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Research Article

Interaction of Nitrogen Fixation and Alginate Synthesis of Azotobacter vinelandii Isolated from Myanmar Mangrove

San San Yu[1,2]*, Matthias Ullrich[2]

[1]*Microbiology Laboratory, Department of Biotechnology Research, Kyaukse, Myanmar*
[2]*Departement of Life Sciences and Chemistry, Jacobs University Bremen, Germany*

***Corresponding author**
San San Yu, Microbiology Laboratory, Department of Biotechnology Research, Kyaukse, Myanmar, Tel: 95-924-01-379; Email: sakurasan2007@gmail.com

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- *Azotobacter vinelandii*; Mangrove rhizospheric soil; Nitrogen fixation activity; Alginate producing activity; Mutation; Kanamycin resistance gene; Biparental mating

Abstract

A nitrogen-fixing bacterium *Azotobacter vinelandii* was isolated from the mangrove rhizospheric soil of Irrawaddy Region, Myanmar. Screening and quantitative determination of the nitrogen fixation activity and alginate producing activity of the isolated strain was studied. The *algD* and *algU* gene fragments of *Azotobacter vinelandii* were isolated by using designated primers. A deletion was engineered in the cloned *algD* and *algU* genes by digestion with suitable restriction endonucleases and kanamycin resistance gene cartridge was inserted. The mutation was subsequently transferred to the host bacteria, *Azotobacter vinelandii* by biparental mating using pEX18 vector and *E .coli* ST 18 under pressure of kanamycin selection. Two mutant strains were developed and confirmed by PCR. The resultant two mutant strains lost their nitrogen fixation activity due to loss of their alginate producing activity were observed.

INTRODUCTION

The Myanmar Coast mangroves are an eco-region in Myanmar, Malaysia, and Thailand where there were once thick forests of mangroves but today most has been cleared, resulting in loss of habitat for wildlife. The mangrove flora consists of three separate regions in Myanmar: the Rakhine mangroves, Irrawaddy mangroves, and Taninthayi mangroves. Mangrove communities are recognized as highly productive ecosystems that provide large quantities of organic matter to adjacent costal water [1]. Mangroves provide a unique ecological niche for different microbes, which play various roles in nutrient recycling as well as in various environmental activities. The highly productive and diverse microbial community living associated with mangrove ecosystems continuously transforms nutrients from dead mangrove material into sources of nitrogen, phosphorous and other nutrients. Those can be used by the plants and in turn plant-root exudates serve as a food source for the microbes [2].

Nitrogen-fixing bacteria identified as members of the genera *Azospirillum, Azotobacter, Rhizobium, Clostridium, Klebsiella, Vibrio* and *Phyllobacterium* have been isolated from the rhizosphere of various mangrove species [1,3,4]. Some of the bacteria found in mangrove roots can be used as PGPB (Plant Growth Promoting Bacteria) to improve the establishment and growth of a mangrove seedling in arid coastal areas. It is possible to enhance mangrove plant growth by addition of terrestrial salt-tolerant plant growth promoting bacteria [2].

Azotobacter vinelandii is a gram-negative bacterium, obligate aerobe capable of fixing nitrogen and to adapt its metabolism to diverse environmental conditions. *Azotobacter vinelandii* forms metabolically dormant cysts after exponential growth or upon induction with specific reagents [5]. Several years ago, *Azotobacter vinelandii* was used as a model for biochemical, physiology and genetic studies, regarding mainly in biological nitrogen fixation studies [6]. On this regard, the reduction of N_2 to ammonia (fixation of nitrogen) by nitrogenase enzyme complex is highly sensitive to oxygen, for which *Azotobacter vinelandii* have particular physiological mechanisms, such as a high respiration rate [7], to protect its nitrogenase enzymes from oxygen inactivation.

A mature cyst consists of a contracted cell known as the central body that is surrounded by a capsule made up of a thin laminated outer layer, called the exine, and a thicker inner layer, the intine [5]. The polysaccharide alginate is also a major component of the cyst capsule and is essential for the differentiation process, since mutations in alginate biosynthesis genes abrogate the formation of cysts resistant to desiccation [8,9].

Alginates are polysaccharides produced by *Pseudomonas* and *Azotobacter* species [10]. Most knowledge about alginate biosynthesis have been particularly well characterized in the opportunistic human pathogen *Pseudomonas aeruginosa,* mainly because alginate plays an important role as a virulence factor during cystic fibrosis [11]. In contrast to *P. aeruginosa, A. vinelandii* is a nonpathogenic bacterium, which has been used for the development of biotechnological process to produce alginate. Alginate is a linear polysaccharide composed of variable amounts of (1-4)-b-D-mannuronic acid and its C- 5-epimer a-L-guluronic

acid, which has a wide range of applications such as thickener, stabilizer, gelling agent and emulsifier in food, as well as textile and pharmaceutical industries. Studies at bioreactor laboratory scale have shown the influence of dissolved oxygen tension (DOT) on alginate production [8,12,13]. In this context, alginate could play a decisive role in protecting the nitrogenase [8], due to higher DOT (20%); alginate forms a capsule on the cell surface, suggesting a possible role as an effective barrier for oxygen.

In *A. vinelandii* all genes involved in the biosynthesis of alginate, except *algC*, are clustered in the chromosome and are headed by *algD*, encoding a GDP-mannose dehydrogenase, an enzyme catalyzing the committed step in the biosynthesis of this polymer [14]. Transcription of *algD* and *algC* is positively controlled by the extra cytoplasmic function sigma factor *algU*. *AlgD* expression is also controlled by the two-component system GacS/GacA, which regulates secondary metabolism in many Gram-negative bacteria [15-17].

In *A.vinelandii*, as in *Pseudomonas aeruginosa*, *algU* is essential for alginate production and is encoded in the *algUmuc ABCD* operon [18,19], *MucA* is an inner-membrane protein that acts as an anti-*algU* sigma factor by directly sequestering *algU* and inhibiting its activity. An *algU* mutant of *A.vinelandii* was unable to synthesize alginate. In contrast, a *mucA* mutation increased *algD* and *algC* transcription and consequently alginate production [17,20].

The major aim of this research work is to create the deactivated gene by antibiotic mutation for studying the interaction of nitrogen-fixation and alginate producing activity of *Azotobacter vinelandii* isolate from mangrove rhizospheric soil.

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers used

Bacterial strains, plasmids and primers used in this study were listed in Table 1,2. *Escherichia coli* DH5α cells were grown overnight at 37ºC on Luria-Bertani (LB) agar plates. *E. coli* ST18 was grown on LB agar plates containing 50 µg/ml 5-aminolevulinic acid because strain ST18 cannot grow without the presence of AVA. *E. coli* transformants were maintained on LB medium supplemented with the appropriate antibiotic.

Isolation and identification of nitrogen-fixing Azotobacter vinelandii strain from mangrove rhizosphere

Soil samples were collected from the rhizosphere of mangrove plants from Irrawaddy region in Myanmar. One gram of soil samples was placed into the sterile test tube. Nine milliliter of normal saline was added to the test tube and vigorous shaking was made. Then the test tube was stand for one hour for setting down all the heavy materials from the sample. 0.5 ml of the upper portion of the sample solution was taken and inoculated into the prepared Jensen's nitrogen free liquid medium with 3% NaCl and BTB and incubated at 37ºC for 5 days (the pH of the medium was adjusted to 7 before inoculating of the sample) [21,29]. After 5 days incubation, the medium which turned green to blue was noted and this tube was selected. A loopful of the broth culture was taken from the selected tube and streaked onto the Jensen's nitrogen free medium with 3% NaCl and BTB and incubated at 37ºC for 5 days. Then the color of the medium was observed and the appeared colony which can turn the medium to blue was chosen. To purify this strain, the selected strain was placed on nutrient agar. After checking contamination, the pure single colony was selected and streaked on the NFG (Nitrogen-Free Glucose Mineral Medium Glucose 10g, $CaCl_2$ 0.1g, K_2HPO_4 1g, $MgSO_4$ 0.25g, NaCl 0.5g, $FeSO_4$ 0.01g, $MnSO_4$ 0.01g, Na2MoO_4 5mg, DW 1L), Alg (Alginate Production Medium Sucrose 20g, $(NH_4)_2SO_4$ 0.5 g, Na_2HPO_4 2g, $MgSO_4$ 0.3 g, Yeast Extract 6 g, DW 1L), MG (Mannitol 10g, L-GlutamicAcid 2g, KH_2PO_4 0.5g, NaCl0.2 g, $MgSO_4$ 0.2 g, DW 1L) and BMS (Burk's Medium Sucrose 20 g, K_2HPO_4 0.8 g, KH_2PO_4 0.2 g, $CaSO_4$ 0.13 g, $FeCl_3$1.45 mg, Na_2MoO_4 0.25 mg, DW 1L) medium. The isolated strain was checked from the different medium by Gram's staining. All the media used in this studied were sterilized at 121ºC for 20 min.

For identification of the isolated strain by 16S rRNA sequencing method, genomic DNA was prepared by boiling 1 colony at 95ºC for 10 min in 15 ml nuclease-free water. The cell lysates were centrifuged and 1 ml of the supernatant containing the genomic DNA was used to PCR amplify the 16S rRNA gene. PCR of this gene was carried out using the 16S rRNA universal primers (5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-GGTTACCTTGTTACTACGT-3'). The amplification product was sequenced on an ABI377 sequencer instrument according to the manufacturer's recommendation (Applied Biosystems). The 16SrRNA sequence was analyzed with the ARB software [4], and the Living Tree Project database provided the reference alignment. The neighbour-joining, maximum-likelihood and maximum-parsimony methods were used to reconstruct the phylogenetic trees [21].

Screening of the nitrogen fixation activity and alginate producing activity of the isolated bacteria

The visual detection of nitrogen fixation activity of the isolated strain was observed by using nitrogen free glucose mineral medium and HGB medium (Medium for Isolation of Marine Beneficial Diazotrophs) with BTB (bromothymol blue) and adjusted to pH 7. Individual purified colony growing on the nitrogen free glucose mineral medium was taken and inoculated into NFG medium and HGB medium containing BTB. After one week incubation, changing the color of the medium was recorded [22].

For screening of the alginate producing activity of the bacteria isolate, LB medium, NFG, Alginate Producing medium and MG (Manitol-Glucose) medium were used. 25µl of the overnight growth of bacteria culture was placed on the agar medium with various concentration of copper sulphate and incubated at 37ºC. Tolerance of this metal and heavily of mucoid colony was recorded as its alginate producing activity [23,24].

Determination of alginate biomass in the culture medium

Biomass and alginate dry weight were determined by gravimetrical methods as follows: 1ml of 0.5M EDTA-sodium salt and 0.5ml of 5M NaCl were added to 25ml sample of culture broth to separate the capsular alginate. After stirring for 5min

Table 1: Bacterial strains used

Bacterial strains	Characteristic	Reference
Escherichia coli		
DH5α	supE44 DlacU169 (F80 *lacZDM15)*	(25)
	hsdR17 recA1 endA1 gyrA96 thi-1 relA1	
ST18	λpirΔhemA*pro thihsdR+* TprSmr	(22)
Chromosome: RP4-2 Ap		
Azotobacter vinelandii		
S-3	Cm^r,Wild type	This study
S-3, *algD*	*algD* mutant containing Km^R cassette	This study
S-3, *algU*	*algU* mutant containing Km^R cassette	This study

Table 2: Plasmid and Primers Used

Plasmid / Primers	Characteristic	Reference
Plasmid		
pGEM-T Easy	Ap^r, vector for cloning of PCR products	Promega
pEX18 Ap	Ap^r, **oriT+ sacB+** gene replacement vector	*(24)*
pFKm	Source of Km cassette flanked with FRT sequences	*(24)*
pGEM-T Easy, *algD*	Ap^r, 1200 bp *algD* gene fragment of S-3 cloned into pGEM-T	This Study
PGEM-T Easy, *algU*	Ap^r, 1600 bp *algU* gene fragment of S-3 cloned into pGEM-T	This Study
pGEM-T Easy, *algD*, Km	Ap^r, Km, 1200 bp *algD* gene fragment of S-3 cloned into pGEM-T	This Study
PGEM-T Easy, *algU*, Km	Ap^r, Km, 1600 bp *algU* gene fragment of S-3 cloned into pGEM-T	This Study
pEX18, *algD*, Km	Ap^r, Km, 1200 bp *algD* gene fragment of S-3 cloned into pEX18	This Study
pEX18, *algU*, Km	Ap^r, Km, 1600 bp *algU* gene fragment of S-3 cloned into pEX18	This Study
Primers	**Sequence(5' 3')**	
8 F	AGAGTTTGATCCTGGCTCAG	(23)
U1492R	GGTTACCTTGTTACTACGT	(23)
algD fwd	ATGCGTATCATTTTCGG	This study
algD rev	TTACCAGCAGATGCCTTCTG	This study
algU fwd	ATCGACTGCGTGTATCTG	This study
algU rev	CGCTCTGGTTATAGAGAC	This study

the sample was centrifuged at 10000 rpm at 20ºC for 30min to precipitate the cells. The cells were washed twice with distilled water, recentrifuged and then dried at 80°C for 24h. The supernatant was cooled and alginate was then precipitated by the addition of 3-volumes of ice cold isopropanol which was then recovered by centrifugation at 10000 rpm at 4ºC for 30min. The precipitate was dissolved in water, precipitated again, centrifuged and then finally dried at 80°C for 24h [24,25].

Detection of nitrogen fixation and nitrogenase activity

The nitrogenase activity assay was carried out according to the method of acetylene reduction. Five ml of the *Azotobacter* broth medium in 12 ml vials was inoculated with ~ 10^4 CFU/ml of each isolate and incubated for 48-120 h at 37ºC. Once visible growth was observed, each vial was sealed with rubber stopper. By means of a disposable plastic syringe, 10% of air from the head space (7 ml) was removed and an equal amount of acetylene was injected into vials. Gas samples (0.7 ml) were removed after 24 h incubation, and were assayed for ethylene production with a gas chromatograph in triplicate (GOW MAC - GM 816 model). The chromatograph was fitted with Poropak N column and a H_2-FID detector. The rate of nitrogen fixation was calculated by Ravikumar formula [26], and values were obtained nmoles C_2H_4 h^{-1} $vial^{-1}$ [27].

Correlation of bacterial growth, alginate production and nitrogenase activity

Time-coursed quantitative measurements were carried out in Erlenmeyer flasks containing 25 ml of broth medium. 100µl of bacterial suspension were inoculated into medium and incubated at 37ºC and 180 rpm. Final population of bacterial suspension was ~10^4 $CFUml^{-1}$. The un-inoculated medium used as control in each case. Sampling was carried out within 120 h. For estimation of the growth rate, 100µl of culture medium were removed every 12 hours, serial dilution were prepared and were contained colonies on solid medium and the growth curve was drawn, while the measurement of simultaneous alginate production and nitrogenase activity were done at 48h, 72h, 96h and 120h.

Isolation of algD and algU gene fragments from Azotobacter vinelandii isolate

According to the published sequences of *algD* and *algU* of *Azotobacter vinelandii* in the GenBank, 1.3-kb sequence encodes a GDP-mannose dehydrogenase and 2.5kb encodes *nadBalgUmucA* operon. The respective primers were designed to amplify for *algD* and *algU* from chromosomal DNA of *Azotobacter vinelandii* isolate.

The *algD* and *algU* gene sequence encoding GDP-mannose dehydrogenase and *algUmucABCD* operon were amplified by PCR. The primers *algD-Fwd* (ATGCGTATCATTTTCGG) and *algD-Rev* (TTACCAGCAGATGCCTTCTG) used to amplify the *algD* gene and *algU-Fwd* (ATCGACTGCGTGTATCTG) and *algU-Rev* (CGCTCTGGTTATAGAGAC) used to amplify the *algU* gene of isolated strain was designed using the *algD* and *algU* gene sequence from a genomic library of *Azotobacter vinelandii*. The PCR was carried out for both *algD* and *algU* gene, in a final volume of 50 µl containing 1 pmol of each forward and reverse primer, 1X ThermoPol reaction buffer (New England BioLabs), 0.2 mM dNTPs (Thermo scientific), 1.25 U Vent DNA polymerase (New England BioLabs) and DNA template (obtained via colony PCR). The thermal programme consisted of 3 min at 94ºC for initial denaturation, 32 cycles of 30 s at 94ºC for denaturation, 1.5 min at 60ºC for annealing and 45 s at 72ºC for extension; 7 min at 72ºC was used for a final extension.

Generation of Azotobacter vinelandii mutant

Genetic accessibility was tested by transformation of bacterial strains using electroporation [28-30], heat shock transformation and conjugation, respectively. In this research work, pGEM-T Easy vector and pEX-18 Ap vector were used**.** To develop *algU* gene mutant and *algD* gene mutant, each of two gene fragments was cloned separately into *E.coli* DH5α with the help of PGEM-T Easy vetor (Thermo Fisher Scientific). A 1.2-kb Kanamycin resistance cassette (KmR) was excised from pFKM [31], and cloned in pGEM-T easy vector with the help of a *SmaI* treatment resulting in an insert fragment of 2.7-kb. Finally, the 2.7-kb fragment was cloned in vector pEX18Ap [32], following a *HindIII* treatment. The resulting mutagenic construct was designated pEX.Km.*algD* and pEX.Km.*algU***.** To obtain *Azotobacter vineladii* mutants, *E. coli* ST18 was heat shock transformed with pEX.Km.*algD* and pEX.Km.*algU* respectively and later used for biparental conjugation with *Azotobacter vineladii S-3.*

Biparental conjugation was prepared by growing *Azotobacter vinelandii* for two days at 37ºC on BMS agar plates and resuspended into 1ml 10mM $MgSO_4$. *E. coli* ST18 Ap containing the mutagenic construct was grown overnight at 37ºC on LB agar plates supplemented with 50 µgml^{-1} 5-aminolevulinic acid (LB.AVA) and 25 µgml^{-1} Km. Bacterial cells were scratched from the plate's thereby taking double amount of isolated strain as compared to strain ST18, resuspended in LB liquid media, and mixed with each other. The mixture was spotted on BMS plus 30% LB.AVA agar plates and incubated overnight at 37ºC. After this mating period cells were scratched from plates, resuspended in MG media for 15 min. The cell suspension was serially diluted and dilutions plated on MG agar supplemented with 25 µgml^{-1} Km [32]. Since strain ST18 cannot grow without the presence of AVA, only *Azotobacter vineladii* transformants containing the KmR from the mutagenic construct transferred by homologous recombination to the bacterial genome will be capable of growing on these agar plates.

RESULTS AND DISCUSSION

Isolation and Identification of nitrogen-fixing Azotobacter vinelandii strain from mangrove rhizosphere

For the isolation of *Azotobacter vinelandii* from the mangrove rhizospheric soil, Jensen's nitrogen free liquid medium with 3%NaCl and BTB was prepared. In this case 3% NaCl was used because seawater has salinity between 3.1% and 3.8% and BTB was used as a pH indicator. Most of nitrogen fixing bacteria is mucoid so the contaminated bacteria can associate with them. To purify this strain, the selected strain was placed on nutrient agar. If other bacteria associated with this strain we can clearly see that different colonies on nutrient agar. Among 7 isolated bacteria which can turn the nitrogen free medium into blue, one isolate was chosen to study in this research work due to the alignment of the sequenced amplicon showed 99% identity to the 16S gene of **Azotobater vinelandii.** The phylogenetic relationship of *S-3* isolate (Accession Number MH048906) and related taxa was shown in (Figure1) as neighbor-joining 16S rRNA gene sequence tree.

Screening of the nitrogen fixation activity and alginate producing activity of the isolated bacteria

Nitrogen fixation activity was observed by using nitrogen free glucose mineral medium and HGB solid and liquid medium supplemented with BTB. After 3 to 5 days incubation, the medium which turned green to blue was noted because it can be assumed that bacteria can fix nitrogen for their growth so the pH of the medium higher than 7.

One function of exopolysaccharides is to protect bacteria from toxic substances. Therefore alginate producing activity of the isolated strain was observed by using various concentration of heavy metal such as copper sulphate as inducer. In this study, LB medium, NFG, Alginate Producing medium and MG (Mannitol-Glucose) medium were used. From the screening method, this strain did not show clearly differences on different media. Then MG (Mannitol-Glucose) medium was selected to study alginate producing activity with various concentration of copper sulphate. On this medium, 0.25 mM, 0.5 mM, 1 mM, 2 mM and 3 mM of the concentration of the copper sulphate was used. The colony from the medium with 0.25 mM copper sulphate showed heavy growth and more mucoid than other 0.5 mM and 1 mM copper sulphate as well as the colony from the MG medium only after one week incubation. No growth was found on the medium with 2 mM and 3 mM copper sulphate (Figure 2).

Correlation of bacterial growth, alginate production and nitrogenase activity

The correlation of bacteria growth, alginate production and nitrogenase activity of *A.vinelandii S-3* was studied (Table 3). This phenomenon provides that the increase in nitrogen fixation dependent on alginate production and the population of bacterial growth because the number of bacteria counts increase, the alginate amount increase and the nitrogenase activity increase as well. Of several methods available for measuring nitrogen fixation, the Acetylene Reduction Assay (ARA) is simpler and faster than the other methods [33]. The results obtained in this research are in agreement with the Rodelas et al. [34], who reported 9.70 to 257.73 nmol C_2H_4 h^{-1} $vial^{-1}$ for ARA rate of *A. vinelandii.*

Clementi et al. [24], assessed the minimum alginate concentration 0.1-0.5 gml^{-1} and speculated that gravimetric

Table 3: The growth, nitrogen fixation and alginate production by *Azotobacter vinelandii S-3* isolate.

Incubation Period (hr)	Bacterial Count ($CFUml^{-1}$)	Nitrogenase activity ($nmol\ h^{-1}\ vial^{-1}$)	Alginate activity (gL^{-1})
0	2.2×10^4		
12	6.2×10^4		
24	4.5×10^5		
36	3.2×10^6		
48	3.4×10^7	125	0.58
60	9.2×10^7		
72	1.2×10^8	150	0.63
84	3.6×10^8		
96	4.4×10^8	250	1.12
108	5.2×10^6		
120	8.4×10^5	160	0.95

method lacks sensitivity because of precipitation of salts and peptones. In a similar study, Sabra et al. [34], reported 0.2-0.9 gL^{-1} for alginate production by gravimetrical method. Fuminori et al. [34], stated that the sucrose-grown cells obtained at the stationary phase produced alginate at 0.3-0.4 gL^{-1}. We obtained alginate production between 0.58-1.12 gL^{-1} by the same method.

Isolation of algD and algU gene fragments from Azotobacter vinelandii S-3 isolate

Genetic Marker of Antibiotic Resistance for the isolated bacteria was tested with the following antibiotics by the agar diffusion assay [35]: ampicillin (50 mg/ml), tetracycline (25 mg/ml), gentamicin (2 mg/ml), chloramphenicol (25 mg/ml), streptomycin (25 mg/ml), carbenicillin (25 mg/ml) and kanamycin (25 mg/ml). Antibiotic stock solution was prepared as 1ml of the stock solution for 1L of nitrogen free glucose medium. The isolated strain *Azotobacter vineladii* was resistant to chloramphenicol and sensitive to all other antibiotics. The two gene fragments *algD* and *algU* were isolated from *Azotobacter vinelandii* by using the designated primers. In this case, the primers for *algU* were designed using the *algU* gene sequence of *Azotobacter vinelandii* DJ. Although the *algU* sequence of *Azotobacter vinelandii* DJ strain contains transposase that is 954-bp, the isolate in this research does not contain transposase, so the resulted band did not show the actual size. If we choose the primers within the base pair of 1.5-kb, normally the size will show at 1.5-kb, but our strain showed at 546-bp (1500-bp minus 954-bp) as the *algU* gene of our strain doesn't contain transposase gene. If we designed primers for *algU* using only the gene sequence of *algU* of *Azotobacter vinelandii* DJ, the sequence was not long enough for cloning. So the primers for *algU* were designed by using *nadB, algU* and *mucA* (2.5Kb). The resultant *algU* gene sequence was 1.6-kb.

After PCR amplification, the isolated gene fragments were checked by electrophoresis and all the two gene fragments showed the correct sites compared with the Gene Ruler DNA Ladder Mix at 1.3-kb for *algD* and 1.6-kb for *algU* (Figure 3).

Then each of two gene fragments was cloned into *E.coli* DH 5*α* with the help of pGEM-T Easy vector. The successful cloning was checked by taking ten colonies from the LB agar supplemented with chloramphenicol (*Azotobacter vinelandii* isolate is resistant to chloramphenicol) and ampicillin (pGEM-T Easy contains Ampicillin resistance gene); the plasmid from the recombinant *E.coli* DH5*α* was extracted by plasmid extraction kit. Then the inserted gene fragment was cut out from the plasmid with restriction enzyme *EcoRI.* The colony which showed the correct gene fragment site was noted and kept at minus 80ºC deep freezer. For *algD*, nine colonies showed the correct insert at 1.3-kb for *algD* and 3.0-kb for pGEM-T easy vector and for *algU*, five colonies showed correct insert at 1.6-kb for *algU* and 3.0-kb for pGEM-T easy vector (Figure 4).

Generation of Azotobacter vinelandii mutant

Mutagenic construct was made by insertion the Km cassette into each of the *algD* and *algU* gene fragments which were cloned into pGEM T Easy vector (Figure 5). Then the vector containing the mutagenic construct was transferred to *pEX-18 Ap* sucide vector and the cloning vector was transformed to *Ecoli* ST18. After that the homologous recombination was done *Azotobacter vinelandii* isolate with recombinant *E.coli ST18* which have sucide vector containing mutagenic construct. To confirm the mutant strain of *Azotobacter vinelandii*, *algD* and *algU* were isolated from the mutagenized strain by using the designed primers of *algD* and *algU.* The resulting gene fragments showed 2.5-kb for *algD* in the place of 1.3-kb and 2.8-kb for *algU* in the place of 1.6-kb because of the two gene fragments carrying Km cartridge, 1.2-kb (Figure 6A) compared with the Gene Ruler 1-Kb DNA ladder. The physical characteristic of the mutagenized strain changed into non-mucoid strain because it might be loss of its alginate producing activity (Figure 6B). The result obtained in this research was consistent with Fuminori et al. [35], who presented that it was easy to select alginate-overproducing mutants because these mutants seemed to form high mucoid colonies.

After biparental mating, the two mutant strains, *algD* negative mutant and *algU* negative mutant showed very slow growth on BMS medium with loss of mucoid. It can be assumed that these two mutant strains have lesser nitrogen fixation activity than the wild type strain. The two mutant strains can grow on MG medium supplemented with kanamycin. The physical appearance of *Azotobacter vinelandii* mutant strains was quite different from the wild type strain and no mucoid was found. The nitrogenase activity and alginate producing activity of two mutant strains were detected after four days incubation because the highest bacterial count, nitrogen fixing amount and alginate amount of wild type observed after four days incubation. Even though the

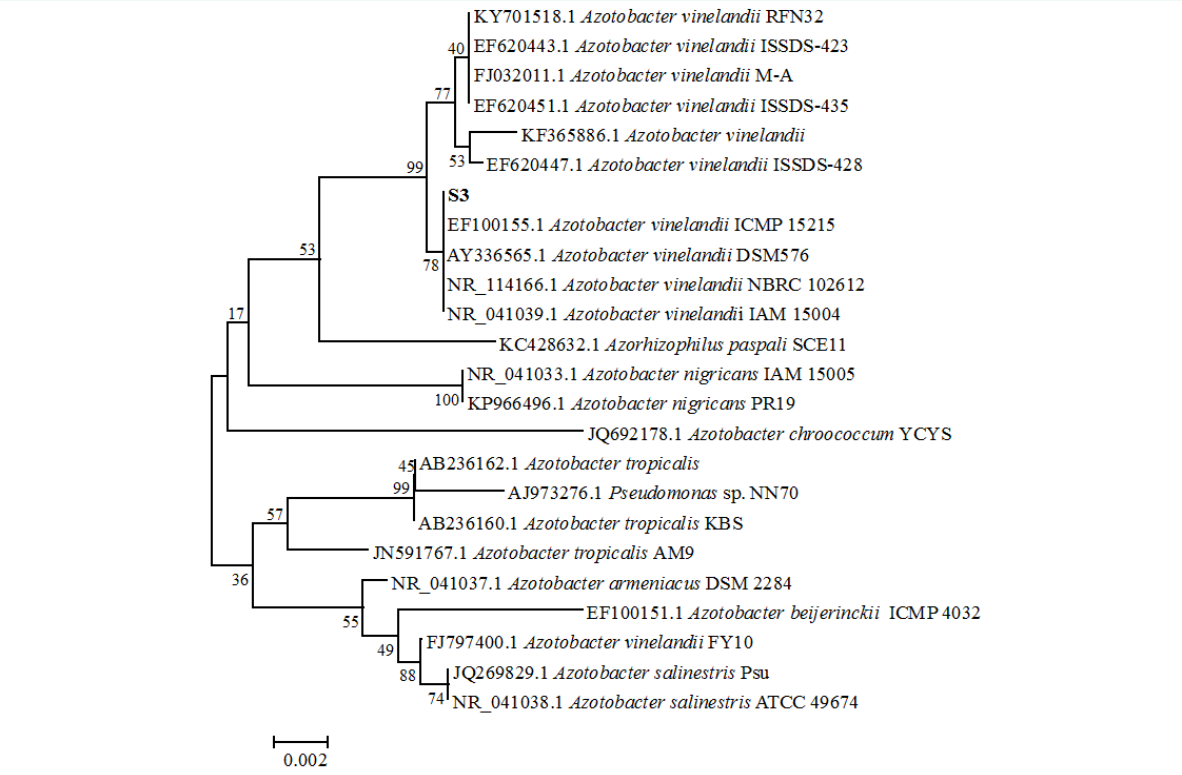

Figure 1 Neighbour-joining 16S rRNA gene sequence tree showing the phylogenetic relationship of S-3(*MH048906*) isolate and related taxa.

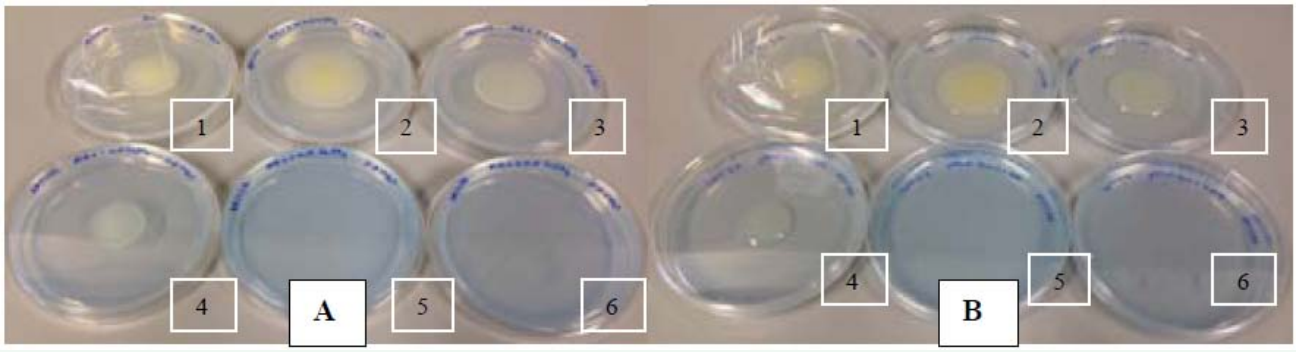

Figure 2 Screening of the alginate producing activity of isolated bacteria on MG medium with various concentration of copper sulphate after one week incubation (A) back view (B) front view.
(1) without $CuSO_4$ (2) 0.25mM $CuSO_4$ (3) 0.5mM $CuSO_4$ (4) 1mM $CuSO_4$ (5) 2mM $CuSO_4$ (6) 3mM $CuSO_4$

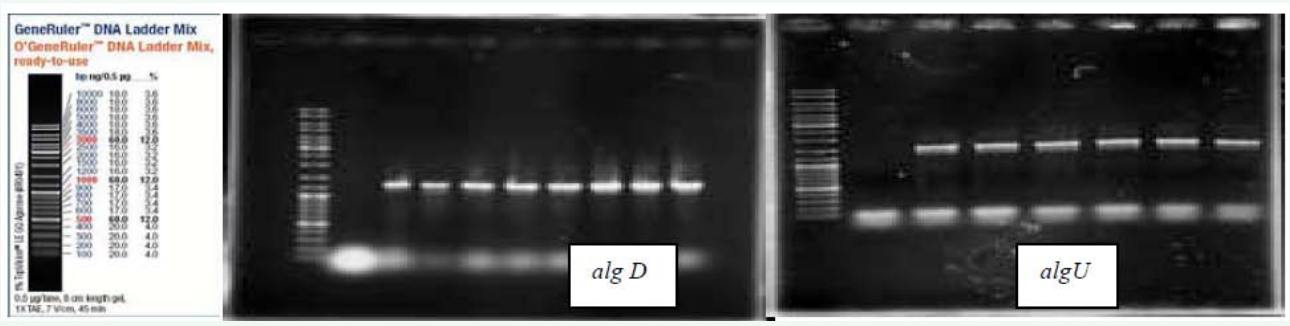

Figure 3 Isolation of *algD* and *algU* gene fragment from *Azotobacter vinelandii* isolate.

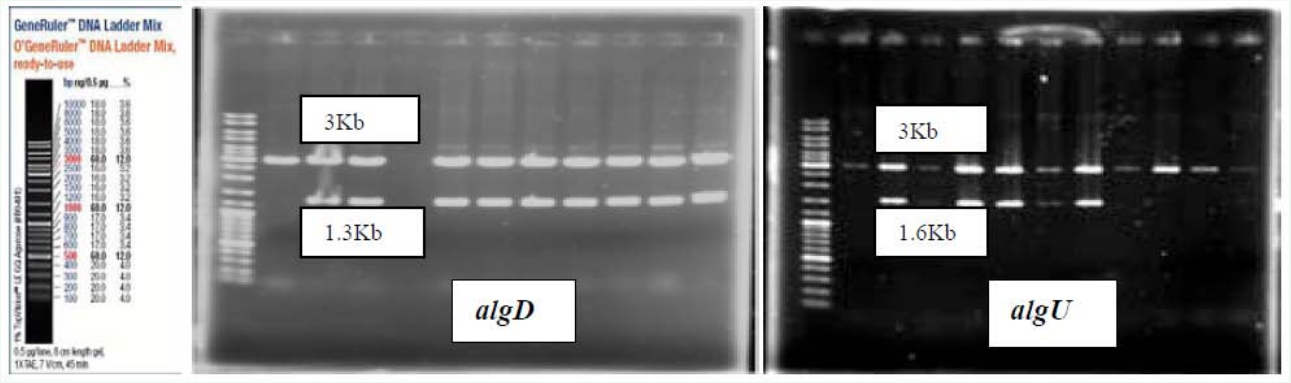

Figure 4 Checking the correct insert of the cloning colony after plasmid extraction and cutting with restriction enzyme *EcoR*I.

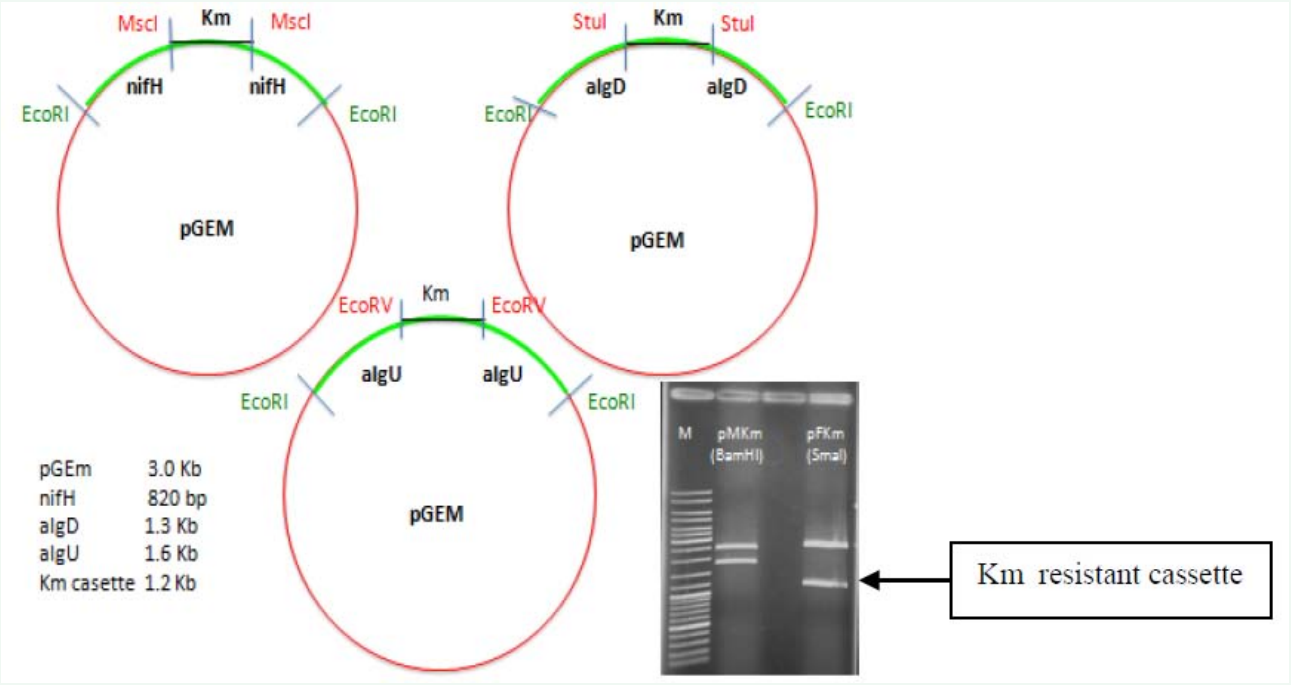

Figure 5 Diagram of mutagenic construct using Km resistant cassette.

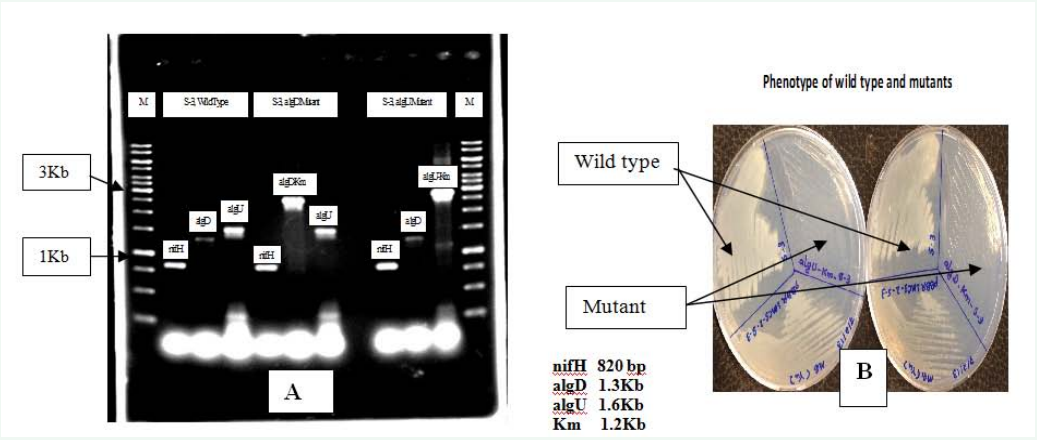

Figure 6 (A) Comfirmation of the mutant strains by PCR (B) Growth of wild type and mutant (*algD*- and *algU*-) strains on MG medium.

two mutants strain could grow very slowly on the nitrogen free medium, none of the nitrogenase amount and alginate amount was detected. Therefore it was observed that the loss of the alginate production activity caused the loss of the nitrogen fixation activity.

CONCLUSION

Azotobacter vinelandii was isolated from the mangrove rhizospheric soil of Irrawaddy region, Myanmar. Two desired mutagenic constructs were made. pEX-18 carrying mutagenic constructs could not be transformed into *Azotobacter vinelandii S-3* isolate by electroporation but two mutagenic constructs could be transformed by conjugation. Mutants were confirmed by PCR. Based on results it was found that the nitrogen fixation activity depends on the alginate producing activity.

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