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## Ammonia Accumulation of Novel Nitrogen-Fixing Bacteria

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### 1. Introduction

Nitrogen is an essential element for many biological processes, including those occurring in plants (Ogura *et al.*, 2006). Despite the abundance of atmospheric nitrogen, production of nitrogen fertilisers by the Harber–Bosch process is increasing annually due to the deficiency of ammonia produced by biological nitrogen fixation—the enzyme-catalyzed reduction of nitrogen gas ( $N_2$ ). Concern over ‘greenhouse’ gasses emitted by the Harber–Bosch process has resulted in a research focus on nitrogen-fixing bacteria, and in particular, their genetic modification to excrete excess ammonia for agricultural purposes (Terzaghi, 1980; Saikia & Jain, 2007).

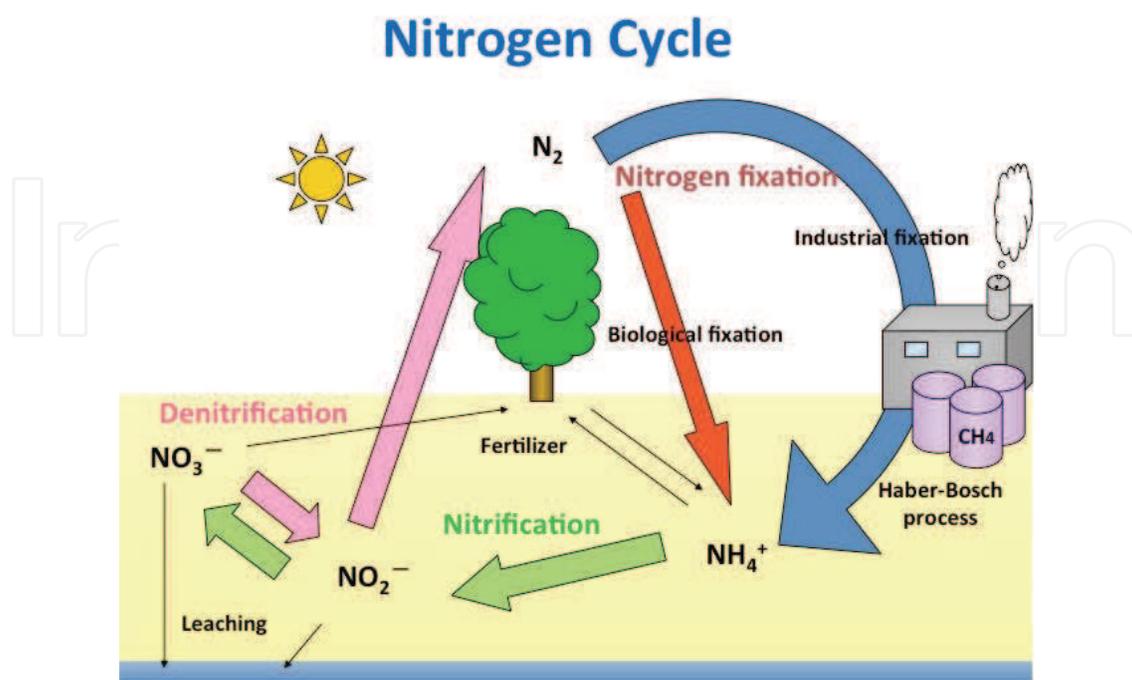


Fig. 1. The nitrogen cycle.

There are three main biological processes in the natural cycle of nitrogen (Fig. 1): fixation, nitrification and denitrification, which involve nitrogen-fixing, nitrifying and denitrifying bacteria, respectively.

Blue arrows indicate nitrogen fixation, including biological and industrial processes. Green arrows indicate microbial nitrification processes involving nitrifying bacteria, and pink arrows indicate microbial denitrification processes involving denitrifying bacteria. Black arrows indicate the flow of each compound in soils. The  $\text{NH}_3$  produced by nitrogen fixation may be assimilated into amino acids and thence to protein and other N compounds, or it may be converted by nitrifying bacteria to  $\text{NO}_2^-$  and  $\text{NO}_3^-$ . In turn,  $\text{NO}_3^-$  may enter metabolism through reduction to  $\text{NH}_4^+$  and subsequent assimilation to amino acids by bacteria, fungi and plants or can serve as an electron acceptor in denitrifying bacteria when oxygen is limiting. Losses from the nitrogen pool occur physically, when nitrogen (especially nitrate) is leached into inaccessible domains in the soils, and chemically, when denitrification releases  $\text{N}_2$ .

## 2. Biological nitrogen fixation

Decomposers use several enzymes to break down proteins in dead organisms and their waste, releasing nitrogen in much the same way as they release carbon. Proteinases break large proteins into smaller molecules. Peptidases break peptide bonds to release amino acids. Deaminases remove amino groups from amino acids and release ammonia.

According to Kneip *et al.* (2007), during biological nitrogen fixation (BNF), molecular nitrogen is reduced (Formula 1) in multiple electron-transfer reactions, resulting in the synthesis of ammonia and release of hydrogen. Ammonium is then used for the subsequent synthesis of biomolecules. This reduction of molecular nitrogen to ammonium is catalysed in all nitrogen-fixing organisms via the nitrogenase enzyme complex in an ATP-dependent, highly energy-consuming reaction (Fig. 2). The nitrogenase complex is composed of two main functional subunits, dinitrogenase reductase (azoferredoxin) and dinitrogenase (molybdoferredoxin). The structural components of these subunits are the Nif (nitrogen fixation) proteins: NifH ( $\gamma_2$  homodimeric azoferredoxin) and NifD/K ( $\alpha_2\beta_2$  heterotetrameric molybdoferredoxin). Three basic types of nitrogenases are known based on the composition of their metal centres: iron and molybdenum (Fe/Mo), iron and vanadium (Fe/V) or iron only (Fe). The most common form is the Fe/Mo-type found in cyanobacteria and rhizobia. Electrons are transferred from reduced ferredoxin (or flavodoxin) via azoferredoxin to molybdoferredoxin. Each mole of fixed nitrogen requires 16 moles ATP to be hydrolysed by the NifH protein. The  $\text{NH}_3$  produced is utilised in the synthesis of glutamine or glutamate for N-metabolism. NifJ: pyruvate flavodoxin/ferredoxin oxidoreductase, NifF: flavodoxin/ferredoxin). An important feature of the nitrogenase enzyme complex is its extreme sensitivity to even minor concentrations of oxygen. In aerobic environments and in photoautotrophic cyanobacteria, in which oxygen is produced in the light reaction of photosynthesis, nitrogenase activity must be protected. This protection is mediated by different mechanisms in nitrogen-fixing bacteria, depending on their cellular and physiologic constitutions. Aerobic bacteria (like *Azotobacter*) prevent intracellular oxygen concentrations from reaching inhibitory levels by high rates of

respiratory metabolism in combination with extracellular polysaccharides that reduce oxygen influx.

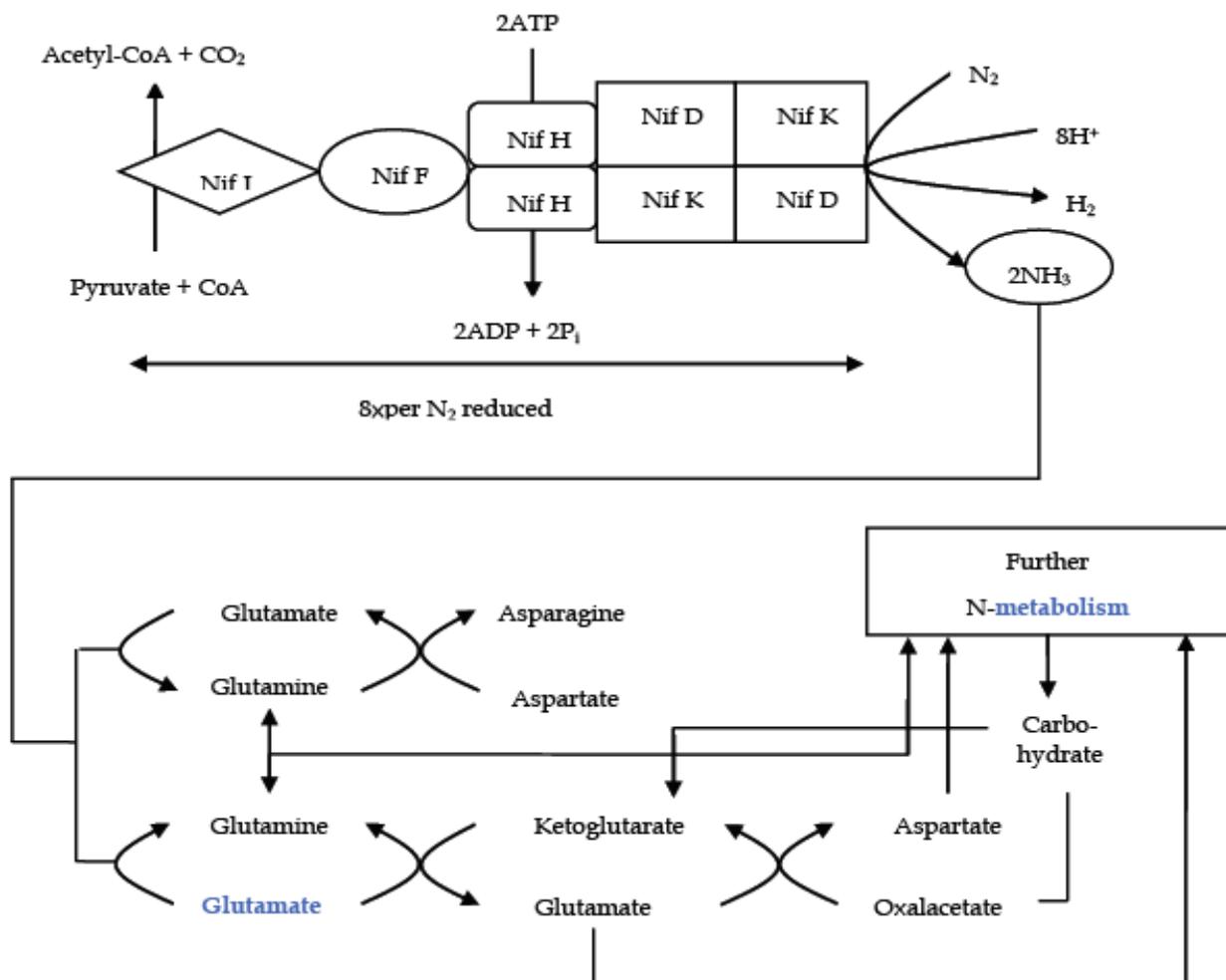
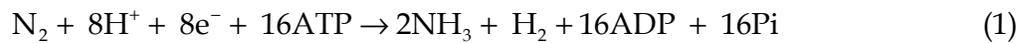


Fig. 2. Reactions and molecular mechanisms of biological nitrogen fixation.

General reaction of molecular nitrogen fixation. Schematic of the structure and operation of the nitrogenase enzyme complex and subsequent metabolism of nitrogen.

*Azotobacter vinelandii*, *Azotobacter beijerinckii* and *Klebsiella pneumoniae* are nitrogen-fixing bacteria commonly used for genetic modification. Metabolic mutants of *A. vinelandii* were first isolated over 50 years ago, but the mutants were unstable and some researchers were unable to mutate this bacterium. However, whether *Azotobacter* was itself difficult to mutate or the selection procedures were inadequate has remained unclear. Such failures have contributed to the continuing studies of this strain mutation.

Ultraviolet mutagenesis, the most easily controllable method of mutation, was thus often the first choice. Ultraviolet irradiation was used to modify *A. vinelandii* and *Azomonas agilis*, but the problems of segregation and mutant stability remained, despite their nitrogen-fixation

activity. Several years later, it became clear that nitrogen fixation by *Klebsiella pneumoniae* is complicated by the presence of biochemically and genetically distinct nitrogenase enzymes, each of nitrogenase enzymes is synthesized under different conditions of metal supply. However, experiments continued and Bali and colleagues (1992) generated the mutant MV376 of *A. vinelandii*, which excreted about 9.3 mM of ammonium in stationary phase cultures. No excretion by the wild type was reported (Bali *et al.*, 1992). Another improvement was achieved by Brewin and colleagues (1999), resulting in production of greater quantities of ammonium. Again, the wild type did not excrete ammonium (Brewin *et al.*, 1999).

The same results arose from mutation of *A. beijerinckii* by chemical mutagens such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) and ethylmethane sulphonate (EMS), together with UV radiation (Owen & Ward, 1985). The same group generated some mutants by means of transposon-insertion mutagenesis several years after the study using chemical mutagens. However, no mention was made of their ammonia-excreting activities, and again, the abnormal growth and instability of putative transposition isolates precluded routine use of the method.

With regard to the carbon sources used to culture these two species, most of the studies described above used Burk's medium, which contains 2% sucrose, or modified Burk's medium (0.5% or 2% glucose) as carbon sources. The latest researches on *A. vinelandii*, *A. beijerinckii* and a new nitrogen-fixing *Lysobacter sp.* have demonstrated that cultures grown in nitrogen-free medium with  $\leq 0.7\%$  glucose resulted in excrete ammonia. This suggests that no modification of these nitrogen-fixing bacteria is required. Even though the mechanisms remain unclear, further research on this topic will contribute greatly to the agriculture industry development (Iwata *et al.*, 2010).

### 3. Screening and identification of nitrogen-fixing bacteria

#### 3.1 Screening of nitrogen-fixing bacteria

To screen for nitrogen-fixing bacteria, 1 g of soil was suspended in 10 mL of sterilized dH<sub>2</sub>O in a 15-mL Eppendorff tube that was left to stand until the soil solution settled. A 1-mL aliquot of supernatant was then added to 200 mL of fresh NFMM or NFMM liquid medium and incubated for 1 week on a rotary shaker at 120 rpm and 30°C. Subculture was carried out twice by adding 2 mL of liquid culture to 200 mL of new C-NFMM medium and incubated as before. Single-colony isolation was performed on NFMM plates. Nitrogen-fixing activity was tested by growing the strains on glucose-NFMM plates substituted with BTB. From the 20 soil samples collected, we obtained four strains that showed a colour change in BTB-containing medium, suggesting excretion of ammonia. These strains were named C4, E4, G6 and G7.

#### 3.2 Identification of nitrogen-fixing bacteria

DNA extraction was performed using a Miniprep DNA Purification Kit (TaKaRa). Bacterial 16S rDNA was amplified over 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 performed using the Agarose Gel DNA Extraction Kit (Roche Diagnostics GmbH). Ligation

was conducted using the DNA Ligation Kit (TaKaRa) and the pT7 Blue T-vector (Novagen) as the plasmid. Transformation used *Escherichia coli* JM109, and plasmid purification was performed according to the manufacturer's protocols. Nucleotide sequences were analyzed using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI).

The nucleotide sequences of C4 and G7 showed high similarity (99%) to *A. beijerinckii*, and E4 and G6 were most similar to *Lysobacter enzymogenes* DMS 2043<sup>T</sup> (99% identity), as recently described. We therefore concluded that E4 and G6 belong to this genus. Subsequently an experiment was performed to determine of ammonia accumulation by *Azotobacter* using the common species *A. beijerinckii*, *A. vinelandii* and *Lysobacter* sp. E4.

### 3.3 Classification of isolated strains

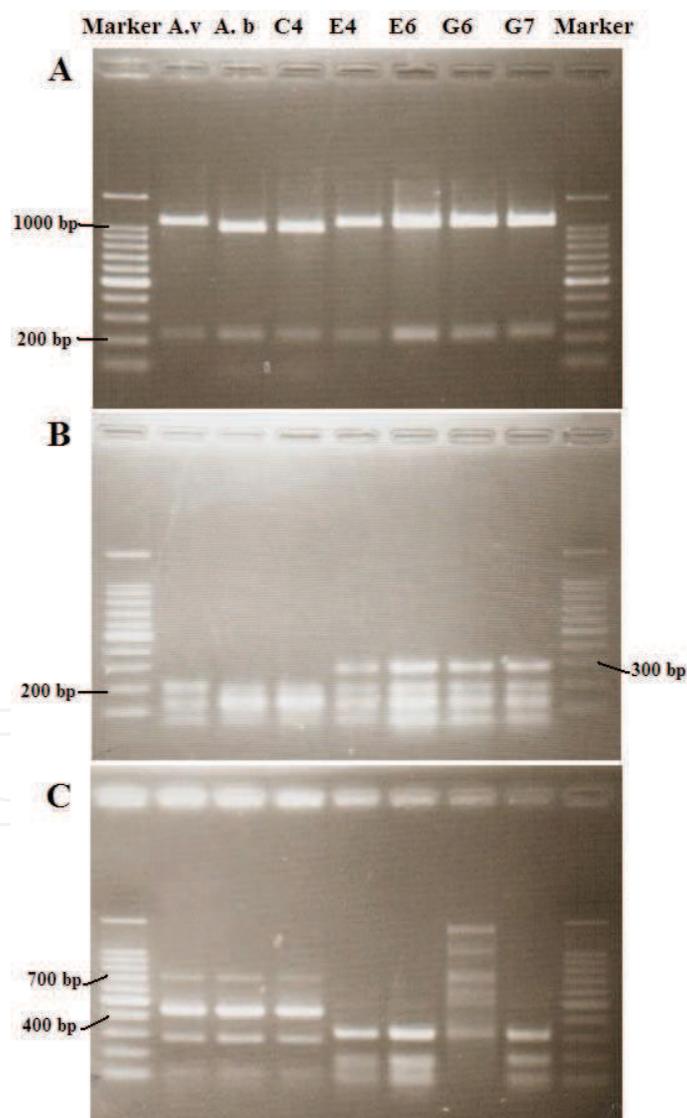


Fig. 3. RFLP analysis of the *nifL* gene of C4, E4, E6, G6, G7, *A. vinelandii* (A.v) and *A. beijerinckii* (A.b). (A) *AfaI*, (B) *HaeIII* and (C) *AluI*.

RFLP of the amplified *nifL* gene of C4, E4, G6 and G7 suggested that these may represent of nitrogen-fixing bacteria. Due to the similarities of strains C4, E4, E6, G6 and G7 to *Azotobacter* species and the amplification of the *nifL* gene from them, RFLP of the amplified *nifL* genes was conducted. Only strain C4 possessed the same restriction fragment pattern as *Azotobacter* species, showing the same length of fragments as both *A. vinelandii* and *A. beijerinckii* for *HaeIII* and *AluI* and as *A. beijerinckii* for *AfaI* (Fig. 3). From this result, it was assumed that the probability of this strain to belong to *A. beijerinckii* was high. E4, E6, G6 and G7 showed the same fragment lengths after digestion with *AfaI* and *HaeIII* but these four strains were divided into two groups by *AluI* digestion; G6 differed from the other three strains (C4, E4 and G7). Additionally, G6 and G7 showed different 16S rDNA RFLP fragment lengths; thus the data suggest that these represent different strains.

#### 4. Mutation of *Azotobacter nif* genes for ammonia accumulation

*Azotobacter* is a free-living nitrogen-fixing microbial genus widely distributed in soil and rhizosphere (Martinez *et al.*, 1985; Kennedy & Tchan, 1992). Considering the possibility of replacing industrially produced ammonia fertilisers, many attempts to modify two species of this diazotroph—*A. beijerinckii* and *A. vinelandii*—were undertaken with the aim of producing an environmentally friendly bacterial fertiliser (Brewin *et al.*, 1999). Generally, regulation of ammonia production by *Azotobacter*, especially *A. vinelandii*, is similar to that achieved by using *Klebsiella pneumoniae*, being regulated by *nifL* and *nifA*. The NifL protein binds to and inactivates NifA when ammonium is present where even at relatively low levels. At higher levels of ammonium, expression of the *nifLA* operon does not occur, and so NifA is not synthesized (Brewin *et al.*, 1999). An idea to mutate *nifL* for enhancing ammonia production by *Klebsiella pneumoniae* for agricultural purposes generated many studies to generate a mutant with a damaged *nifL* gene. Various methods of mutation were tested on *A. beijerinckii*, including UV radiation and chemical mutagenesis using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) and ethylmethane sulphonate (EMS). However, no ammonia-excreting mutants were isolated, even using the mating approach (Owen & Ward, 1985). This may have been due to the production by *Azotobacter beijerinckii* of polysaccharide that surrounds the cell (Danilova *et al.*, 1992), rendering mutation problematic. However, for *A. vinelandii* a mutation in *nifL* (upstream of and regulatory to *nifA*) was successfully produced. This mutant was named MV376, and it secreted significant quantities of ammonium during diazotrophic growth (Bali *et al.*, 1992). According to Bali *et al.* (1992), the mutant strain MV376, but not the wild type, showed ammonium production up to 10 mM when grown in Burk's sucrose medium.

#### 5. Accumulation of ammonia by wild-type strains

When wild-type *A. beijerinckii* and *A. vinelandii* were cultured in Glucose-Nitrogen Free Mineral Medium (G-NFMM) and Fructose-Nitrogen Free Mineral Medium (F-NFMM), respectively, both strains showed ammonium accumulation. This indicates that the concentration, as well as the nature, of the carbon source might influence ammonium accumulation; here we report a correlation of carbon source concentration with ammonium accumulation by both *Azotobacter* species.

## 6. Ammonia detection and estimation

Ammonia concentration was estimated using the Visocolor Alpha Ammonia Detection Kit (Macherey-Nagel). After centrifugation at 13,000 rpm for 10 min at room temperature (RT), supernatant (1 mL) was transferred into a test tube. Two drops of NH<sub>4</sub>-1 were added to the sample and mixed well, after which one-fifth of a spoonful of NH<sub>4</sub>-2 was added. After mixing, the sample was left at RT for 5 min. One drop of NH<sub>4</sub>-3 was then added, mixed well and left at RT for 5 min.

Ammonia concentration was also estimated using ion chromatography. After centrifugation at 13,000 rpm for 10 min at RT, the supernatant was passed through a 0.2- $\mu$ m filter and the ammonium concentration determined using an 861 Advanced Compact Ion Chromatography (Metrohm). The cation eluent used was 4 mM H<sub>3</sub>PO<sub>4</sub> with 5 mM 18-crown 6-ether. The separation column was an IC YK-421 (Shodex) and the guard column was an IC-YK-G (Shodex). Standard ammonium solution was prepared from (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; the concentration was adjusted to 1000 parts per million (ppm) and diluted appropriately to obtain a standard curve. All experiments were performed in triplicate.

## 7. Cultivation of nitrogen-fixing *Lysobacter* sp.

*A. beijerinckii*, *A. vinelandii* and *Lysobacter* sp. were grown on 0.5% G-NFMM plates for 2 days and then inoculated into 6 mL G-NFMM or F-NFMM liquid media, respectively, containing various glucose and fructose concentrations. These species were then incubated for 2 (*Azotobacter*) or 3 (*Lysobacter*) days. Optical density (OD), pH and ammonium concentrations were then measured to examine the relationship between the carbon source concentration and ammonia accumulation. Best concentration was chosen for examining the correlations among incubation time, ammonia accumulation and carbon uptake. *A. beijerinckii*, *A. vinelandii* and *Lysobacter* sp. were pre-cultured in 6 mL of 0.5% G-NFMM and 0.25% F-NFMM, respectively, for 2 days and 2 mL was then transferred to 200 mL fresh media in 500-mL baffle flasks. Samples of cultures were taken at different times for measurement of OD, pH, ammonium ion and concentration of carbon source. All incubation periods were carried out aerobically at 30°C with shaking (200 rpm). Culture samples were centrifuged and filtered (0.2  $\mu$ m) before being ammonium assayed by Nessler's reagent; ammonium concentration was estimated by ion chromatography. The cation eluent used for ion chromatography was 4 mM H<sub>3</sub>PO<sub>4</sub> added to 5 mM 18-crown 6-ether. The residual carbon concentration in media was assayed by Somogyi-Nelson method. All experiments were performed in triplicate.

## 8. Effect of carbon concentration

The optimum carbon source concentration was used to determine the correlations among incubation time, ammonia accumulation and carbon uptake. *Azotobacter beijerinckii* and *A. vinelandii* were pre-cultured in 6 mL G-NFMM and F-NFMM, respectively, for 2 days and 2 mL was transferred to 200 mL fresh medium in 500-mL baffle flasks. The OD, pH, ammonium ion and residual sugar levels in cultures were determined. All incubation periods were carried out aerobically at 30°C on a rotary shaker at 200 rpm. Experiments were performed in triplicate. For *A. vinelandii*, almost no ammonium accumulation was

detected in culture broth containing glucose as the carbon source (Table 1). However, ammonium accumulation was detected with fructose (Table 2). Similar to *A. beijerinckii*, ammonium accumulation started 16 h after incubation. At this time, the fructose level in the medium had decreased, and no fructose was detected using the Somogyi–Nelson method after 20 h incubation.

Glucose concentration		0.10%	0.25%	0.50%	0.70%	1.00%	2.00%
<i>A. beijerinckii</i>	OD	0.145	0.486	1.109	1.406	1.698	1.522
	pH	7.0 (7.0)*	7.0 (7.0)*	6.8 (7.1)*	6.6 (7.1)*	6.4 (7.1)*	6.3 (7.1)*
	NH <sub>4</sub> <sup>+</sup>	0.062	0.117	0.202	0.080	0.026	0.001
<i>A. vinelandii</i>	OD	0.189	0.478	0.950	1.391	1.710	1.948
	pH	7.1 (7.1)*	6.8 (7.1)*	6.1 (7.1)*	4.9 (7.1)*	4.7 (7.1)*	4.7 (7.0)*
	NH <sub>4</sub> <sup>+</sup>	0.010	0.024	0.020	0	0	0

OD: optical density (600 nm). \*Figures in parentheses show the value before incubation.

Note: ammonium ion concentration is in mM. Presence of ammonium was primarily tested using Nesler's reagent before the concentration was determined by ion chromatography.

Table 1. OD, pH and ammonium accumulation by *A. beijerinckii* and *A. vinelandii* in G-NFMM liquid medium of various glucose concentrations after 2 days incubation.

		Glucose	Fructose	Galactose	Mannose	Sucrose	Citrate	Succinate
<i>A. beijerinckii</i>	OD	0.518	0.739	0.564	0.029	0.656	0.005	0.212
	pH	7.3 (7.0)*	7.2 (7.0)*	7.1 (7.1)*	7.1 (7.1)*	7.1(7.1)*	7.4	8.6 (7.2)*
	NH <sub>4</sub> <sup>+</sup>	0.296	0.315	0.201	0.041	0.192	(7.0)*	N. D.
<i>A. vinelandii</i>	OD	0.442	0.704	0.573	0.122	0.655	0.361	0.361
	pH	7.0 (7.0)*	7.2 (6.9)*	7.1 (7.0)*	7.1 (7.1)*	7.2(7.0)*	8.4	8.8 (7.2)*
	NH <sub>4</sub> <sup>+</sup>	0.026	0.179	0.025	0.017	0.63	(7.0)*	N. D.

N.D.: not determined, OD: optical density (600 nm). \*Figures in parentheses show the value before incubation.

Note: ammonium ion concentration is in mM. Presence of ammonium was primarily tested using Nesler's reagent before the concentration was determined by ion chromatography.

Table 2. OD, pH and ammonium accumulation by *A. beijerinckii* and *A. vinelandii* in G-NFMM liquid medium containing various carbon sources after 2 days incubation.

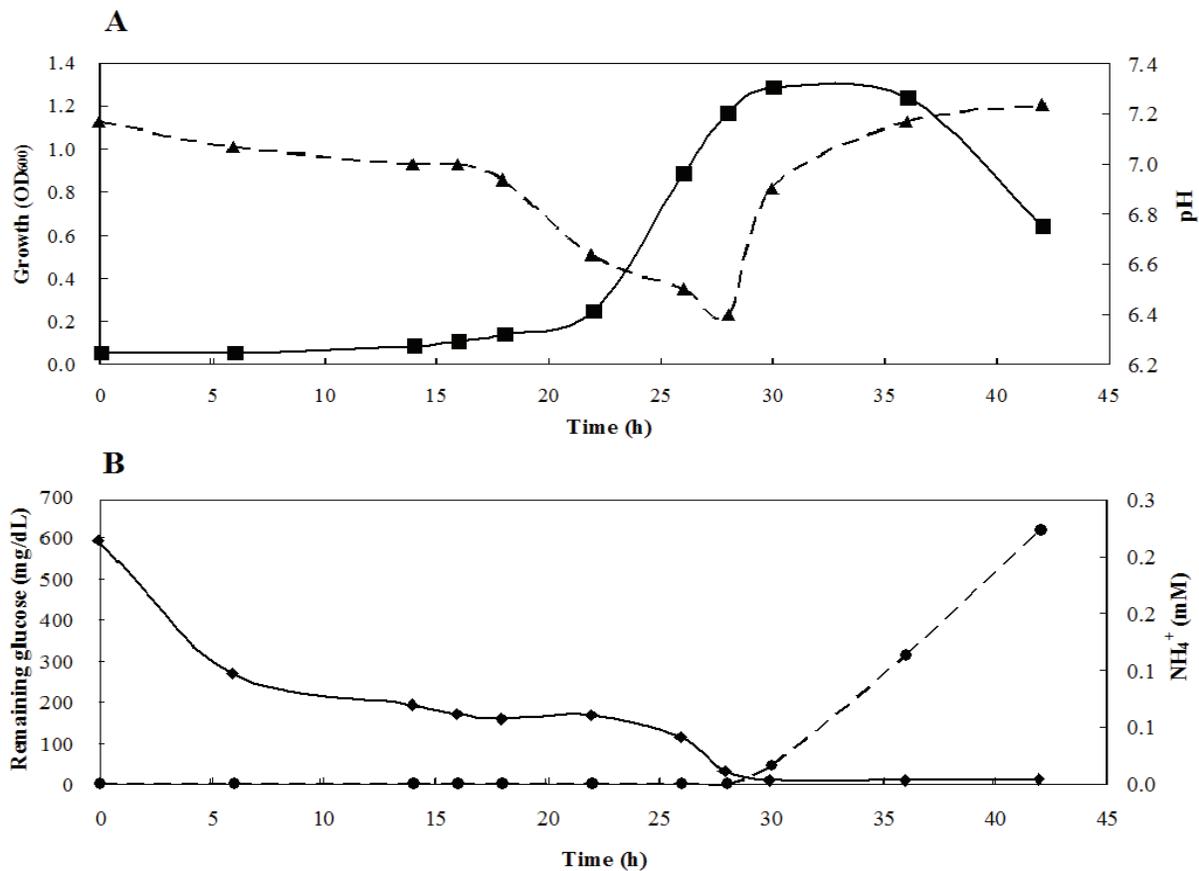


Fig. 4. A: Growth (■), pH (▲), ammonium concentration of *Azotobacter beijerinckii*. (●) B: remaining glucose concentration (◆) in cultures of *Azotobacter beijerinckii*. Samples were removed for analysis at the indicated times.

## 9. Time course of ammonia accumulation

As the *A. beijerinckii* population increased, medium pH decreased slowly due to production of acidic substances from glycolysis; a sharp decrease to pH 6.4 occurred after 16 h (Fig. 4A). Medium pH began to increase at the end of the log phase or early stationary phase due to production of ammonium around 30 h after inoculation. Medium pH remained steady at 7.1–7.2 beginning in the middle of stationary phase, whereas the amount of ammonium gradually increased to 0.46 mM after 54 h incubation (Fig. 4B).

## 10. Time course of ammonia accumulation by *Lysobacter* sp.

Time-course experiments suggested that ammonia accumulation began upon glucose depletion. In the 0.30% medium, no glucose remained after incubation for 3 days, resulting in ammonia accumulation. In media with higher glucose concentrations, residual glucose was present after 3 days. As a result, no ammonia accumulation occurred; longer incubation times may have resulted in production of detectable levels of ammonia (Fig. 5A).

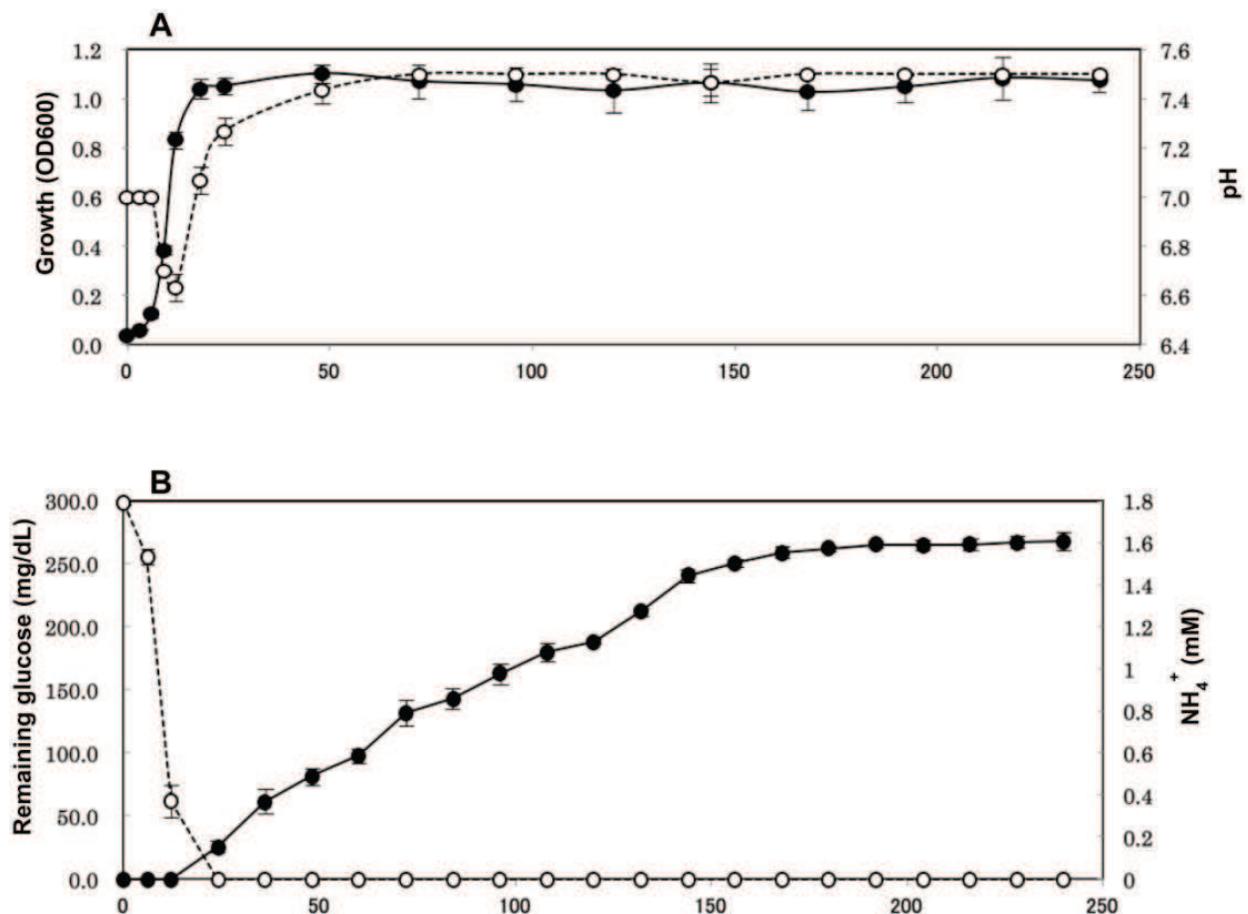


Fig. 5. A: Growth (■), pH (▲), ammonium concentration of *Lysobacter* sp. E4. (●) B: remaining glucose concentration (◆) in cultures of *Lysobacter* sp. E4. Samples were collected for analysis at every ten hours.

## 11. Effect of remaining sugar on ammonia accumulation

Residual sugar levels were determined using a glucose detection kit, according to the manufacturer's protocol (Miwa et. al., 1972). For *A. beijerinckii*, the concentration of glucose slowly decreased. Almost no glucose remained in the medium after 30 h incubation, at which point ammonia began to accumulate.

These data suggest that ammonia accumulation by strain E4 is dependent on sugar concentration. Glucose is required for bacterial growth until the middle of the logarithmic phase, and fixation of nitrogen during this period likely supports bacterial growth. Ammonia starts to accumulate when no more glucose remains in the culture, as shown by glucose and ammonia determinations after 14 h incubation (Fig. 5B).

For *A. vinelandii*, as for *A. beijerinckii*, bacterial growth and medium pH decreased slowly due to production of acidic substances from glycolysis; a sharp decrease to pH 6.4 occurred after 8 h. Medium pH began to increase at the end of log phase or early stationary phase due to production of ammonium approximately 16 h after inoculation. Medium pH remained neutral at 7.1–7.2 beginning in the middle of stationary phase, whereas ammonium levels gradually increased, reaching 0.1 mM after 28 h.

Thus, in both strains, ammonia began to accumulate at the end of log phase or in early stationary phase; no carbon source could be detected in the medium at this time. Higher ammonia levels in the medium will likely be detected after more than 30 hours, longer incubation times, suggesting that the mechanism of nitrogen fixation might be influenced by sugar levels in the medium.

E4 strain grew well at pH 7.0 and produced the highest concentration of ammonia (~0.4 mM). Although media at pH 8.0 resulted in the greatest growth, ammonia accumulation was lower than at pH 7.0, suggesting that accumulated ammonia at the higher pH value may have been used for bacterial growth (Fig. 5B).

Ammonia was detected in E4 cultures incubated at 30°C, but not at 20°C. Ammonia may accumulate at 20°C after longer incubation times, since some glucose remained after 3 days incubation.

## 12. Conclusions

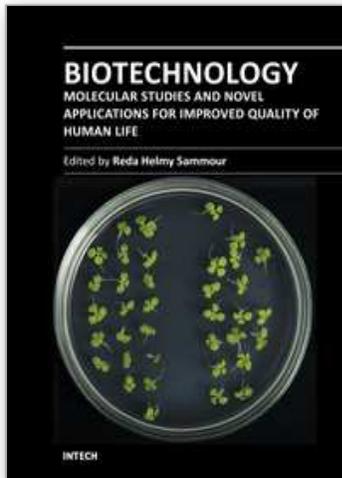
From the above, the following conclusions could be drawn. Firstly, the ammonium accumulation is clearly dependent on the carbon source concentration. Higher ammonium accumulation occurred in media with lower concentrations of the carbon source. Glucose was required for growth of *A. beijerinckii* until late logarithmic phase. Fixation of nitrogen during this time likely supports bacterial growth. Ammonium starts to accumulate after glucose depletion as determined by the Somogyi-Nelson method after 30 h incubation, which suggests that regulation of *nifL* and *nifA* genes might not be functioning when the medium contains less than 2.0% glucose. Normally, in the presence of excess ammonium or ammonia, *nifL* is expressed, resulting in repression of *nifA* and cessation of ammonia production. However, when glucose levels drop to 2.0% or less (0.5% for this experiment), we consider believe that the lowered glucose concentration renders the *nifL* system nonfunctional. This results in continuing *nifA*-mediated extracellular ammonium production and accumulation in the medium.

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## **Biotechnology - Molecular Studies and Novel Applications for Improved Quality of Human Life**

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