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ACCUMULATION OF AMMONIA IN CULTURE BROTH BY WILD-TYPE NITROGEN-FIXING BACTERIUM, *STENOTROPHOMONAS MALTOPHILIA*

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ABSTRACT: *Stenotrophomonas maltophilia* was isolated from paddy field of Kyaukse District, Mandalay Division, Myanmar. Nitrogen fixing activity of *S. maltophilia* was detected in nitrogen free mineral medium supplemented with different carbon sources. *S. maltophilia* accumulated highest amounts of ammonia in glucose supplemented medium. But their nitrogen fixing activity was best at 0.7% glucose concentration. When 0.5609 ppm of glucose still remained in the medium after 24 hours incubation, no ammonia was detected. After incubation of 32 hours, ammonia began to accumulate and the amount of 0.5 ppm of ammonia was detected but no sugar remained in the medium. Highest amount of ammonia (2 ppm) was accumulated in the medium after 48 hour incubation. So we found that ammonium accumulation began when no sugar remained in the medium, supposing that sugar plays a key role in ammonia accumulation.

Keywords: Ammonium accumulation, carbon sources, nitrogen fixing bacteria, *Stenotrophomonas maltophilia*

INTRODUCTION

Bacteria and blue green algae are able to reduce atmospheric nitrogen to ammonia. Some organisms (microorganisms) can fix atmospheric N_2 by nitrogenase enzyme. But some plants assimilate N_2 by the help of accompanying microorganisms capable of assimilating N_2. Mostly, nitrogen fixing bacteria are present around roots, or inside plant tissues, and fix nitrogen and contribute to plants nitrogen nutrition. Nitrogen fixing strains have also been reported for *Acetobacter*, *Azotobacter*, *Campylobacter*, and *Pseudomonas*. But if new strains for nitrogen fixation are found, taxonomic identification of several unknown nitrogen-fixing organisms can be accomplished through sequencing of the nifH gene, which is also useful their genetic potential for the nitrogen fixation (Zehr et al., 1995). NifH genes can be employed as markers for the detection and study of the genetic diversity of diazotrophic organisms in microbial communities, like those in rice roots (Ueda et al., 1995) or forest soil (Widmer et al., 1999). Putative nitrogenase amino acid sequences revealed that more than half of the nifH products were derived from methylotrophic bacteria, such as Mthylocella spp. The next most frequent sequence types were similar to those from Burkholderia (Izumi et al., 2006).Several of them do even live in symbiosis, or in loose association with green plants. The nodule bacteria (e.g. *Rhizobium*) of leguminosae are best known. A number of free-living soil bacteria, e.g. bacteria of the genera *Azotobacter*, *Closterium*, *Klebsiella* and *Rhodospirillum* belong to the nitrogen reducing species. The genetic basis of nitrogen fixation is largely known. In nitrogen fixation, the nitrogenase complex takes up a key position. The encoding and the regulation of this protein are controlled by a certain DNA region, the nif region.

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 (Elo and Haahtela, 1999). *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}.

In this study, we screened nitrogen fixing activity of *S. maltophilia* in nitrogen free mineral medium supplemented with different carbon sources. After screening, we report the accumulation of ammonia in wild type nitrogen fixing bacteria, the correlation with the carbon source in the medium, and the time course.

MATERIALS AND METHODS

Materials

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

Sample Collection

Soil samples were collected from various paddy fields from Kyaukse District, Mandalay Division in central Myanmar.

Isolation of Nitrogen Fixing Bacteria

Nitrogen-free mineral medium (NFMM) was used to isolate nitrogen fixing bacteria. The composition of the isolated medium 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 $MnSO_4 \cdot 5H_2O$ and a carbon source was glucose (20 g/L). Solid medium was produced by adding 2% agar.

Screening of Nitrogen Fixing Activity

The visual detection of nitrogen fixing activity was observed by using Nitrogen Free Mineral Medium with 0.7% and 2% Glucose and BTB. The media were prepared both agar and broth. After 3 to 7 days incubation, changing the color of the medium was recorded. To detect nitrogen fixing activity from the broth culture, the reagents of ammonium test kit were added and the appeared color was noted by comparing the color chart on the test kit. To study the relationship between the carbon source and ammonia accumulation, the medium used was the same as the screening medium, but glucose was replaced with fructose, sucrose, mannitol, arabinose, cellulose or succinic acid at the same concentration.

16S rDNA Identification

DNA extraction was performed using the Miniprep DNA Purification Kit (TaKaRa, Tokyo, Japan). Bacterial 16S rDNA was amplified using 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 using the Agarose Gel DNA Extraction Kit (Roche Diagnostics GmbH, Mannheim, Germany). Ligation was conducted using the DNA Ligation Kit (TaKaRa) and the pT7 Blue T-vector (Novagen) as the plasmid. Transformation was carried out using *E. coli* JM109, and plasmid purification was performed according to the manufacturer's protocol. Nucleotide sequences were analyzed using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and BLAST on the NCBI BLASTN.

Ammonia Accumulation by *Stenotrophomonas maltophilia* with Different Carbon Sources

Isolated bacterium was cultured on 0.5% G–NFMM plates with BTB at 30°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 30°C for approximately 2 days. Samples were taken at days 0 and 2 to determine the OD_{600}, pH, and concentration of ammonia in the medium. Each experiment was replicated three times.

Effect of Carbon Concentration on Ammonia Accumulation by *Stenotrophomonas maltophilia*

S. maltophilia was grown on 0.5% glucose-nitrogen free mineral medium (G–NFMM) plates for 2 days and then inoculated into 6 ml of G–NFMM with various concentrations of glucose and 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. Three replicates of each experiment were performed.

Time Course of Ammonia Accumulation by *Stenotrophomonas maltophilia*

The best carbon source concentration was chosen to examine the correlations among incubation time, ammonia accumulation, and carbon uptake. *S. maltophilia* was pre-cultured in 6 ml of G–NFMM 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 30°C on a rotary shaker at 200 rpm. Three replicates of experiment were performed.

Detection of Ammonia Accumulation in Culture Broth

The concentration of ammonia was estimated using the Visocolor Alpha Ammonia Detection Kit. After centrifuging the sample at 10,000 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 colour of the sample: yellow indicated 0 mg/L, whereas green indicated 3 mg/L of ammonium.

Estimation of Remaining Sugar

Remaining glucose in the medium was tested using a Glucose C2 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 AND DISCUSSION

Screening of Nitrogen Fixing and Ammonia-Producing Bacteria and 16S rDNA Identification

Isolated strain showed colour changes to blue in the GNFM-medium containing BTB, suggesting the excretion of ammonia into the agar medium. The nucleotide sequences of this strain showed high similarity (99%) to *S. maltophilia*. Therefore, an experiment was subsequently performed to determine the generality of ammonia accumulation by *S. maltophilia.*

Ammonia Accumulation by *Stenotrophomonas maltophilia* with Different Carbon Sources

S. maltophilia grew well in glucose, fructose, mannitol, and sucrose media, but no growth was observed in succinic acid medium and only a small amount of growth occurred in arabinose and cellulose media shown in Table 1. The bacteria accumulated ammonium in only glucose, sucrose and mannitol media. The highest quantity of ammonium was accumulated in glucose medium.

Ammonia Accumulation in Culture Broth with Different Glucose Concentrations

For the isolated strain, growth increased with higher concentrations of the carbon source in the medium shown in Table 2. Ammonia accumulation by *S. maltophilia* cultured in G–NFMM 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 0.7%; no ammonium was detected in 1.0 and 2.0% G–NFMM shown in Table 2.

pH decreased gradually as the concentration of the carbon source increased, except for 0.7% glucose. A reduction in pH suggests that more acidic substances were produced when the carbon concentration was higher.

Ammonium accumulated from the wild type strain in medium that had carbon concentrations <1.0% glucose. The optimum concentration of carbon for ammonia accumulation was 0.7%. A decrease in the number of viable cells from cell lysis was not observed during ammonia accumulation. These results suggest that ammonia accumulation by wild-type *S. maltophilia* is correlated with the concentration of the carbon source in the medium.

Time Course of Ammonia Accumulation by *Stenotrophomonas maltophilia* in Glucose Medium

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 6.4 was observed after 24 hours of incubation shown in Fig. 1. 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. The amount of ammonium gradually increased to 2 ppm after 60 h of incubation and it was shown in Fig. 1. Higher ammonia accumulation was observed in medium after longer incubation times.

Estimation of Remaining Sugar

The concentration of glucose slowly decreased. Almost no glucose remained in the medium after 24 h of incubation, when ammonia began to accumulate in the medium. Ammonium clearly began to accumulate in the medium when all of the carbon had been used. In this study, 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 32 h of incubation. It may seem that *S. maltophilia* cannot fix atmospheric nitrogen in the presence of carbon source.

Table 1. OD, pH and ammonium accumulation in NFMM liquid medium with various carbon sources by *S. maltophilia* after 2 days incubation

Carbon sources	NH_4^+ (ppm)	OD	pH
Glucose	2	1.155	6.7
Sucrose	1	1.415	6.4
Fructose	0	1.127	6.3
Mannitol	0	1.223	5.9
Arabinose	0	0.884	5
Cellulose	0	0.341	5.4
Succinic acid	0	0.011	5.2

Table 2. OD, pH and ammonium accumulation in NFMM liquid medium with various glucose concentrations by *S. maltophilia* after 2 days incubation

Glucose concentration (%)	NH_4^+ (ppm)	OD	pH
0.1	0.1	0.428	6.6
0.25	0.2	0.718	6.3
0.5	1	0.574	6.3
0.7	2	0.574	6.6
1	0	1.611	5.1
2	0	1.440	4.5

Table 3. pH, OD, Viable Count, Glucose remaining and ammonium concentration in 0.7% G-NFMM liquid medium by *S .maltophilia*

Incubation time (h)	pH	OD	Viable Count (CFU/ml)	Glucose remaining (ppm)	NH_4^+ (ppm)
0	7.6	0.0615	5.0×10^4	1.667	0
8	7.23	0.0871	6.1×10^4	2.0051	0
16	7	0.1263	2.2×10^5	1.3663	0
24	6.4	1.0595	9.0×10^7	0.5609	0
32	7.4	1.3382	1.1×10^8	0	0.5
40	7.7	1.287	3.0×10^7	0	1
48	7.76	1.3005	6.0×10^7	0	1.5
60	7.53	1.2723	2.9×10^8	0	2

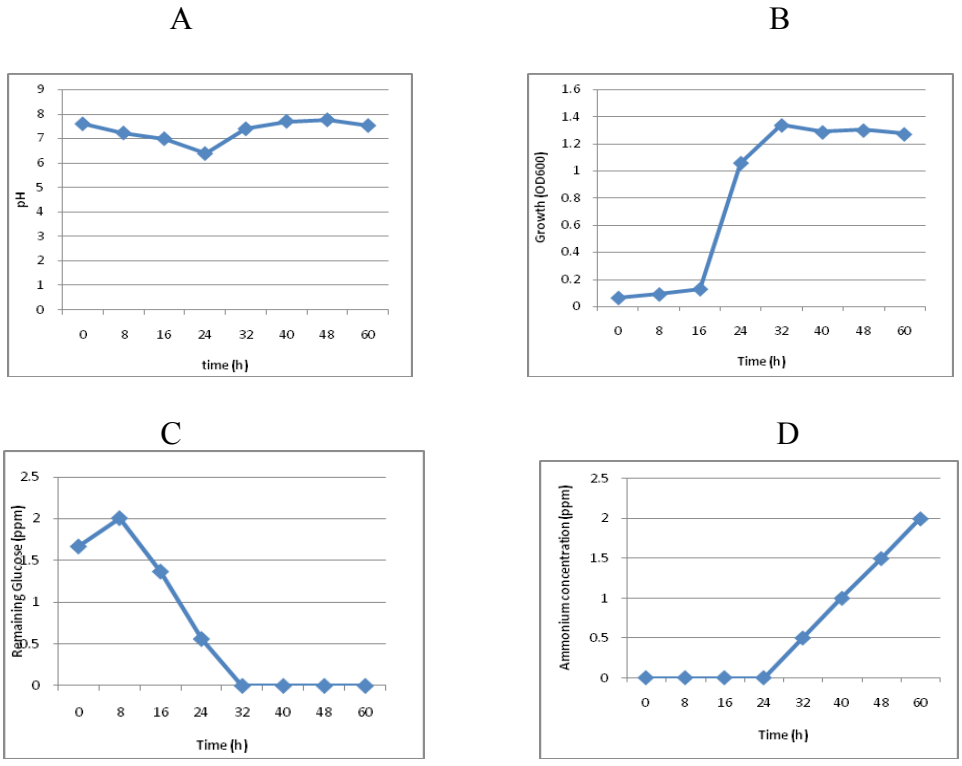

Figure1. (A) pH, (B) OD, (C) Remaining Glucose Concentration and (D) Ammonium Concentration in culture of *S. maltophilia.* Samples were removed for analysis at the indicated times.

Conclusion

In this study, we have found that *S. maltophilia* accumulated highest amount of ammonia in glucose supplemented medium when tested with difference carbon sources. *S. maltophilia* accumulated approximately 2 ppm of ammonia after 60 h incubation in 0.7% glucose-NFMM. No carbon source was detectable in the medium at these times. Higher ammonia accumulation was observed in the medium after longer incubation times, suggesting that the mechanism of nitrogen fixation might be influenced by sugars in the medium.

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