. 37,No.11
8. Terzaghi, B., E. (1980) A method of isolation of Azotobacter mutants derepressed of Nif. Journal of General Microbiology 118, 275-2
9. Iwata, K., Azlan, A., Yamakawa, H. & Omori, T. (2010). Ammonia accumulation in culture broth by the novel nitrogen-fixing bacterium, Lysobacter sp. E4. Journal of Bioscience and Bioengineering 110 (4), 415-418