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Enhancement of Cellulolytic Nitrogen Fixing Activity of *Alcaligenes* sp. by MNNG Mutagenesis

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ABSTRACT: For effective degradation of agricultural residues into more useful forms, cellulolytic nitrogen fixing bacteria were isolated from soil samples of central region of Myanmar. Among 32 isolated strains, six best isolates (three strains of *Azomonas agilis*, two strains of *Azotobacter chroococcum*, and one strain of *Alcaligenes* sp.) were selected. Best strains were selected by their nitrogen fixing activities. Nitrogen fixing bacteria cannot excrete significant amount of ammonia into their environment. To improve cellulolytic nitrogen fixing activities, *Alcaligenes* sp. among six isolates was muatgenized with chemical mutagen, MNNG. From treatment of *Alcaligenes* sp. with three concentrations of MNNG (7.5 ppm, 10 ppm and 12.5 ppm), six potential mutant colonies were obtained. After screening of nitrogen fixing activities of wild type and mutagenized strains, four out of six mutant strains excreted higher amount of ammonium concentration than wild type strain. Although wild type strain of *Alcaligenes* sp. excreted 46.64 ppm of ammonium concentration, 101.35 ppm of ammonium concentration was excreted by mutant strain (M2-F) of *Alcaligenes* sp. According to results obtained, 10 ppm of MNNG was more effective for obtaining better mutant strain for nitrogen fixing activity. Although nitrogen fixing activities of mutant strains were increased, cellulolytic activities were decreased than those of wild type strain. Reducing sugar concentrations produced by all mutant strains were decreased using cellulose and CMC as substrates than wild type strain.

KEYWORDS: Agricultural residues, cellulose, nitrogen fixation, MNNG mutagen, *Alcaligenes* sp.

1 INTRODUCTION

Agricultural residues are produced in plentiful every year. Approximately one kg of residues is produced for each kilograms of grains harvested [1]. The annual crop of corn, wheat, and soybean produce about 16, 10 and 10 billion ton of residues respectively and most of this material is left in the field after grain harvest. In some developing countries, these residues are used as a major source of cattle feed. In developed countries, these agricultural residues are not allowed to use as cattle feed because of the presence of various pesticide, herbicide, and insecticide residues. Utilization of biomass resources such as starchy and cellulosic materials of plant origin for production of energy and chemicals by microorganisms has attracted considerable interest in recent years ([2, 3]). Cellulose is the most abundant renewable resource on the earth (100 million dry tons per year). It is the primary products of photosynthesis in the environment [4]. This study was aimed for effective conversion of these residues into more useful forms by microorganisms.

There are many nitrogen fixing bacteria that possess cellulose activities, such as *Sinorhizobium fredii* [5,6], *Bacillus spharricus* [7], *Bacillus circulans* [8], *Paenibacillus azotofixans* [9], *Gluconacetobacter* [10], *Azospirillum* [11]. Although there are many reports about cellulolytic nitrogen fixing activities of other bacteria, there are few reports about *Alcaligenes* sp. for

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studying of these activities. So, this research work leaded to study the cellulolytic nitrogen fixing activities of *Alcaligenes* sp. and to enhance these activities by MNNG mutagenesis.

Alcaligenes is a genus of Gram-negative, aerobic, rod-shaped bacteria. The species are motile with one or more peritrichous flagella. *Alcaligenes* species have been used for the industrial production of non-standard amino acids; *A. eutrophus* also produces the biopolymer polyhydroxybutyrate (PHB).They are rods, coccal rods, or cocci sized at about 0.5-1.0 x 0.5-2.6. They are obligately aerobic, but some can undergo anaerobic respiration if nitrate is present. They tend to be colorless. They typically occur in the soil and water, and some live in the intestinal tract of vertebrates. Samples from blood, urine, feces, discharge from ears, spinal fluid and wounds have produced this type of bacteria [12]. The diazotrophic *Alcaligenes faecalis* is capable of entering rice roots [13].

Use of nitrogen fertilizer is of great importance in rice production, as nitrogen is the major factor limiting growth under most conditions [14]. Since agriculture is expected to move toward environmentally sustainable methods [15], much attention has been paid to natural methods of biological nitrogen fixation. Several diazotrophic bacteria, including *Klebsiella oxytoca*, *Enterobacter cloacae* [16], *Alcaligenes* [13], and *Azospirillum* [17], have been isolated from the rhizosphere of wetland rice.

Free living diazotrophs fix dinitrogen sufficient for their own needs and do not generally excrete significant amounts of ammonia into their environment. In nitrogen fixing bacteria, nitrogen fixation is controlled at the transcriptional level by the regulatory proteins encoded by Nif A. Nif L inhibits Nif A function in response to ammonium, high oxygen concentration and reduced energy charge. To inhibit nif transcription, Nif L binds to Nif A, and normal regulation occurs only when the proteins are present in approximately stoichiometric amounts [18]. For these problems, nitrogen fixing bacteria cannot excrete significant amounts of ammonium into their environment. So, it is necessary to produce significant amounts of ammonia into their environment for production of N-rich biofertilizers using these bacteria and effective conversion of agricultural residues into more useful forms.

After isolation and detection of cellulolytic nitrogen fixing activity of isolated *Alcaligenes* sp., cellulolytic nitrogen fixing activities of this strain was enhanced by MNNG mutagenesis.

2 MATERIALS AND METHODS

2.1 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).

2.1.1 SOIL SAMPLING

Soil samples were collected from rhizosphere of rice and under decayed rice straw around Patheingyi Township, Mandalay Region, Myanmar.

2.1.2 CULTURE MEDIA

Cellulolytic nitrogen fixing bacteria were isolated on Cellulose Nitrogen Free Mineral Medium (C-NFMM) by serial dilution method. The C-NFMM contained (g/L): KH_2PO_4 1.0, $CaCl_2$ 1.0, $MgSO_4.7H_2O$ 0.25, NaCl 0.5, $FeSO_4.7H_2O$ 0.01, $MnSO_4.H_2O$ 0.01, Na_2MoO_4 0.01, Cellulose powder 7.0, Agar 20.0.

2.2 METHODS

2.2.1 ISOLATION OF CELLULOYTIC NITROGEN FIXING BACTERIA

Cellulose Nitrogen Free Mineral Medium (C-NFMM) was used to isolate cellulolytic nitrogen fixing bacteria and carbon source was cellulose (7 g/L). Solid medium was produced by adding 2% agar.

2.2.2 SCREENING OF NITROGEN FIXING ACTIVITY

Nitrogen fixing activities of isolated strains were tested by growing the strains on Glucose Nitrogen Free Mineral Medium (G-NFMM) and C-NFMM plates substituted with Bromothymol Blue (BTB) as an indicator. The strains that change the color of the BTB containing media to blue were marked as nitrogen fixers, suggesting excretion of ammonia.

2.2.3 DETECTION OF EXCRETED AMMONIA CONCENTRATION

Excreted ammonium concentration by isolated strains was estimated using the Viscolor Alpha Ammonium Detection Kit (Macherey-Nagel). Single colony of isolated strains were inoculated in G-NFMM or C-NFMM broth and incubated for one week. After one week incubation, culture broth was centrifuged at room temperature (RT) and supernatant (1 ml) was transferred into a test tube. Two drops of NH_4-1 were added to the sample and mixed well, after which one-fifth of a spoonful of NH_4-2 was added. After mixing well, the sample was left at RT for 5 min. One drop of NH_4-3 was added, mixed well and left at RT for 5 min. The color development of supernatants was observed and the ammonium concentration was recorded by comparing with the color chart from the Viscolor Alpha Ammonium Detection Kit [19].

Ammonium concentration was also estimated by constructing standard curve of ammonium sulphate solution reacted with the same reagent using UV-vis spectrophotometer.

2.2.4 SCREENING OF CELLULOLYTIC ACTIVITY OF ISOLATED STRAINS

Cellulolytic activity of isolated strains were studied by growing the single colony of strains on C-NFMM or Sodium Carboxylmethyl Cellulose-NFMM (CMC-NFMM) plates and then the plates were incubated at 37°C for one week to allow for the excretion of cellulase. After incubation, cellulolytic activity was detected by flooding the agar media with an aqueous solution of Congo red solution (0.1% w/v) for 10 min. After then, the solution was poured off, and the plates were washed with 1M NaCl solution for two or three times. The formation of clear zones around bacterial colonies indicated cellulose degradation.

2.2.5 QUANTITATIVE DETERMINATION OF CELLULOLYTIC ACTIVITY

Cellulolytic activities of isolated strains were also detected quantitatively by the 3,5-dinitrosalicylic acid (DNS) method [20]. The bacterial strains were preliminary inoculated in C-NFMM broth and the culture broth was incubated at 37°C in water bath shaker. After incubation, culture broth was centrifuged and supernatant (1 ml) was transferred into a test tube. Cellulose as substrate was added into supernatant and it was allowed to react. After reaction time, cellulase activity was measured by DNS method.

2.2.6 IDENTIFICATION OF ISOLATED STRAINS

Isolates were identified through its morphological and some biochemical characteristics according to Bergey' Mnaual of Systematic Bacteriology [21] and 16s rDNA sequencing. Sequencing reactions were performed using the Big Dye Terminator Cycle Sequencing Kit (v.3.1) and the results were analysed in a GA 33130 sequencer. Nucleotide sequences were analysed using BLAST on the NCBI BLAST and Greengenes.IBI.gov.

2.2.7 ENHANCEMENT OF NITROGEN FIXING ACTIVITY BY N-METHYL-N-NITRO-N-NITOSOGUANIDINE (MNNG) MUTAGENESIS

MNNG mutagenesis was performed by [22, 23].The wild type bacterial strain grown in G-NFMM broth at 37°C for 24 hr was harvested after centrifugation and washed twice with phosphate buffer. The cells were resuspended in phosphate buffer and various concentrations of MNNG (7.5 ppm, 10 ppm, 12.5 ppm) were added. And then, it was allowed to react for 30 min and 1 hr. After reaction time, the cells were centrifuged again and washed with phosphate buffer for two to three times. The treated cells were resuspended in NFMM broth and diluted serially. After dilution, 20 µl of sample was spread on G-NFMM plates containing BTB. The culture plates were incubated at 37°C for one week and the development of mutant colonies was observed by changing the color of BTB containing plates.

3 RESULTS AND DISCUSSION

3.1 ISOLATION OF CELLULOYTIC NITROGEN FIXING BACTERIA

32 strains were isolated on C-NFMM media from collected soil samples. As these all strains were isolated from various soil samples, their growth rates and colonial morphology were different.

3.2 SCREENING OF NITROGEN FIXING ACTIVITY

All isolates were screened for their nitrogen fixing activities, but six isolates showed a color change in BTB containing media from dark green to blue. Although other isolates did not change the color of media, they may have nitrogen fixing activities. Nitrogen activity of *Azotobacter beijerinckii* and *Lysobacter enzymogenes* DMS 2043^T and rhizospheric bacterial isolates were screened by changing the color of BTB containing nitrogen free media, and these strains showed a color change in BTB containing media, suggesting excretion of ammonia [19], [24].

3.3 DETECTION OF EXCRETED AMMONIUM CONCENTRATION

On detection by Viscolor Alpha Ammonia Detection Kit, six isolates that showed a color change on plate screening also showed the highest color development after one week incubation, assuming that these six isolates excreted above 3 ppm of ammonium concentration when compared with the color chart. Although these isolated strains showed nitrogen fixing activity by giving color development during one week incubation, the highest color development was given after one week incubation. Free-living diazotrophs fix dinitrogen sufficient for their own needs and do not generally excrete significiant amounts of ammonium into their environment: fixed nitrogen is released after death and lysis of bacteria [25]. Among best nitrogen fixers, *Alcaligenes* sp. was selected for further study.

3.4 SCREENING OF CELLULOLYTIC ACTIVITY OF ISOLATED STRAINS

All isolates grew well on C-NFMM and CMC-NFMM plates and gave clear zones around their colonies when detected by pouring Congo red solution. But, the best strains for cellulolytic activity could not be selected by observing clear zone formation. So, six isolates that gave highest ammonium concentration were also selected for quantitative determination.

3.5 QUANTITATIVE DETERMINATION OF CELLULOLYTIC ACTIVITY BY DNS METHOD

By using Cellulose and CMS as substrates, reducing sugar concentrations in terms of cellulose activity were measured by DNS method. Table 1 showed the reducing sugar concentrations produced by six isolates. Reducing sugar concentration converted from cellulose and CMC substrates by six isolates were not obviously different among them.

Table 1. Reducing Sugar Concentration Produced by Six Selected Strains Using Cellulose and CMC as Substrates by DNS Method

Strains	Reducing sugar concentration (mg/ 0.5 ml) (using cellulose substrate)	Reducing sugar concentration (mg/ 0.5 ml) (using CMC substrate)
M-1	0.429	0.493
M-2	0.590	0.456
M-3	0.402	0.461
M-4	0.498	0.439
M-5	0.525	0.439
M-6	0.413	0.300

3.6 IDENTIFICATION OF SELECTED SIX STRAINS

Some biochemical characteristics of six isolates were studied according to Bergey's Manual of Systematic Bacteriology. From this study, selected six strains could not be identified by studying these characteristics. But according to 16s rDNA sequencing analysis, M-1, M-3 and M-4 were *Azomonas agilis*, M-2 was *Alcaligenes* sp. and M-5 and M-6 were *Azotobacter chroococcum*. Table 2 showed some biochemical characteristics of *Alcaligenes* sp.

3.7 STRAIN IMPROVEMENT BY MNNG MUTAGENESIS

Wild type *Alcaligenes* sp. was subjected to successive mutagenic treatment using MNNG. After mutagenic treatment, no better mutant colonies for ammonium excretion were obtained from three treatments of 30 min. So, reaction time was increased to 1 hr. Six potential mutant colonies were obtained from three treatments based on colour change of medium around their colonies. Figure 1 showed the colour change of the BTB containing media from the growth of wild type and selected mutant colonies of *Alcaligenes* sp. Survival rates decreased when MNNG concentration was increased. Reference [26] said that survival rates decreased with increasing MNNG concentration from studying of Ultraviolet irradiation and MNNG mutagenesis of *Acetobacter* species for enhanced cellulose production.

Table 2. Some Biochemical Characteristics of Alcaligenes sp. (M-2)

Biochemical Tests	*Alcaligenes* sp.
Motility	+
TSI	+
Nitrate reduction	+
Citrate utilization	+
Indole test	+
Methyl red	+
Voges proskauer	-
Catalase	+
Starch hydrolysis	+
Gelatin agar test	+

Table 3 showed survival rates of *Alcaligenes* sp. from three treatments of MNNG for 30 min and 1 hr. Among six selected potential mutant colonies, four colonies excreted higher ammonium concentration than wild type strain. Table 4 showed the excreted ammonium concentration of wild type and mutant strains of *Alcaligenes* sp. Among six selected potential mutant strains, two strains excreted lower amount of ammonium concentration than wild type strain. Mutant strains (M-2E and M-2F) excreted highest amount of ammonium concentration.

In this study, it was found that 10 ppm MNNG concentration for 1 hr reaction time was suitable for obtaining better mutant nitrogen fixing bacteria. According to higher ammonium excretion by selected potential mutant colonies of *Alcaligenes* sp., it was assumed that mutant strains of *Alcaligenes* sp. for higher ammonium excretion were obtained by MNNG mutagenesis. Although various methods of mutation were tested on *Azotobacter beijerinckii*, including UV radiation and chemical mutagenesis using N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and ethylmethane sulphonate (EMS), no ammonia-excreting mutants were isolated, even using the mating approach [27]. This may have been due to the production by *A. beijerinckii* of polysaccharide that surrounds the cell [28], rendering mutation problematic. However, mutant strain (MV376) of *A.vinelandii* was successfully produced, secreting significiant quantities of ammonium during diazotrophic growth [25]. Figure 2 showed that color development of six potential mutant and wild type *Alcaligenes* sp. for excreted ammonium concentration from detection by Viscolor Alpha Ammonium Detection Kit.

Table 3. Concentration of MNNG, Reaction Time and Survival Rates of Alcaligenes sp. (M-2) for Mutagenesis

Strain	MNNG Concentration (ppm)	Reaction Time	Survival Rate	Nitrogen Fixing Activity
M-2	7.5	30 min	0.688%	< wild type
M-2	10	30 min	0.45%	= wild type
M-2	12.5	30 min	0.15%	< wild type
M-2	7.5	1 hr	0.83%	> wild type
M-2	10	1 hr	0.53%	> wild type
M-2	12.5	1 hr	0.43%	< wild type

Table 4. Comparison of Excreted Ammonium Concentration of Wild Type and Mutant Alcaligenes sp. (M-2)

Strain	MNNG Concentration (ppm)	Ammonium Concentration (ppm) by Spectrophotometric Method
M-2 (wild type)	Without treatment	46.64
M-2A	7.5	9.66
M-2B	7.5	57.52
M-2C	7.5	69.85
M-2D	7.5	43.37
M-2E	10	71.98
M-2F	10	101.35

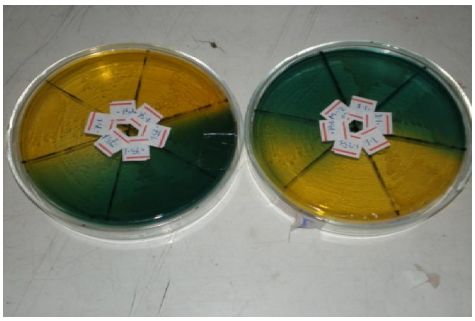

Fig. 1. Screening of Nitrogen Fixing Activities of Six Potential Mutant Colonies and Wild Type Alcaligenes sp.

Fig. 2. Screening of Nitrogen Fixing Activities of Six Potential Mutant Colonies and Wild Type Alcaligenes sp.

3.8 CELLULOLYTIC ACTIVITY OF WILD TYPE AND MUTANTS *ALCALIGENES* SP .

Two mutant strains (M-2E and M-2F) were studied for their cellulase activities by comparing with wild type strain. Table 5 showed reducing sugar concentration produced by wild type and mutant *Alcaligenes* sp. Although these two mutant strains excreted higher amount of ammonium concentration than wild type, their cellulase activities were lower than wild type. Although the aim of this study is to enhance cellulolytic nitrogen fixing activities of *Alcaligenes* sp., the resulted mutant strains did not possess higher cellulase activities than wild type. So to get better strain for dual activities, it may need to study using various MNNG concentrations, various reaction times for mutagenesis and reaction time for cellulase activity, pH of medium and various incubation temperatures.

Mutagenesis by UV and MNNG caused changes in cellulose production or secretion. However, enhanced cellulose production of *Acetobacter* species by UV irradiation and MNNG mutagenesis [26] enhanced cellulose production of *Cellulomonas* sp. TSU-03 by UV mutagenesis and MNNG mutagenesis [23] improvement of cellulase production of fungal strains using repeated and sequential mutagenesis were studied. They reported that mutant strains possessed more cellulase activities than their parental strains. In this study, mutant strains of *Alcaligenes* sp. were selected based on ammonium excretion, not mainly on cellulolytic activity. So, as mutant strain selection was based on only nitrogen fixing activity, cellulolytic activity of mutant strains was not better than wild type strain.

Table 5. Comparison of Reducing Sugar Concentration of Wild Type and Mutant Alcaligenes sp. (M-2)

Alcaligenes sp.	Reducing Sugar Concentration (mg/0.5ml) (cellulose as substrate)	Reducing Sugar Concentration (mg/0.5 ml) (CMC as substrate)
M-2 (Wild type)	0.590	0.456
M-2E	0.504	0.386
M-2F	0.402	0.370

4 CONCLUSION

Although six best nitrogen fixing isolates were selected from 32 isolates, only one *Alcaligenes* sp. was obtained. Isolated *Alcaligenes* sp. possessed cellulolytic and nitrogen fixing activities. Attempts were made to enhance cellulolytic nitrogen fixing activities of this strain by MNNG mutagenesis. According to this study, although nitrogen fixing activity of *Alcaligenes* sp. was higher after treatment with 10 ppm of MNNG for 1h, cellulase activity was decreased than wild type strain. But, resulted mutant strains still possessed dual activities.

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