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ORIGINAL RESEARCH ARTICLE

Characterization and Mutational Analysis of Some Cellulolytic Nitrogen Fixing Bacteria for Nitrogen Rich Biofertilizer Production

Zaw Ko Latt *[1], San San Yu [2], Tin Mar Lynn[1], Mya Mya Oo[1]

1. Department of Biotechnology, Mandalay Technological University, Mandalay, Myanmar
2. Guest Scientist, Molecular Life Science Research Center, Jacobs University, Bremen, Germany

ABSTRACT

32 strains of cellulolytic nitrogen fixing bacteria were isolated from various soil sources on Avicel Cellulose or CMC N-free medium, but seven isolates were selected based on their nitrogen fixing activity. The nitrogen fixing activity of isolates was assayed by VISCOLOR Alpha Ammonium reagent, and the ammonium concentration was estimated from the ammonium standard curve, assayed with the same reagent. All seven isolates excreted significant quantities of ammonium (3.643 ppm – 60 ppm). When cellulolytic activity was detected on Cellulose or CMC medium by pouring Congo-red solution, all isolates can degrade Cellulose or CMC by showing clear zone. For significant ammonium excretion, when four isolates were mutated by chemical mutagenesis using various NTG concentrations, Z-1 mutant strain excreted ten times ammonium concentration than the wild type strain, and the ammonium excretion of M-2 strain increased from 40 ppm to about 91.5 ppm. All selected strains can also solubilize phosphate on Pikovakaia's and NBRIP media. Four out of seven isolates can produce Indole Acetic Acid. When antifungal activities of isolated strains were tested with Fusarium, Rhizotonia and Pythium, M-2 isolate can inhibit the growth of Fusarium and Rhizotonia, and Z-4 and G isolate can inhibit Rhizotonia and Pythium. Identification of isolated strains indicated that all isolates were Azotobacter species.

Keywords: Nitrogen fixing bacteria, cellulose degradation, chemical mutagenesis.

INTRODUCTION

Agricultural residues are produced in plentiful. Approximately one kg of residues is produced for each kilogram of grains harvested (Fahey et al., 1991).[1] The annual crop of corn, wheat, and soybean produce about 16, 10 and 10 billion of tons of residues, respectively a

nd most of this material are left in the field after grain harvest. In some developing countries, these residues are used as a major source of cattle feed.

Address for correspondence:
Zaw Ko Latt *
Department of Biotechnology, Mandalay Technological University, Mandalay, Myanmar
E-mail: zaw.zk23@gmail.com

In developed countries, these agricultural residues are not allowed to use as cattle feed because of the presence of various pesticides, herbicide, and insecticide residues. Farmers have to manage the residues efficiently in time for subsequent year's crop and also economically to save cost. Farmers usually burnt these residues after harvest. Today, burning is still seen as the most effective method of clearing waste stubble from fields in preparation for the succeeding planting seasons. One major problem with residue is the emission of smoke and other pollutants into the atmosphere and their effects on ambient air quality and public health. One study has shown that burning one ton of dry rice straw emits about seven pounds of particulate matter, eighty pounds of carbon monoxide and ten pounds of hydrocarbons. Burning of agricultural residues has many disadvantages. So, scientists have studied various methods for effective degradation of agricultural residues into more useful forms through microbial activity.

Kalininskaya et al.[2] and Yoneyama et al. [3] both describe increased nitrogen fixation in waterlogged soils amended with rice straw. Roper found significant levels of nitrogenous activity in several soils amended with wheat straw, and Lynch and Harper[4] observed significant nitrogen fixation associated with the breakdown of straw after inoculation with a cellulolytic fungus, Penicillum corylophilum, and the nitrogen-fixing anaerobe Clostridium butyricum. However, there have not been any reports of pure cultures of diazotrophs able to use straw as an energy source for nitrogen fixation.

Goul[5] has shown that treatment of lignocellulosic materials, such as crop residues, with a dilute solution of alkaline hydrogen peroxide solubilizes 50 % or more of the lignin present. The treatment also appears to significantly decrease the crystallinity of residual cellulose, making it particularly susceptible to microbial digestion. But the problem is that if they are used as biofertilizers, exogenous nitrogen must be applied. Dorothy and Garcia [6, 7] studied that degradation of wheat straw with combination of cellulomonas strains and nitrogen fixing strains. One problem with this process was that the process must be separated in either time or space from the process of nitrogen fixation, because the growth condition is different. Germida also studied that development of microbial inoculant to enrich N-content of organic residues by a combination of N2 fixing bacteria and cellulose degrading bacteria. But he also reported the similar problem.

One way to solve above problem is to isolate the cellulolytic nitrogen fixing bacteria for beneficial use of agricultural residues by converting them to organic fertilizers through microbial activities. But, 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.[8] To inhibit nif transcription, Nif L binds to Nif A, and normal regulation occurs only when the proteins are present in approximately stoichiometric amounts.[9] For these problems, nitrogen fixing bacteria can't excrete significant amounts of ammonium into their environment. So, it is necessary to produce significant amounts of ammonia into their environment by cellulolytic nitrogen fixing bacteria if agricultural residues are considered to produce as N-rich biofertilizers using these bacteria. So this research work was studied in the development of effective mutant strain for nitrogen fixation using mutagenic chemical, NTG (N-methyl-N-Nitro-N-nitrosoguanidine).

MATERIAL AND METHOD

Isolation of Cellulolytic Nitrogen-Fixing Bacteria

To isolate the cellulolytic nitrogen-fixing bacteria, soil samples were collected from various paddy fields under cultivation and cultivated condition and natural compost from Kyaukse District, Mandalay Division in Myanmar. Strains of both cellulolytic nitrogen-fixing characteristics were selected by using a nitrogen-deficient medium wherein the sole carbon sources are Avicel Cellulose and Sodium Carboxymethyl Cellulose (CMC).

One gram of soil samples was added to 100ml sterile nitrogen free cellulose medium and nitrogen free CMC medium in each 250 ml conical flasks. The flasks were shaken once and incubated on a water bath shaker at room temperature for a week. Then the broth culture was placed on solid nitrogen cellulose medium and CMC medium. The plates were labeled and incubated for a week. After incubation, they were analyzed and colonies with different morphologies were subcultured onto new plates. The isolated colonies were characterized for their morphological and biochemical characters and were also detected for nitrogen fixation and enzyme production activities.

Detection of Nitrogen-Fixing Activity

The visual detection of nitrogen-fixing activity was observed by using Glucose Nitrogen Free Mineral Agar Medium as well as Broth Medium and Ammonia Test Kit. Individual purified colony growing on nitrogen free Cellulose medium was taken and inoculated into GNFMM containing BTB (bromothymol blue solution) and without BTB. After one week incubation, changing the color of the BTB containing medium was recorded. To detect nitrogen-fixing activity of the broth culture without BTB, the regents of ammonia test kit were added and the appeared color was noted by comparing with the color chart from the test kit.

The quantitative analysis was done by using Spectrophotometric method.

Detection of Cellulase Producing Activity

The purified colony of each isolates was planted on 0.2% Avicel Cellulose and Sodium Carboxymethyl Cellulose media and incubated at room temperature for a week. Cellulose activity and CMCase activity were recognized by zones of clearing after flooding with 0.1% Congo-red solution for 1 hour and washed with 1M NaCl. A clear zone was evident around the colony while the hydrolyzed portions remain colorless. Cellulose enzyme activity was also measured by the ditrosalicyclic calorimetric method (DNS) as described by Miller (1959).[10]

Screening on Anti-Fungal Activity

Fusarium, Rhizotonia and Pythium were used as target soil borne pathogenic fungi. One loopful of target fungus was spread on the Potato Dextrose Agar Medium. Then tested strains were placed onto the fungus medium and incubated at room temperature for a week. After incubation the inhibition zones appeared around colony were recorded.

Screening on Phosphate Solubilizing Activity

Detection and estimation of the phosphate solubilizing ability of microorganisms have been possible using plate screening methods. Phosphate solubilizers produced clearing zones around the microbial colonies on the medium containing insoluble mineral phosphate such as tricalcium phosphate. Also the bromothymol blue method was used for detection of pH drop-in activity that was caused by the releasing of organic acids and showing yellow halos around the tested colonies.

Screening on Plant Growth Hormone (IAA) Producing Activity

Single bacterial colonies were inoculated in 200ml of broth with tryptophan (0.5g/l) and incubated at 28±2˚C for 15 days in a shaker incubator. Bacterial cells were separated from the supernatant by centrifugation at 3000 rpm for 15 minutes. And then 1ml of supernatant mixed with one drop of orthophosphoric acid and 2ml of Solkowski's reagent.

Strain Development by Chemical Mutagenesis (N-methyl-N'-Nitro-N nitrosoguanidine)

Strain development was done on bacterial isolates by mutagenesis using NTG. The purified single colony was inoculated into 20ml of GNFMM and incubated at 37˚C for a week. After incubation the broth culture was centrifuged at 10000 rpm for 10 minutes and decant off supernatant and the cell pellet was washed with phosphate buffer for 3 times. Then the cell was resuspended in 4ml phosphate buffer. 0.9 ml of the broth culture solution was added to Tube A and 1ml of broth culture was added to Tube B as a control. 0.1ml of

NTG solution (1mg NTG / 1ml phosphate buffer) was added to Tube A, and incubated at 37˚C for variable time. After incubation, the reaction solution was centrifuged, decants off supernatant, washed the cell with phosphate buffer for 3 times and responded the cells in 1ml phosphate buffer. Serial dilution was made for both Tubes and spread it on GNFMM containing BTB. The plates were incubated at room temperature to observe changing the color of the medium and the survival rate of the isolate. The effective strain was recognized by changing of the medium green to blue as cell excreted ammonium.

RESULTS AND DISCUSSION

Isolation and Selection of Strains

32 strains were isolated from various soil sources and tested their nitrogen fixing activities. But six strains were selected based on their nitrogen fixing activities. Nitrogen fixing activities of six selected strains were described in Table 1.

Table 1: Nitrogen Fixing Amount of six selected strains by calculation from a standard curve and from visual detection from a reference on Ammonium Test Kit Reagent

No	Strains	Nitrogen Fixing amount (ppm)	Test Kit Result (mg/L)
1	M-2	46.64	> 3 mg/l
2	M-4	89.03	> 3 mg/l
3	Tw	55.00	> 3 mg/l
4	G	12.57	2-3 mg/l
5	Z-4	36.51	> 3 mg/l
6	Z-1	3.931	1-2 mg/l

Detection of Cellulase Production Activity

Six strains grew well on 0.2 % CMC or Cellulase Nitrogen Free Mineral medium and provided a clear zone around their colonies when detected by pouring 0.1 % Congo-red solution. But, when cellulose production activities were detected by the dinitrosalicyclic acid calorimetric method (DNS), all six strains produced different amount of reducing sugar and described in Table 2.

Table 2: Calculation of reducing sugar production activity of six selected strains using cellulose and CMC as substrate by DNS method from a standard curve at 540 nm

No	Strains	Glucose concentration (mg/ 0.5 ml) (using cellulose substrate)	Glucose concentration (mg/ 0.5 ml) (using CMC substrate)
1	M-2	0.60	0.46
2	M-4	0.52	0.44
3	Tw	0.33	0.36
4	G	0.42	0.35
5	Z-4	0.39	0.41
6	Z-1	0.40	0.49

Identification of Isolated Bacterial Strains

Biochemical characteristics of six isolated strains were described in Table 3. According to biochemical characteristics, all six strains are cocci, gram-negative, Azotobacter species. (Table 3)

Table 3: Biochemical characteristics of six selected bacterial strains

Biochemical Tests	M-2	M-4	Tw	G	Z-1	Z-4
TSI	+	+	+	+	+	+
MR	-	+	-	+	-	-
Citrate	+	+	+	+	+	+
Nitrate	+	+	+	+	+	+
Motility	+	+	+	+	+	+
Gelatin	+	+	-	+	+	+
Starch hydrolysis	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Indole	+	+	-	+	+	+
Gram's stain	-	-	-	-	-	-

(+) = positive results of respective tests, (-) = negative results of respective tests

Screening on P-solubilizing Activity

All six strains can solubilize phosphate on Pikovskaia's and NBRIP media by measuring the media's pH and clear zone diameter. Activity was different among six strains by the differences of pH and clear zone diameter. So, it can be noted that these strains have a P-solubilizing activities. (Table 4)

Table 4: P-solubilizing activity of six selected strains by measurement of clear zone and pH after 3 days incubation

No	Strains	Clear zone diameter of NBRIP medium (mm)	pH in NBRIP broth media (initial pH-8.3)
1	M-2	31	2.6
2	M-4	14	4.9
3	Tw	33	2.6
4	G	15	6.5
5	Z-4	23	2.8
6	Z-1	35	2.7

Screening on IAA production activity

Four out of six bacterial strains produced IAA and they also gave positive results to Indole test.

Screening on Antifungal Activity

When antifungal activity was screened with Fusarium, Pythium, and Rhizotonia as target soil borne pathogenic fungi, M-2 isolate can inhibit the growth of Fusarium and Rhizotonia, and Z-4 and G isolate can inhibit Rhizotonia and Pythium.

Mutagenesis of Bacterial strains with NTG

When strain development was done on four strains by using various NTG concentrations, Z-1 and M-2 strains were mutagenized. Ammonium production of Z-1 mutant strain was higher than wild type at 1 % NTG concentration and produced ten times ammonium concentrations. And ammonium

excretion of M-2 mutant strain increased from 46.64 ppm to 69.85 ppm at 0.75 % NTG concentration and from 46.64 ppm to 91.5 ppm at 1 % NTG concentration. The survival rate of M-2 mutant strain by 0.75 % NTG was higher than mutant strain by 1 % NTG concentration. And also, antibiotic sensitivity patterns were different between wild type and mutant strains (Table 5).

Table 5: Antibiotic sensitivity patterns of wild type and mutagenized strains (M-2)

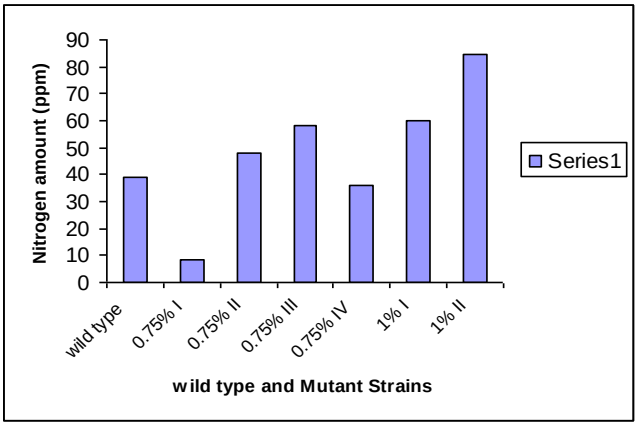
No	Antibiotics	Wild type	M-2 mutant (0.75%)	M-2 mutant (1%)
1	Gentamycin	S (26mm)	S (21 mm)	S (21 mm)
2	Tetracycline	S (27 mm)	S (22 mm)	S (21 mm)
3	Cipromycin	S (16 mm)	S (11 mm)	S (10 mm)
4	Ampicillin	S (20 mm)	R	R
5	Streptomycin	R	S (12 mm)	S (21 mm)
6	Chloramphenicol	S (25 mm)	S (21 mm)	S (20 mm)

1 % NTG was better for strain development in nitrogen fixation but the survival rate of strains was lower. (Table 6)

Table 6: Survival rate and N2 fixing rates of wild type and mutagenized strains (M-2)

Strain	NTG concentration	Incubation Time	Survival rate	Ammonium Test Kit	Spectrophotometric method (ppm)
M-2	0	1 hr	100 %	> 3 mg/l	46.64
Mutant (0.75 %)	0.75 %	1 hr	0.83 %	> W	69.85
Mutant (1%)	1%	1 hr	0.53 %	>W	91.50

Figure 1: Comparison of nitrogen fixing amount of wild type and mutagenized strain (M 2)



CONCLUSION

Six selected strains were the best strains for nitrogen fixation in this study. They excreted significant amounts of ammonia into their environment after one week incubation. For cellulose production activity, they utilized cellulose and CMC well but production of reducing sugar was different among six strains. When mutagenesis was done on four strains, two mutant strains for more efficient nitrogen fixation were obtained by varying NTG concentrations and incubation time. Activity was observed by visual detection of color development.

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