

# Improvement of cellulolytic activity in cellulolytic nitrogen-fixing bacteria by transposon mutagenesis

## Abstract

Strain improvement of four cellulolytic nitrogen-fixing bacterial isolates were generated by transposon mutagenesis using recombinant *Escherichia coli* S17 strain carrying cellulase gene from *Rhizobium Leguminosarum*. Four transconjugant strains produced higher amount of reducing sugar concentration on cellulose substrate but their nitrogen fixing activities were nearly the same. After detection of the cellulolytic activity, wild type and mutant strains (H3, H3m and H7m) produced the highest concentration of reducing sugar (0.125µg/ml, 0.312µg/ml and 0.248µg/ml) on 0.2% cellulose substrate after 9th day incubation period. The other strains (H6m, H8 and H8m) reached the maximum growth rate and produced the highest amount of reducing sugar in 6th day incubation period. Reducing sugar concentration converted from the five types of agricultural cellulose waste for H3m were two times higher than H3. Reducing sugar production of H8m was also higher than H8 by using different types of agricultural cellulose waste as substrates.

**Keywords:** cellulolytic nitrogen-fixing bacteria, cellulase gene, transposon mutagenesis, reducing sugar, agricultural cellulose waste

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## Introduction

As cellulose is the most abundant renewable natural product in the biosphere, cellulolytic microorganisms are fundamental for the transformation of cellulose into sugars that are essential nutrients for various organisms and for bio-fuel<sup>1</sup>. Since the annual production of cellulose is estimated at 4.0x10<sup>7</sup> tons, large quantities of industrial and agricultural cellulosic waste have accumulated due to inefficient use.<sup>2</sup> Cellulosic biomass comprising forestry, agricultural and agro-industrial wastes are abundant, renewable and inexpensive energy sources. Such wastes include a variety of materials such as sawdust, poplar trees, sugarcane bagasse, waste paper, brewer's spent grains, switch grass, and straws, stems, stalks, leaves, husks, shells and peels from cereals like rice, wheat, corn, sorghum and barley, among others. Plant fiber is a complex natural composite consisting of various different chemical substances and mainly composed of cellulose, hemicellulose and lignin with minor amount of pectin.<sup>3</sup> Due to its main chemical composition, plant fibers are also called as cellulosic or lignocellulosic fibers.<sup>4</sup> The cellulosic waste from crop residues and other agricultural wastes contain 31–60% cellulose, 11–38% pentose, and 12–28% lignin.<sup>5</sup> Myanmar is the agricultural country and produced enormous amount of agricultural waste every year but the microbiological research on these biomass is still rare.

Nitrogen-fixing microorganisms are used to reduce dependence on nitrogenous chemical fertilizers.<sup>6</sup> The decomposition of the cellulose is inhibited by a nitrogen limitation and this could be overcome by decomposer organisms which combined both cellulolytic and nitrogen-fixing function. Since 1986, Dorothy studied that degradation of wheat straw with combination of *Cellulomonas* strains and nitrogen fixing strains. But the process must be separated in either time or space on cellulolytic and nitrogen fixing activity because growth condition was difference.<sup>7</sup> The cellulolytic nitrogen-fixing bacteria can be used in agricultural residues degradation by converting them into organic fertilizers through microbial activities. The role of nitrogen-fixing bacteria on cellulose degradation may be useful in developing technologies to convert solid waste into nitrogen

rich compost. Cellulase activities have been seen in many nitrogen-fixing bacteria such as *Stenotrophomonas spp*, *Bacillus spp*, *Pseudomonas spp*, *Paenibacillus azotofixans*, *Gluconacetobacter*, *Azospirillum spp*.

Using a combination of genetic and DNA technology approaches, a few strains with increased specific activities have been reported by Sheir-Neiss & Montencourt et al.<sup>8</sup> Transposon mutagenesis involves the use of mobile genetic elements. The most commonly employed of these are a sub-class called transposon, short pieces of DNA that replicate by inserting into other piece of DNA (plasmid, chromosome, viruses). They encode two sets of functions. One set is involved in regulating and performing the movement of the transposon from one piece of host DNA to the next (transposition function). The other set of functions encode genes that may provide an advantage for the host of the transposon, for example, antibiotic resistance, ability to utilize new metabolites etc. In this research, *E. coli* S17 with modified transposon that has had the normal drug resistance gene of Tn5 and cellulase gene from *Rhizobium leguminosarum* was used as donor strain. Plasmid DNA has been introduced by conjugation using an *Escherichia coli* strain expressing transfer functions. Conjugation is a process that involves direct cell-to-cell contact. Virtually every recipient that comes into contact with a donor receives the donor's plasmid. *E. coli* S17 that contains *tra* gene is very useful for bacterial conjugation, the transfer of genetic material between bacteria through direct cell to cell contact.<sup>9</sup> The aim of this research work was to improve cellulolytic activity in cellulolytic nitrogen-fixing bacteria via transposon mutagenesis using transformed *E. coli* S17 carrying cellulase gene from *Rhizobium Leguminosarum*.

## Materials and methods

### Sample collection, media and growth condition

Bacterial strains were isolated from rice rhizospheric soil around Pathingyi Township, Mandalay Region, Myanmar and inoculated in the Glucose- Nitrogen Free Mineral Medium (GNFMM) at 37°C. The compositions of G-NFMM are: KH<sub>2</sub>PO<sub>4</sub> 1.0g, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.25g, NaCl 0.5g, FeSO<sub>4</sub>·7H<sub>2</sub>O 0.01g, MnSO<sub>4</sub>·H<sub>2</sub>O 0.01g, Na<sub>2</sub>MoO<sub>4</sub> 0.01g,

glucose 20g, Agar 12.0g (g/1liter). *Escherichia coli* strain, (having the normal drug resistance gene (Kanamycin resistance gene) of Tn5 and cellulase gene from *Rhizobium Leguminosarum*) was obtained from Open Research Center, Shibaura Institute of Technology, Japan and was maintained on 2xYT medium at 37°C. The compositions of 2xYT medium are: tryptone 16g, yeast 10g, NaCl 5g, agar 15g (g/1liter).

### Primary screening of nitrogen-fixing activity

Isolated strains were selected based on their nitrogen-fixing activity by inoculating on G-NFMM medium with BTB (bromothymol blue) as an indicator and incubated at 37°C for a week. G-NFMM liquid medium without BTB was prepared and incubated on a shaker for detecting the nitrogen fixation amount by ammonium test kit.

### Primary screening of cellulase producing activity

The preliminary qualitative analysis for cellulolytic activity was conducted by using Congo red dye.<sup>10</sup> The bacteria were grown on Cellulose agar containing 0.2% C-NFMM (Cellulose instead of Glucose) and incubated at 37°C for one week and then flooded with Congo red (0.1% w/v) solution. The ratio of the clear zone diameter to colony diameter was measured to select for the highest cellulase activity producer.

## Identification of the selected bacterial strain

### Detection via biochemical characteristics

The selected bacterial strains were characterized by gram staining, colony morphology and biochemical tests according to the instructions of Bergey's Manual of Determinative of Bacteriology.<sup>11</sup>

### 16s r DNA identification

DNA extraction was performed using the MicroSEQ bacterial identification kit (Thermo Scientific). Bacterial 16S r DNA was amplified by using universal primers. Each cycle consisted of denaturation for 1min at 94°C, annealing for 30s at 60°C, and extension for 4min at 72°C. DNA purification was done using MicroSEQ ID purification kit (Thermo Scientific). Nucleotide sequences were performed using the Big Dye Terminator Cycle Sequencing Kit (v.3.1) and analyzed in the ABI 3130 Genetic Analyzer (Applied Biosystems). Nucleotide sequences were analysed using BLAST on the National Center Biotechnology information; NCBI website (<http://www.ncbi.nlm.nih.gov/>) and Greengenes.lbl.gov.

### Antibiotic activity testing for selection purpose

Antibiotic susceptibility test of the isolated strains and *E. coli* were inoculated by agar well diffusion technique<sup>12</sup> using seven different antibiotics. Different concentration of Kanamycin and Chloramphenicol were used as antibiotic marker for conjugation. To obtain Minimum Inhibitory Concentration (MIC) values, various concentrations was added to the culture media and then, donor and recipient strains were cultivated by streaking and incubated at 37°C for 24 hours.<sup>12</sup>

### Bacterial conjugation

The transfer of the cellulase gene from *E. coli* was carried out by bacterial conjugation process described by Notani et al.<sup>13</sup> with a few modifications.<sup>13</sup> After one day incubation of the separated culture of donor and recipient strains, samples were mixed (1:1) and 25µl of the mixture were spread onto 30mm of 0.45µm sterile millipore filter

paper. After 24hr incubation, the filter was removed and put it into 10ml of LB broth and vortexed thoroughly until all the bacteria were off the filter. Then the conjugation mixtures were spread onto suitable selective media (containing two types of antibiotics) and incubated for 24hr at 37°C. After incubation, growth of transconjugant strains was observed and studied their activities.

### Detection of ammonium concentration

The ammonium concentration of the bacterial strains was detected by Indophenols method. The bacterial strains were inoculated in G-NFMM liquid medium and incubated at 37°C. All the chemical and reagents were prepared as according to the new approach to indophenols blue method by Nikolaos et al.<sup>14</sup> 1 ml of sample was centrifuged at 8000rpm for 10minutes. Ammonium excreted in supernatants was added with 0.5ml of EDTA solution and 0.5ml of P-buffer in the flask. The solution was neutralized with 1M NaOH by the indication of methyl red color reagent changed from red to yellow, and then 2.5ml of nitroprusside reagent was added immediately and stirred well. Finally, 2.5ml of 10% hypochlorite solution was added and then the volumetric flasks were kept at room temperature or at 30°C for 3 hr or longer. The optical absorption at 625nm was measured.

### Detection of cellulolytic activity

Reducing sugars were estimated spectrophotometrically with 3, 5-dinitrosalicylic acid.<sup>15</sup> The isolated bacterial strains were incubated in C-NFMM broth (0.2% cellulose). Cultures were harvested 3 days interval and centrifuged at 5000rpm for 20min. 0.5ml of supernatants was added to 1ml of 0.05M citrate buffer at pH 4.8 in the test tube and incubated in water bath shaker at 80-85rpm, 50°C for 1hr. After incubation, 3ml DNS containing 40% potassium sodium tartrate (Rochelle salt) solution is added. The mixture was heated at 90°C for 5-15 minutes to develop the red-brown colour. After cooling at room temperature in a cold water bath, 0.2ml of mixture is added with 2.5ml of water and then the reducing sugar was measured by optical density method at 540nm.

## Cellulolytic activity of bacterial strains in various agricultural residues

Agricultural residues such as rice straw, corn stalk, pea husk, sugarcane bagasse and rice husk residues were used as substrates in this study. All agricultural wastes were collected and dried at room temperature and sterilized at 121°C for 15 minutes. Then 25g of the agricultural residues were ground into powder using a blender. The bacterial strains were cultured in NFM liquid media supplemented with 0.2% of agricultural residues as a carbon source and incubated at 37°C. Cellulolytic activity of wild type and transconjugant mutant strains were also detected in measuring the reducing sugar concentration by DNS method for 3 days interval.

## Results and discussion

### Isolation of cellulolytic nitrogen-fixing bacteria

In this study, 10 bacterial strains were isolated from the rice rhizospheric soil source around Pathaingyi Township, Mandalay, Myanmar and cultured on G-NFMM containing BTB as indicator. Other reported that cellulolytic nitrogen-fixing bacteria have been isolated from diverse effluent treatment plants and wastewater<sup>16</sup> and from a Brazilian sugar cane variety.<sup>17</sup>

## Screening of nitrogen fixing activity

Primary screening of the nitrogen fixing activity of the isolated bacterial strains were carried out on G-NFMM media and all the isolated bacteria produced ammonium by changing the colour of the media from blue to green. By detection of Viscolor Ammonium test kit, five out of ten isolated strains (H2, H3, H6, H7 and H8) excreted the highest amount of ammonium concentration (>3ppm) in G-NFMM broth. But in C-NFMM broth only four strains (H3, H6, H7 and H8) excreted more ammonium concentration than the others. The result of the reducing sugar concentration produced by selected bacterial strains was shown in Table 1.

**Table 1** Ammonium concentrations excreted by isolated strains in g-nfmm and c-nfmm

Isolated strains	Ammonium concentration in G-NFMM (ppm)	Ammonium concentration in C-NFMM (ppm)
H-1	~2.5	1-2
H-2	>3	~2
H-3	>3	~2
H-4	~0.5	~3
H-5	ND	~1
H-6	~3	~3
H-7	>3	~3
H-8	>3	2-3
H-9	ND	ND
H-10	ND	ND

ND, not detected

## Screening of cellulase producing activity

The primary screening of cellulolytic activity of the selected 4 bacterial strains were determined in the plate screening method with 0.2% cellulose-NFMM and determined their cellulolytic activity by DNS method at 540 nm. The two strains (H6 and H7) did not show cellulolytic activity in plate screening assay and also in DNS method. The remaining two strains (H3 and H8) showed clear zone formation on C-NFMM medium and produced reducing sugar in the concentration of 0.125µg/ml and 0.100µg/ml respectively and the result were shown in Table 2.

**Table 2** Reducing sugar concentrations produced by selected strains on cellulose substrates

Bacterial strains	Reducing sugar Concentration (µg/ml)
H3	0.125
H6	ND
H7	ND
H8	0.1

ND, not detected

## Identification of the selected bacterial strain

According to the biochemical analysis, isolated bacterial strains were predicted as the *Pseudomonas* and *Stenotrophomonas spp.*

The 16S r DNA partial gene sequence analysis proved that the isolated bacteria H3 and H7 belong to 99.6% identity with that of *Stenotrophomonas malophila spp.* and H6 and H8 were 96% similarity to *Pseudomonas sp.* compared with the data sequences from NCBI BLAST and Greengenes.lbl.gov.

## Antibiotic activity testing for selection purpose

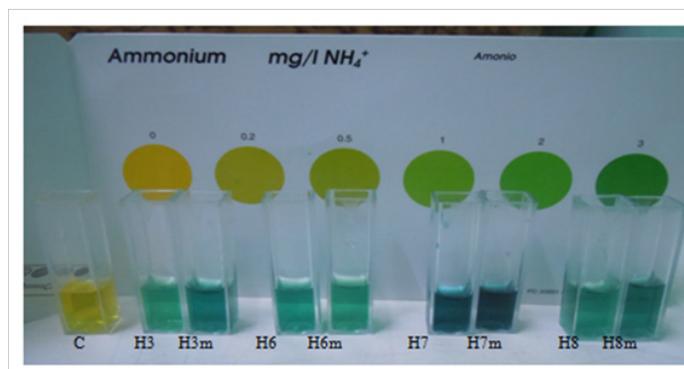
Antibiotic sensitivity patterns of the selected strains and *E. coli* S17 on seven different antibiotics were shown in Table 3. Among them Kanamycin and Chloramphenicol were selected to be used in conjugation process. According to MIC, the isolated bacteria were detected by culturing on G-NFMM with 30mg/ml Chloramphenicol but no growth on Kanamycin, the *E. coli* S17 was also growth on 2xYT medium with Kanamycin 20mg/ml, not in Chloramphenicol respectively. This was the main point in conjugation process because antibiotics were used as a marker to select the conjugated colonies.

**Table 3** Antibiotics sensitivity patterns of the selected strains (Recipients) and *E. coli* S17 (Donor)

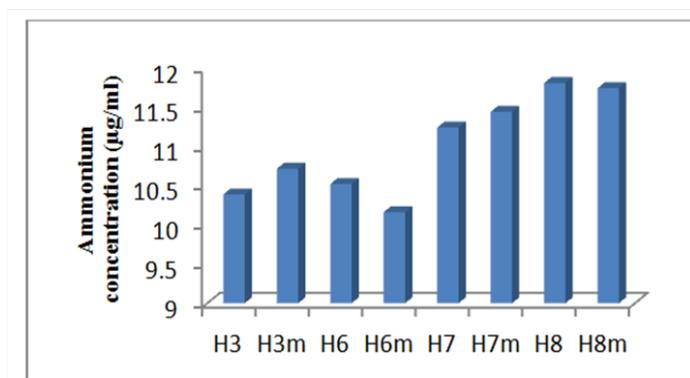
Antibiotics	Concentration (µg/ml)	H-3	H-6	H-7	H-8	<i>E. coli</i> S17
Chloramphenicol	20	+	+	+	+	-
Penicillin G	0.5 U	+	+	+	+	+
Ampicillin	20	+	+	+	+	+
Tetracycline	40	+	+	+	+	+
Kanamycin	15	-	-	-	-	+
Rifampicin	10	+	+	+	+	-
Ciprofloxacin	1	+	+	+	+	-

## Detection of ammonium concentration after conjugation

After conjugation experiment, the nitrogen fixing activities of the wild type and transconjugant mutant strains were measured again by Vis color Ammonium Test Kit and Indophenols method. There was not quite difference before and after conjugation and all excreted the ammonium concentration above 3ppm. Figure 1 showed the color development of wild type and transconjugant mutant strains for nitrogen-fixing activity on detection by Ammonium Test Kit. Excreted ammonium concentrations of wild type and mutant strains were shown in Figure 2.



**Figure 1** Screening the nitrogen fixation activity of wild type and mutant strains by ammonium test kit

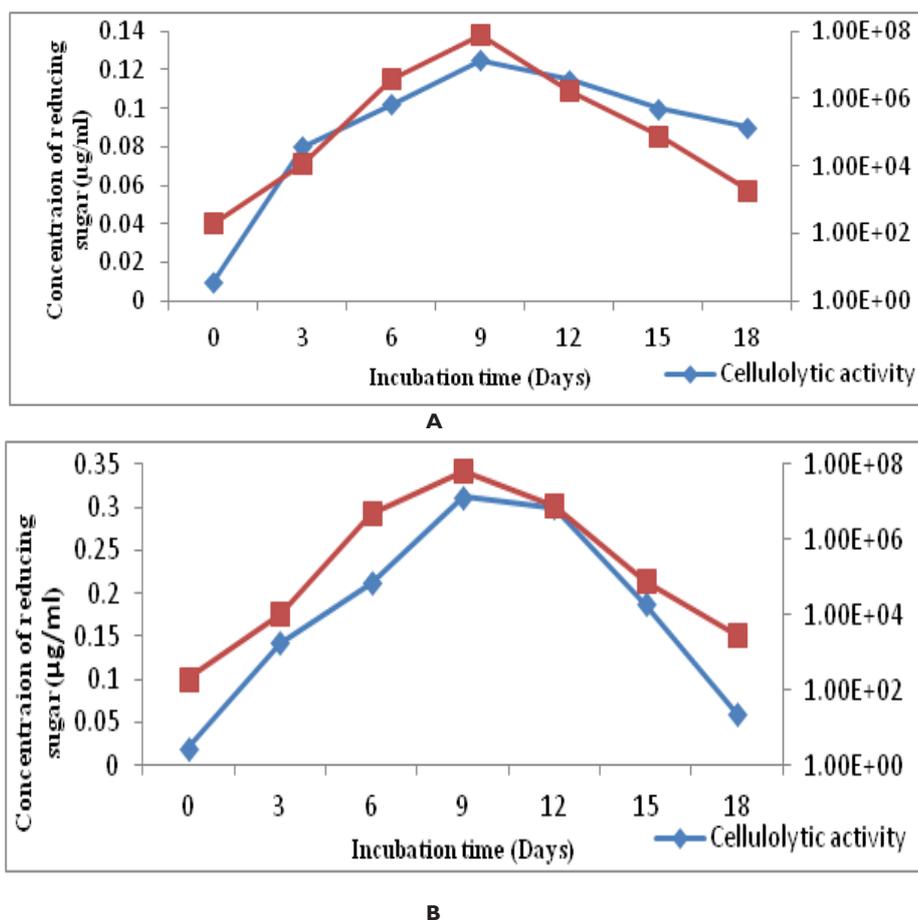


**Figure 2** Quantitative determination of ammonium concentrations excreted by wild type and transconjugant mutant strains in G-NFMM by indophenols method

### Correlation of ammonium concentration and growth rate of wild type and mutant strains

The present study was also determined the correlation between the cellulolytic activity and growth rate of wild type strains and transconjugant mutant strains by using 0.2% cellulose as substrate for

3 days interval and the results were shown in Figures 3–6 respectively. H3 and H3m reached the maximum growth rate and produced the highest amount of reducing sugar (0.125, 0.312 µg/ml) in 9th day incubation period in cellulose substrate. But H8 and H8m got the peak in 6th day incubation period and produced (0.192, 0.432 µg/ml) amount of reducing sugar concentration respectively. Although the remaining two wild type strains (H6 and H7) did not produce reducing sugar, their transconjugant mutants (H6m and H7m) reached the maximum growth rate in 9th day and 6th day incubation period respectively and produced the highest amount of reducing sugar (0.273, 0.248 µg/ml) at their maximum growth rate. This analysis was shown that conversion of cellulose substrate into reducing sugar by cellulase enzyme of wild type (H3, H8) and all mutant strains (H3m, H6m, H7m and H8m) were highest in their maximum growth rate. Using a combination of genetic and DNA technology approaches, a few strains with increased specific activities have been reported Sheir-Neiss & Montencourt et al.<sup>8</sup> Moreover, there were some reports that the mutant stains produced more reducing sugar concentration than the wild types strains. A heterologous expressed *Cellulomonas fimi* exoglucanase with increased specific activity from *E. coli* was overproduced.<sup>10</sup> In this study, it was observed that the cellulolytic activity of transconjugant mutants were two times higher than the wild type strains at their maximum growth rate by using cellulose substrate.



**Figure 3** Growth curve and reducing sugar concentration produced by H3 & H3m using cellulose substrates (A) H3 (B) H3m

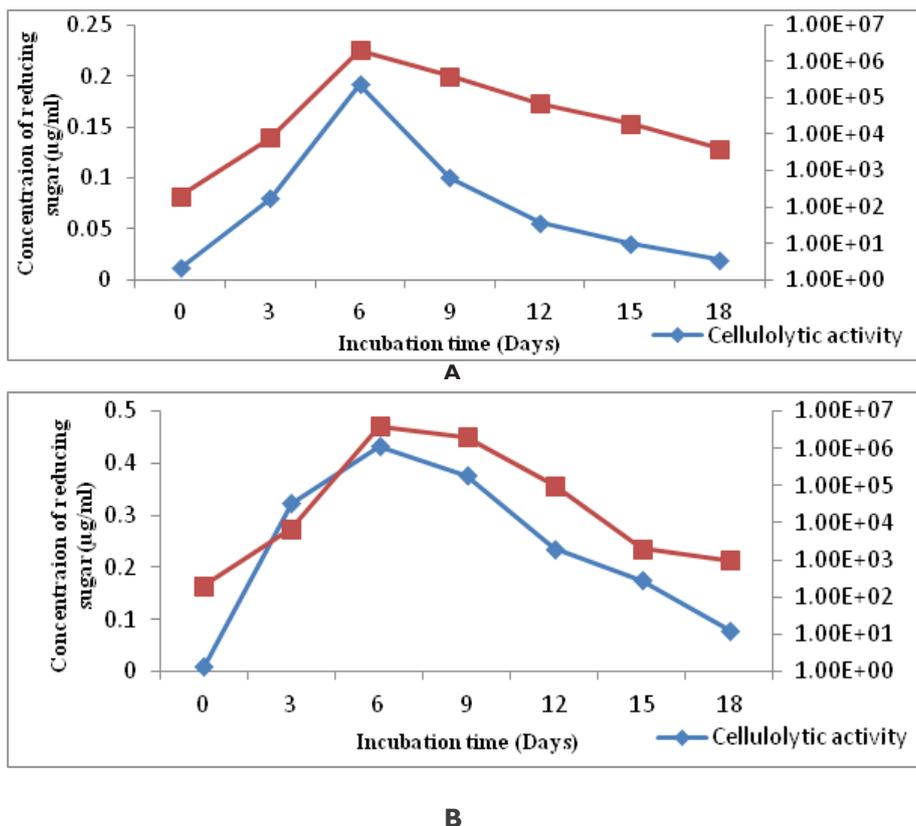


Figure 4 Growth curve and reducing sugar concentration produced by H8 & H8m using cellulose substrates (A) H8 (b) H8m

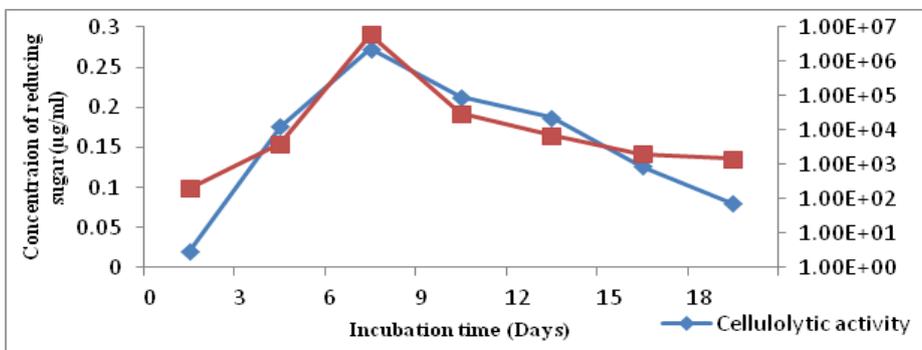


Figure 5 Growth curve and reducing sugar concentration produced by H6m using cellulose substrates

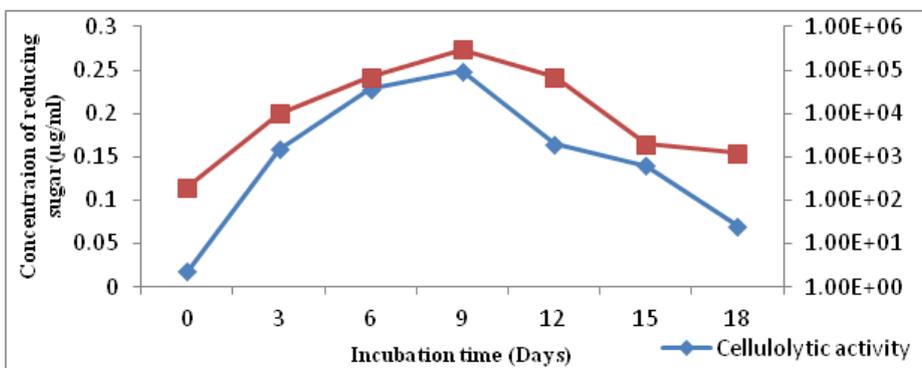


Figure 6 Growth Curve and Reducing Sugar Concentration Produced by H7m Using Cellulose Substrates

## Cellulolytic activity of bacterial strains in various agricultural residues

In the present study, five types of agricultural residues (rice straw, corn stalk, pea husk, sugarcane bagasse and rice husk) were used to determine their cellulolytic activity. Soni et al.<sup>18</sup> reported a maximal production of endoglucanase in rice straw when comparing different sources of carbon during cultivation of *A. fumigatus*.<sup>18</sup> Gupte and Madamwar found an endoglucanase activity of 14.55 IUg<sup>-1</sup> when using sugarcane bagasse, in comparison to other substrates during co

cultivation of *Aspergillus ellipticus* and *A. fumigatus*.<sup>19</sup> The reducing sugar concentration was differed depending on the substrates. It can be observed that the nature of the carbon source in the culture medium had a significant influence on endoglucanase production. In this study, rice straw was found to result in maximal cellulose producing activity, followed by Corn stalk, Pea husk, Sugarcane bagasse and Rice husk. The reducing sugar production was determined in wild types and transconjugant mutant strains by using agricultural cellulose wastes as substrates and their reducing sugar concentration were shown in Table 4.

**Table 4** Reducing sugar concentration converted from agricultural substrates by transposon mutagenized strains and wild type strains

Substrates	Reducing sugar Concentration (µg/ml)							
	H3	H3m	H8	H8m	H6	H6m	H7	H7m
Rice Straw	0.122	0.292	0.09	0.402	ND	0.302	ND	0.289
Corn stalk	0.119	0.28	0.078	0.37	ND	0.274	ND	0.204
Pea husk	0.08	0.258	0.064	0.3	ND	0.212	ND	0.176
Sugarcane bagasse	0.11	0.201	0.047	0.27	ND	0.198	ND	0.134
Rice husk	0.038	0.128	0.027	0.207	ND	0.125	ND	0.092

The highest production reducing sugar by using all five types of substrates was achieved after 6th day and 9th day of incubation period respectively. After this period, there was a decrease in cellulolytic activity. Although H8 produced a slight amount of reducing sugar (0.09, 0.078, 0.06, 0.064, 0.047 and 0.027µg/ml) by using 0.2% of each of the agricultural substrates, H8m produced higher amount of reducing sugar concentration than H8. H6 and H7 did not produce the reducing sugar in all substrates of agricultural residues. Cellulolytic activities of H6m and H7m were also provided on agricultural residues.<sup>20-24</sup> H6m produced (0.302, 0.274, 0.212, 0.198 and 0.125µg/ml) and H7m produced (0.289, 0.204, 0.176, 0.134 and 0.092µg/ml) of reducing sugar concentration respectively. The present investigation revealed that different agricultural substrates produced different amount of reducing sugar concentration.

## Conclusion

The present investigation revealed the improvement of the cellulolytic activity in cellulolytic nitrogen fixing bacteria by transposon mutagenesis. Cellulolytic nitrogen fixing bacteria were isolated and determined their nitrogen fixing activity and cellulolytic activity. The improvement of cellulolytic activity was carried out by transposon mutagenesis using antibiotics as selective markers. The nitrogen fixing activity of the transconjugant mutants strains were not differed from the wild type strains. In the case of cellulolytic activity, the transposon mutagenized strain degraded cellulose more than the wild type strains at their maximum growth rate. Using the agricultural cellulose waste as substrates, the reducing sugar producing of transposon mutagenized strains was higher than the wild type strains. But depending on the agricultural cellulose waste used, the amount of reducing sugar producing were differed. This attributes will be of great importance for future applications in biofertilizer preparation, genetic study of cellulase genes and genetic diversity of cellulolytic nitrogen fixing bacteria.

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## Conflict of interest

Authors declare that there is no conflict of interest.

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