



Research Article

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Development of cellulolytic nitrogen-fixing activity of *Azotobacter* spp. by transposon mutagenesis

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Abstract

The chemical mutagenized microorganisms used in this study were nitrogen-fixing bacteria; *Azotobacter chroococcum* and *Azotobacter benjerinkii* which were collected from the Microbiology Laboratory, Department of Biotechnology, Kyaukse. Transposon mutagenesis was studied on these strains to get dual activities such as nitrogen-fixing and cellulolytic activities by using recombinant *Escherichia coli* S17 carrying cellulase gene from *Rhizobium leguminosarum*. The targeted strains that carry the transposon were selected on the Glucose Nitrogen Free Mineral Medium which contains chloramphenicol (0.6 mg/20ml) and kanamycin (0.4mg/20ml). Nitrogen-fixing activity of *Azotobacter* spp. was detected by the colour changes in Glucose Nitrogen Free Mineral Medium (G-NFM) containing trace amount of Bromothymol Blue (BTB) as indicator and ammonium test kit. Cellulolytic activity of *Azotobacter* spp. before and after transposon mutagenesis was detected by both plate screening method and by Dinitrosalicylic colorimetric method. The nitrogen-fixing activities of the strains were almost the same before and after transposon mutagenesis. Cellulolytic activity measured in Glucose Nitrogen Free Mineral Medium was the highest in 5 days incubation.

Keywords: Nitrogen fixing bacteria, *Azotobacter* spp., Transposon mutagenesis, Cellulase gene, Cellulolytic activity.

INTRODUCTION

Transposons, also called transposable elements or jumping genes, are stretches of deoxyribonucleic acid (DNA) that can move around an organism's chromosome. These "transpositions" occur at a very low frequency. A transposon can contain one gene or a set of genes, and transposons are found in both eukaryotes and prokaryotes. The transposon encodes enzymes that cut the transposon from the DNA sequence and reinsert it elsewhere. This cutting and pasting requires short DNA segments at either end that are inverted repeats of each other called insertion sequences. These insertion sequences are duplicated by the transposon enzymes at the insertion site, also called the target site. No particular DNA sequence serves as the target site for transposons. However, during insertion each transposon duplicates a set number of nucleotides at the chromosomal target site [1].

Prokaryotic transposons may replicate DNA as well as cut and paste it. Transposons in eukaryotes do not replicate DNA. They move either by cutting and pasting, or by creating a ribonucleic acid (RNA) intermediate. These so-called retrotransposons are thought to be related to retroviruses whose genetic material is RNA. Retrotransposons are also thought to have created the repetitive *Alu* sequences that make up a very large fraction of human chromosomes.

Although transposition occurs at a low frequency, evolution has provided ample time in which to transpose elements. In addition to the *Alu* sequences in humans, about 3 percent of the fruit fly *Drosophila melanogaster* genome is made up of transposable element DNA [1].

In the 1940s, Barbara McClintock first discovered mobile genetic elements in corn that caused differences in gene expression, resulting in kernels containing dots of different colors against a background predominant color. Because transposons can be inserted anywhere in a chromosome, they can cause genetic mutations by disrupting whole genes, which they do in pigment genes in corn. They can also disrupt expression of genes downstream of the target site by inserting between the regulatory and the expressed parts of a gene. If two transposons end up flanking a gene, the ends can work together as one large transposon, duplicating that gene within the genome. Gene duplication is a mechanism of

evolution. One copy of the gene can mutate further, perhaps resulting in a new function, while the other ^[1]. Many bacterial transposons offer an alternative, simpler strategy. They were originally isolated as they carry drug resistant genes - in fact they are the reason that medical doctors are reluctant to prescribe antibiotics until they are certain of the diagnosis - since drug resistance spreads far too easily among bacterial species. So it is possible to chop up the chromosome of the mutant, clone the fragments into an appropriate vector and screen the transformed *E. coli* cells for the acquisition of drug resistance by including the relevant antibiotic in the media. This is kanamycin in the case of Tn5.

Bacterial conjugation is the transfer of genetic material between bacteria through direct cell-to-cell contact ^[2]. Discovered in 1946 by Joshua Lederberg and Edward Tatum, conjugation is a mechanism of horizontal gene transfer as are transformation and transductional though these mechanisms do not involve cell-to-cell contact ^[3].

Conjugation is a convenient means for transferring genetic material into a variety of targets. In the lab, successful transfer has been reported from bacteria into yeast ^[4], plants, mammalian cell ^[5, 6], and isolated mammalian mitochondria ^[7]. Conjugation has advantages over some other forms of genetic transfer for engineering purposes, namely minimal disruption to the target's cellular envelope and the ability to transfer relatively large amounts of genetic material. In plant engineering, *Agrobacterium*-like conjugation is complementary to other standard vehicles, such as tobacco mosaic virus (TMV). While TMV is capable of infecting many plant families, these are primarily herbaceous dicots. *Agrobacterium*-like conjugation is also primarily used for dicots, but monocot recipients are not uncommon.

Triparental mating is a form of bacterial conjugation where a conjugative plasmid present in one bacterial strain assists the transfer of a mobilizable plasmid present in a second bacterial strain into a third bacterial strain. Plasmids are introduced into bacteria for such purposes as transformation, cloning, or transposon mutagenesis. Triparental mating can help overcome some of the barriers to efficient plasmid mobilization. For instance, if the conjugative plasmid and the mobilizable plasmid are members of the same incompatibility group they do not need to stably coexist in the second bacterial strain for the mobilizable plasmid to be transferred.

Process requirements are as follow:

- A *helper strain*, carrying a conjugative plasmid (such as the F-plasmid) that codes for genes required for conjugation and DNA transfer.
- A *donor strain*, carrying a mobilizable plasmid that can utilize the transfer functions of the conjugative plasmid.
- A *recipient strain*, you wish to introduce the mobilizable plasmid into.

Five to seven days are required to determine if the plasmid was successfully introduced into the new bacterial strain and confirm that there is no carryover of the helper or donor strain.

MATERIALS AND METHODS

Sample Collection

Strains used throughout this research work (recombinant *E. coli* S17 carrying cellulase gene from *Rhizobium leguminosarum*, *Azotobacter benjerinkii* and *Azotobacter chorococcum*) were collected from the Microbiology Laboratory, Department of Biotechnology, Kyaukse.

Antibiotic Sensitivity Test

Antibiotic sensitivity test was conducted by culturing *Azotobacter* spp. and recombinant *Escherichia coli* S17 on appropriate medium with respective antibiotics.

Antibiotic used for recombinant *E. coli* S17 was kanamycin and concentration is (0.4mg/20ml). Antibiotic used for *Azotobacter* spp. was chloramphenicol and concentration of this antibiotic was (0.6mg/20ml). The resistance or sensitive to respective antibiotic was determined according to the growth and non-growth on the medium.

Bacterial Mating

Azotobacter strains were inoculated on Glucose Nitrogen Free Mineral Medium (GNFM) broth with chloramphenicol (0.6mg/20ml) at 30°C for 48 hours. Then, recombinant *E. coli* donor cells were grown in 2xYT broth with kanamycin (0.4mg/20ml).

Samples of donor and acceptor cells (1:1ratio) were mixed and incubated on shaker for 1 hour. Then, the mixture was centrifuged at 13000 rpm for 10 minutes and removed the tube from the centrifuge and decanted the liquid, being careful not to dislodge the pellet of cells.

The pallet was resuspended with 300 µl 2 x YT broth by vortexing the tube until pellet disappeared. Spread bacteria were placed onto 30mm sized sterile millipore filter paper on 2 x YT media and the plate was incubated overnight at 37°C. This is the point where mating take place. Now the bacteria have mated and the filter was removed with flamed forceps and put it into 10ml of 2xYT broth and vortexed thoroughly until all the bacteria were off the filter.

The suspension was centrifuged and discarded the supernatant and resuspended in 2xYT media. The resuspended pellet was inoculated onto suitable selective media (containing two types of antibiotics) and grown over night. Bacteria that can stand two antibiotics on GNFM medium were selected and it can be defined as transposon mutagenized strains.

Detection of Nitrogen Fixing Activity

Azotobacter spp. was cultured on G-NFMM broth medium at 37°C on a shaker for a week. After one week incubation, broth culture was centrifuged at 13000 rpm for 10 minutes and the pellets were removed.

And then, two drops of ammonium test kit reagent (I) were added into 1 ml supernatant. And, one fifth of reagent (II) was added and stands for 5 minutes. After 5 minutes, one drop of reagent (III) was added and stands again for 5 minutes. And then, the color development was observed and compared with the color chart from the ammonium test kit reagent.

Detection of Cellulase Producing Activity

By Plate Screening Method

The plate screening analysis for cellulolytic activity was conducted by using congo red dye. The bacteria were grown on cellulose containing nitrogen free mineral medium and pH was adjusted to 7.0 with 1M NaOH.

The cellulose agar plates were incubated at 37°C for one week to allow for the secretion of cellulose. At the end of the incubation, the agar

medium was flooded with an aqueous solution of congo red (0.1% w/v) for 15 minutes.

The Congo red solution was then poured off, and the plates were further treated by flooding with 1M NaCl for 15 minutes. The formation of a clear zone of hydrolysis indicated cellulose degradation.

Determination of Cellulolytic Activity of *Azotobacter* spp. before and after Transposon Mutagenesis by Dinitrosalicylic Colourimetric Method

The purified colony of *Azotobacter* spp. was incubated in Cellulose-NFM broth with 1% cellulose as a carbon source and 1 ml of sample was taken each day and measured cellulolytic activity for 7 days by the dinitrosalicylic colorimetric method (DNS).

Biochemical Characteristics of *Azotobacter* spp. before and after Transposon Mutagenesis

Characteristics of *Azotobacter* spp. before and after Transposon Mutagenesis were analyzed by some biochemical tests.

RESULTS AND DISCUSSION

Antibiotic Sensitivity Pattern of *Azotobacter* spp. and Recombinant *E. coli* S17

The antibiotic susceptibilities of *Azotobacter* spp. and recombinant *E. coli* S17 were detected by culturing on GNFM media with (0.6mg/20ml) chloramphenicol and on 2 xYT medium with Kanamycin (0.4mg/20ml) respectively. The resistance or sensitive to respective antibiotic was determined according to the growth and non-growth on the medium.

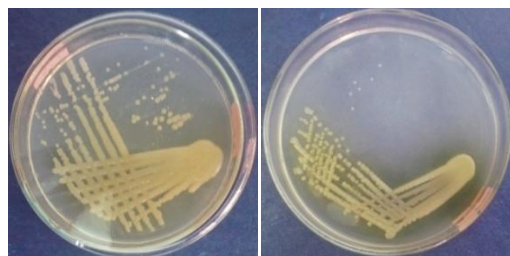


Figure 1: Growth of *Azotobacter chroococcum* (M2M) and *Azotobacter benjerinckii* (M4M) on G-NFM Medium Containing Chloramphenicol (0.6mg/20ml)

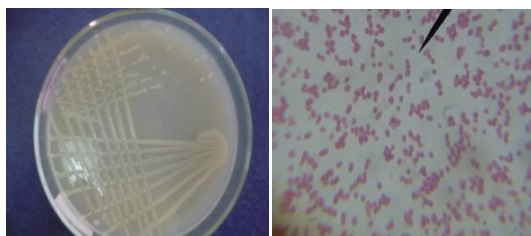


Figure 2: Growth and Microscopic Morphology of Recombinant *E. coli* S17 on YT Medium Containing Kanamycin (0.4 mg/20ml)

Transposon Mutagenesis of *Azotobacter* spp. and Recombinant *E. coli* S17

After doing conjugation experiment the transposon mutagenized strain was selected according to the antibiotic resistance activity. The colonies grown on that medium were selected as the transposon mutagenized strain.

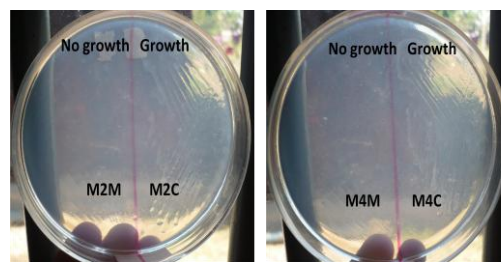
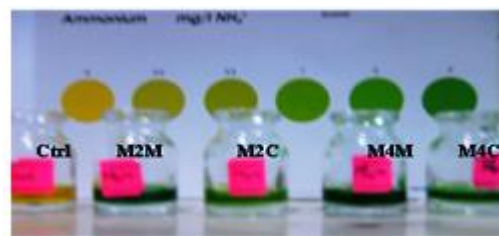


Figure 3: Growth Study of Comparison of Transposon and Chemical Mutagenized Strains on G-NFM Medium Containing Kanamycin and Chloramphenicol

Nitrogen-Fixing Activity of *Azotobacter* spp. before and after Transposon Mutagenesis

Nitrogen fixation activity was detected by ammonium test kit in G-NFM broth medium. After screening, nitrogen-fixing activity of transposon mutagenized strains fell a little. This may be due to growth rate of transposon mutagenized strains.



M2M =Chemical Mutagenized *Azotobacter chroococcum*
M2C =Transposon Mutagenized *Azotobacter chroococcum*
M4M =Chemical Mutagenized *Azotobacter benjerinckii*
M4C =Transposon Mutagenized *Azotobacter benjerinckii*

Figure 4: Detection of Nitrogen-Fixing Activity of (M2M) and (M4M) before and after Transposon Mutagenesis by Ammonium Test Kit

Detection of Cellulase Producing Activity by Plate Screening Method

All *Azotobacter* spp. grew well on 1% cellulose containing nitrogen free medium (C-NFMM) and 1% sodium carboxyl methyl cellulose containing nitrogen free medium (CMC-NFMM) and provided clear zone around their colonies when detected by pouring 0.1% congo-red solution for one hour. Diameter of clear zone was not different among them on two media. Recombinant *E. coli* S17 also grew well on 1% cellulose containing YT medium and provided clear zone around their colonies when detected by pouring 0.1 % congo red solution for one hour.

Detection of Cellulolytic Activity of *Azotobacter* spp. before and after Transposon Mutagenesis by Dinitrosalicylic Colorimetric Method (DNS)

In this research, cellulase gene from *E. coli* S 17 was transferred to the recipient *Azotobacter* spp. Therefore, in testing cellulolytic activity of *Azotobacter* spp. by dinitrosalicylic colorimetric method, it can be found cellulolytic activity was increased after transposon mutagenesis. *E. coli* S17 had the cellulolytic activity of 0.89 mg/ml at 5 day incubation.

Analyzing the results, cellulolytic activity of *Azotobacter* spp. after transposon mutagenesis was a little higher than before transposon mutagenesis.

Investigating the relationship between bacterial count and cellulolytic activity, it can be when bacterial count decreased, the cellulolytic activity of *Azotobacter* spp. before and after transposon mutagenesis increased.

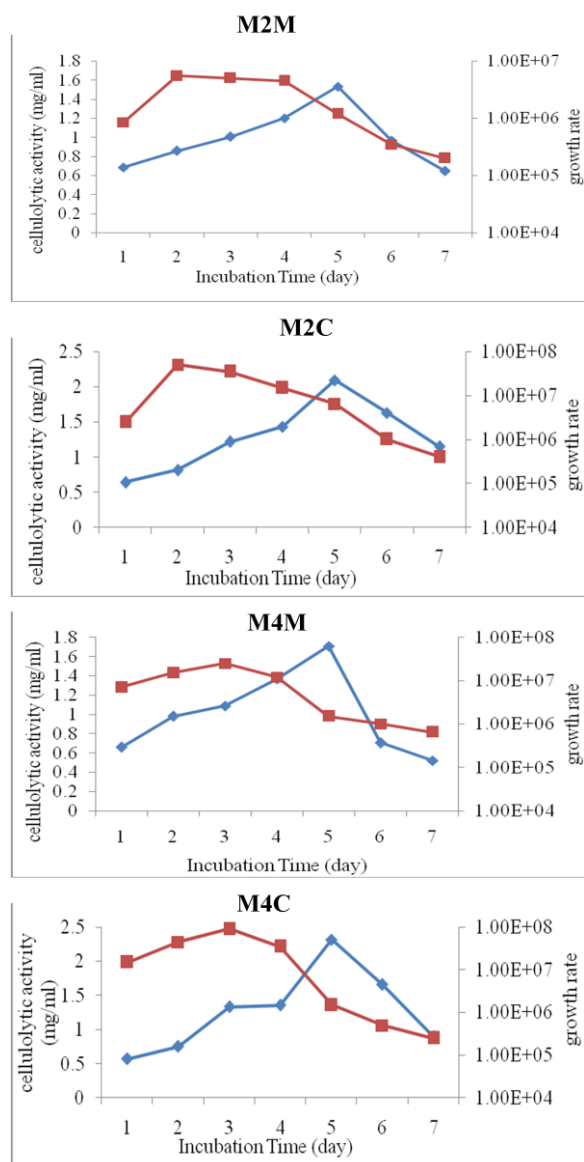


Figure 5: Detection of Cellulolytic Activity of *Azotobacter* spp. before and after Transposon Mutagenesis by Dinitrosalicylic Colorimetric Method (DNS)

Identification of *Azotobacter* spp.

Azotobacter spp. before and after transposon mutagenesis were identified by standard bacteriological methods described in Bergey's Manual of Determinative Bacteriology. Biochemical characteristics of *Azotobacter chorococcum* and *Azotobacter benjerinckii* before and after transposon mutagenesis were not so much changed. *Azotobacter chorococcum* and *Azotobacter benjerinckii* were different in motility test. The colonial morphology of *Azotobacter chorococcum* is pale yellow, mucoid and 1 mm in diameter. Microscopic morphology of this species is 1.5-2.0 μm , rod to coccoid and it can be found in pairs, sometimes in chains of varying length and it can not produce endospore but form cysts. The colonial morphology of *Azotobacter benjerinckii* is yellow, mucoid and 1 mm in diameter. Microscopic morphology of this species is 1.5-2.0 μm , rod to coccoid and it can be found in pairs, sometimes in chains of varying length and it cannot produce endospore but form cysts.

The colonial morphology of transposon mutagenized strains changed from cream colour to transparent and microscopic morphology also changed. Microscopic morphology of these species were 1.0-1.5 μm , rod to coccoid, in pairs, sometimes in chains and cannot form endospore.

Table 1: Biochemical characteristics of *Azotobacter* spp. before and after transposon mutagenesis

Biochemical characteristics	Strains			
	M-2C	M-2M	M-4C	M-4M
Colonial morphology	Transparent, Mucoid, 1mm in diameter	Pale yellow, mucoid, 1mm in diameter	Transparent, Mucoid, 1mm in diameter	yellow, mucoid, 3-4mm in diameter
Microscopic morphology	1.0-1.5 μm , rod to coccoid, in pairs, in chains, form cyst	1.5-2.0 μm , rod to coccoid, in pairs, in chains, form cyst	1.0-1.5 μm , rod to coccoid, in pairs, in chains, form cyst	1.5-2.0 μm , rod to coccoid, in pairs, in chains, form cyst
Gram reaction	-	-	-	-
Motility	+	+	-	-
Catalase	+	+	+	+
Starch hydrolysis	+	+	+	+
Gelatin liquefacien	+	+	+	+
Nitrate reduction	+	+	+	+
Citrate utilization	+	+	+	+
Methyl red	-	-	-	-
Indole test	+	+	+	+

CONCLUSION

In this research work, two *Azotobacter* spp. were conjugated with recombinant *E. coli* S17 that contain cellulase gene from *Rhizobium leguminosarum* and two transposon mutagenized strains were obtained.

When testing the nitrogen fixing activity of *Azotobacter* spp. before and after transposon mutagenesis by ammonium test kit, nitrogen-fixing activity of transposon mutagenized strains fell a little.

Cellulolytic activity was higher after transposon mutagenesis and the highest amount of cellulase produce at 5 days incubation period in cellulose nitrogen free mineral medium.

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