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Biodegradation of Cellulose by New Recombinants of *Rhizobium* Harboring Cellulase Genes

Mervat I. Kamal*; K. A. Zaied; Mervat G. Shrabia and A. H. Abd El–Hadi

Department of Genetics, Faculty of Agriculture, Mansoura University, Egypt

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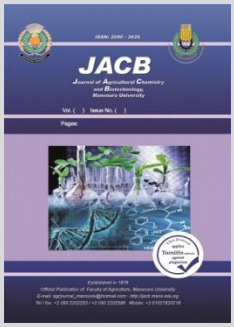

ABSTRACT

Plant biomass is efficiency decomposed by a great number of different microbial enzymes. The enzyme systems in cellulolytic bacteria have been genetically improved via plasmid transfer and genome shortening. In this study six conjugations were done between six parental strains carrying the opposite genetic markers from which 18 transconjugants were evaluated for biodegradation of cellulose. Three strains of *Rhizobium leguminosarum bv. trifolii* were used as recipients against *Bt* and *Serratia* strains expressing cellulose activity as donors. The wild type strains of *Bacillus thuringiensis* and *Rhizobium* encoded significant cellulose biodegradation after genome shortening. In addition, all transconjugants resulted from the mating between Bt_1 and Rh_{11} expressed significant cellulase activities after curing. In constrant, all transconjugants results from the mating between Bt_2 x Rh_6 and Bt_2 x Rh_{11} appeared insignificant differences in cellulase activity before and after curing. Meanwhile, the parental strains Rh_6 and Rh_{11} expressed significant cellulase activity after curing. Furthermore, all transconjugants resulted from the mating between *Sm* x Rh_6 and *Sm* x Rh_{15} encoded significant cellulase activity after curing. This work provides efficiently encoded cellulases in genome shortening mutants than in the wild type. The enzymes encoded in this study represented that cellulase expressing genes were located on the bacterial chromosome and plasmids. Though, plant infection of legumes was affected by *Rhizobia* cellulases which considered as important determinant in nodulation process.

Keywords: Plasmid curing, cellulose biodegradation, genome shorting.

INTRODUCTION

About half of the bacteria containing cellulase genes, hemicellulases and pectinases which are efficiently degrading the wastes of plant biomass (Medie *et al*. 2012). However, only a small number of bacteria strains possess more than three genes for b-1,4-glucanases (cellulases), which were effective for biodegradation of natural cellulose (Schwarz *et al*. 2004).

Truly cellulolytic microorganisms had great importance for providing the genetic information on the production of cellulolytic and hemicellulolytic enzymes that can be used for application in many industrial and biotechnological processes. Recently the genomic sequences of a number of truly cellulolytic species became publicly available (Schwarz *et al.* 2011). Cellulolytic activity in bacteria is regulated on the transcriptional level. The regulatory mechanisms for the synthesis of extracellular cellulolytic enzymes are still poorly understood. The presence of cellulose in the medium might function as an inducer (Nataf *et al.* 2010). Cellulases (CelS) transcription is induced under conditions of carbon or nitrogen limitation (Dror *et al*. 2003).

Cellulose is the most plentiful molecule on the earth. Most plants are composed of cellulose and hemicellulose polymers along with minor amounts of other sugar polymers such as starch and pectins (Han and Rowell, 1996). Cellulose is organic polymer and is the main component of plant biomass that provides stability. Cellulose is a linear polysaccharide containing thousands of D-glucose residues per individual cellulose unit (Russell *et al.* 2008). Cellulose is a stable macromolecule and is more resistant to hydrolysis. Molecular weight of cellulose ranges from 200,000 to 2,000,000, corresponding to 1250-12,500 glucose molecules per residues (Bashir *et al.* 2013). Cellulose requires temperature beyond 32°C to attain the amorphous state which can be digested by the application of strong acid and produces nano-crystalline cellulose after the breakdown (Zhang and Zhang 2013).

The biological lysis of cellulose is an enzymatically controlled synergistic process. Three types of glycoside hydrolases: endo -β-1,4-glucanases, exo-β 1,4 cellobiohydrolases and β- glucosidases are involved in cellulose 1,4- hydrolysis (Willis *et al*. 2010). To decrease the level of polymerization of the cellulose chain, β-1,4 glycosidic bonds of the cellulose strands are randomly broken down by endo-β-1,4- glucanases. Further, exo-β-1,4-cellobiohydrolases releases cellobiose or glucose by removing subunits at both reducing and non-reducing ends of the cellulose chain. To complete the biodegradation of cellulose, glucose is produced through hydrolysis β-glucosidases from cellobiose or water soluble cellodextrin. Several factors play important roles in the successful bioconversion of cellulose that include, nature of cellulose, source of cellulolytic enzymes, optimal condition for catalytic activity (like temperature, presence or absence of oxygen) and production of enzymes (Chandra et al. 2007). Large diversity of microorganisms including bacteria can synthesize cellulases during their development on cellulosic materials (Kuhad *et al*. 2011).

When cellulose, xylan or pectin was added as carbon source to the cultures, a high-level expression of various cellulase, hemicellulase, and pectin lyase genes was induced. This suggests a coordinated expression in transcriptional units of cellulose and hemicellulase genes and an influence of hemicellulose on cellulose degradation (Demain *et al*. 2005).

* **Corresponding author.**
E-mail address: mervat_y2007@yahoo.com - Tel : 002 – 01008665560
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Moreover, the effective degradation of cellulose has been considered as one of the most critical processes in degrading corn stover (Liang *et al.* 2010). Adding cellulose-degrading microorganisms is a valid way to boost cellulose degradation (Kato and Miura 2008). In consideration of the rapid growth, research has focused on a variety of cellulose-degrading bacteria (Jeong *et al.* 2017). Knockout mutants lacking the *CelC2*-encoding gene (CelC), are unable to penetrate the cell wall (Robledo *et al.* 2008). Furthermore, inoculation with a $CelC_2$ overexpressing ($CelC_2$+) derivative leads to aberrant symbiotic phenotypes in *Trifolium. repens* (Robledo *et al.* 2011). Inoculation of *Medicago truncatula* with *Sinorhizobium meliloti* heterologously expressing CelC2 also altered signalling and nodulation (Robledo *et al.* 2018). *Bacillus subtilis* and *Bacillus safensis* showed significant cellulolytic activity on carboxymethyl cellulose agar plates (Melo *et al.* 2014).

Several studies appeared that rhizobia produce enzymes capable of degrading plant cell-wall polymers which constitute the primary barrier for host infection (Oldroyd *et al.* 2013) . In particular, *Rhizobium leguminosarum bv trifolii* synthesize a ß-(1–4)-endoglucanase, called cellulase $CelC_2$, that has been biochemically and functionally characterized. $CelC_2$ is involved in the establishment of the symbiosis between this bacterium and, white clover.

This study will provide valuable information on the process of cellulose biodegradation by *Rhizobium* strains and their transconjugants to improve the biotechnological applications of cellulose- degrading strains.

MATERIALS AND METHODS

Bacterial strains

Wild type isolates of *Rhizobium meliloti* were isolated as previously described on yeast extract mannitol medium at 28˚C (Karanja and Wood 1988). These isolates were isolated from root nodules of clover plants grown in the farm of Genetic Department , Faculty of Agriculture , Mansoura University through the winter season of academic year 2015/2016. All bacterial strains used in this study, as well as, their references, are listed in Table 1.

Growth conditions

Yeast agar medium (YEMAM) was used as a full synthetic medium according to Allen (1959).However, YEMAM with Cango –red was used according to Vincet (1970).While Peptone glycerol medium PG It was used for *Serratia* strains according to Palleroni (1984) . In addition, TGY medium was used for *Bt* according to Mazza *et al.* (1992). Furthermore, Yeast extract cellulose agar medium (YECAM) was used for screening cellulase producing bacteria which was performed on cellulose agar medium and incubated at 28°C. This was done according to Somasegaran and Hoben (1985).

Table 1. The bacterial strains were used in this study and their sources.

Strains	Source or reference	Designation
Rhizobium meliloti	Root nodules of clover plants	Rm_6, Rm_{11} and Rm_{15}
Serratia marcescens	Microbiology Dept., Soil, Water and Environmental Research Institute, Agricultural Research Center (ARC).	*Sm*
Bacillus thuringiensis	Microbiology Dept., Soil, Water and Environmental Research Institute, Agricultural Research Center (ARC).	Bt_1
Bacillus thuringiensis	*Bacillus* Genetics Stock Center, Biochemistry Dept., Ohio University, Columbus, USA.	Bt_2

Genetic marking based on antibiotic susceptibility assays

Antibiotic susceptibility was measured by plate diffusion method according to Collins and Lyne (1985) using different antibiotics listed in Table 2.

Table 2. Different antibiotics used for genetic marking *Rhizobial* strains.

Antibiotics	Symbol	Concentration (µg/ml)
Xithrone	Xt	0.01
Tobrin	Tb	0.03
Amikacin	Am	0.05
Neomycin	Nm	0.03
Uvamine	Um	0.01
Ciprofloxacin	Cp	0.05
Ospen	Op	0.05

The conjugation between different bacterial strains harboring the oppsite genetic markers was done according to Grinsted and Bennet (1990) between Bacillus and Serratia strains as a donors against *Rhizobium* strains as a recipients as shown in Table 3.

Table 3. Conjugation between *Serratia* and *B. thurinogiensis* as a donor strains against *Rhizobium* strains *as a recipient strains*.

Mating	Revelant geneotype	Selective markers	Number of transconjugant (Tr) colonies isolated
Bacillus thuringiensis (Bt_1)	XT^-,AM^+,CP^+		Tr_1
X	*X*	XT^+,AM^+,CP^+	Tr_2
Rhizobium meliloti (6)	XT^+AM^-,CP^-		Tr_3
Bacillus thuringiensis (Bt_1)	UM^+,OP^-	UM^+,OP^+	Tr_4
X	X	-	Tr_5
Rhizobium meliloti (11)	UM^-, OP^+		Tr_6
Bacillus thuringiensis (Bt_2)	XT^-, AM^+,UM^-,CP^+		Tr_7
X	*X*	XT^+, AM^+,UM^+,CP^+	Tr_8
Rhizobium meliloti (6)	XT^+ AM^-,UM^+,CP^-		Tr_9
Bacillus thuringiensis (Bt_2)	XT^- ,AM^+, CP^+		Tr_{10}
X	*X*	XT^+ ,AM^+, CP^+	Tr_{11}
Rhizobium meliloti (11)	XT^+ ,AM^-, CP^-		Tr_{12}
Serratia marcescens (Sm)	TB^- , NM^+ , UM^+ , CP^-		Tr_{13}
X	X	TB^+ , NM^+ , UM^+ , CP+	Tr_{14}
Rhizobium meliloti (6)	TB^+ , NM^- , UM^- , CP^+		Tr_{15}
Serratia marcescens (Sm)	UM^- , CP^-, OP^-		Tr_{16}
X	X	UM^+ , CP^{+-}, OP^+	Tr_{17}
Rhizobium meliloti (15)	UM^+ , CP^{+-}, OP^+		Tr_{18}

Screening cellulase activity

For enrichment cellulose - producing bacteria, a cellulose agar medium (YMCAM) containing cellulose as a sole carbon and energy source was used. Cellulase activity was assayed qualitatively on agar plates by observing the size of the halo zones formed around the colonies after seven days of incubation at 28°C according to Morales *et al*. (1984).

Genome shortening

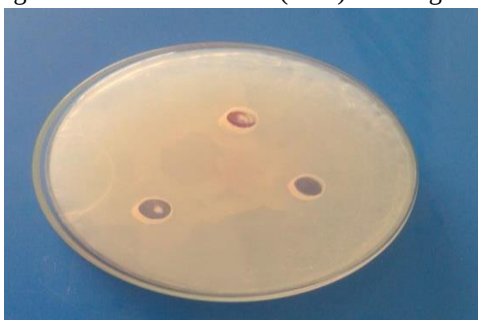
Approximately 10^9 cells from a log phase culture of *Rhizobium* isolates were starved by growing in a medium free of carbon as mentioned by Thorne and Williams (1997). Starvated cultures are shifted to incubated at 40°C for one day and then maintained at 28°C for two days. Single colonies from the cultures heat treated were selected and retested for antibiotic resistance pattern. The inabitily of *Rhizobium* to grow on the antibiotic supplemented medium indicating that plasmid was cured (Baldani *et al*. 1992).

Statistical analysis

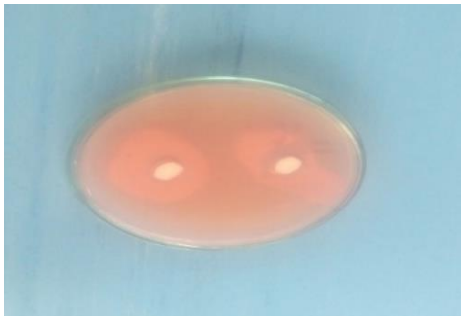
The data were subjected to the analysis of variance according to Snedecor and Cochran (1955). Least signification difference (L.S.D) was used to compare between means if the F-test was significant.

RESULTS AND DISCUSSION

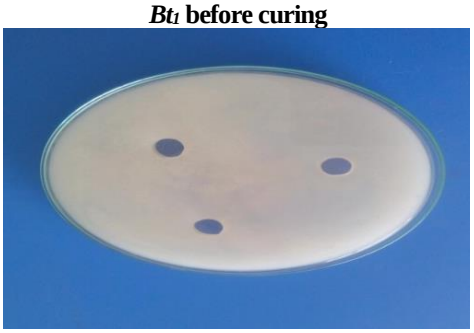
The data summarized in Table 4 and Figure 1 illustrated that all transconjucants resulted from the mating between *Bacillus* (Bt_1) and *Rhizobium* (Rh_6 , Rh_{11}) appeared insignificant differences between different genotypes in cellulose biodegradation after curing, while they were appeared significant differences before curing. On the other hand, the wild type strains Bt_1 and Rh_6 expressed significant cellulose biodegradation after curing. While, all transconjucants Tr_4, Tr_5 and Tr_6 resulted from the mating between Bt_1 *and* Rh_{11} produced significant amounts of cellulase after curing. This result agreed with Mateos *et al*. (2001), who suggested a complementary function of rhizobial cell- bound hydrolytic enzymes exists in the initial host infection. This indicated that genome shortening may delete some of suppressor genes which extrachromosomal located that may decrease the level of cellulase production.



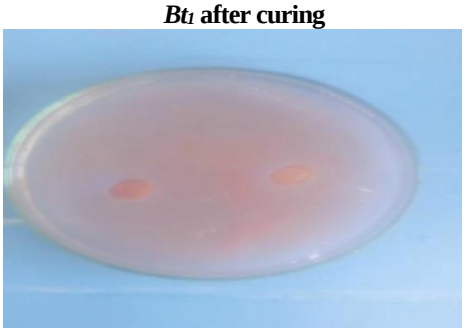
Bt_1 before curing



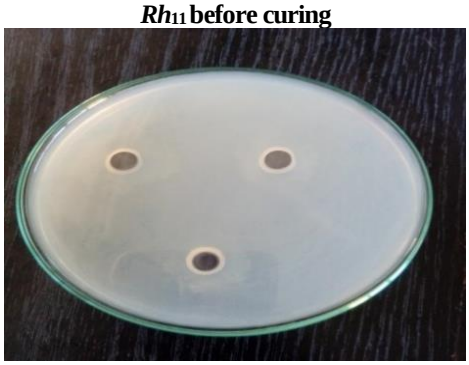
Bt_1 after curing



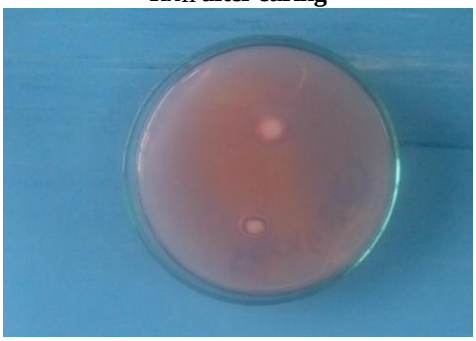
Rh_{11} before curing



Rh_{11} after curing



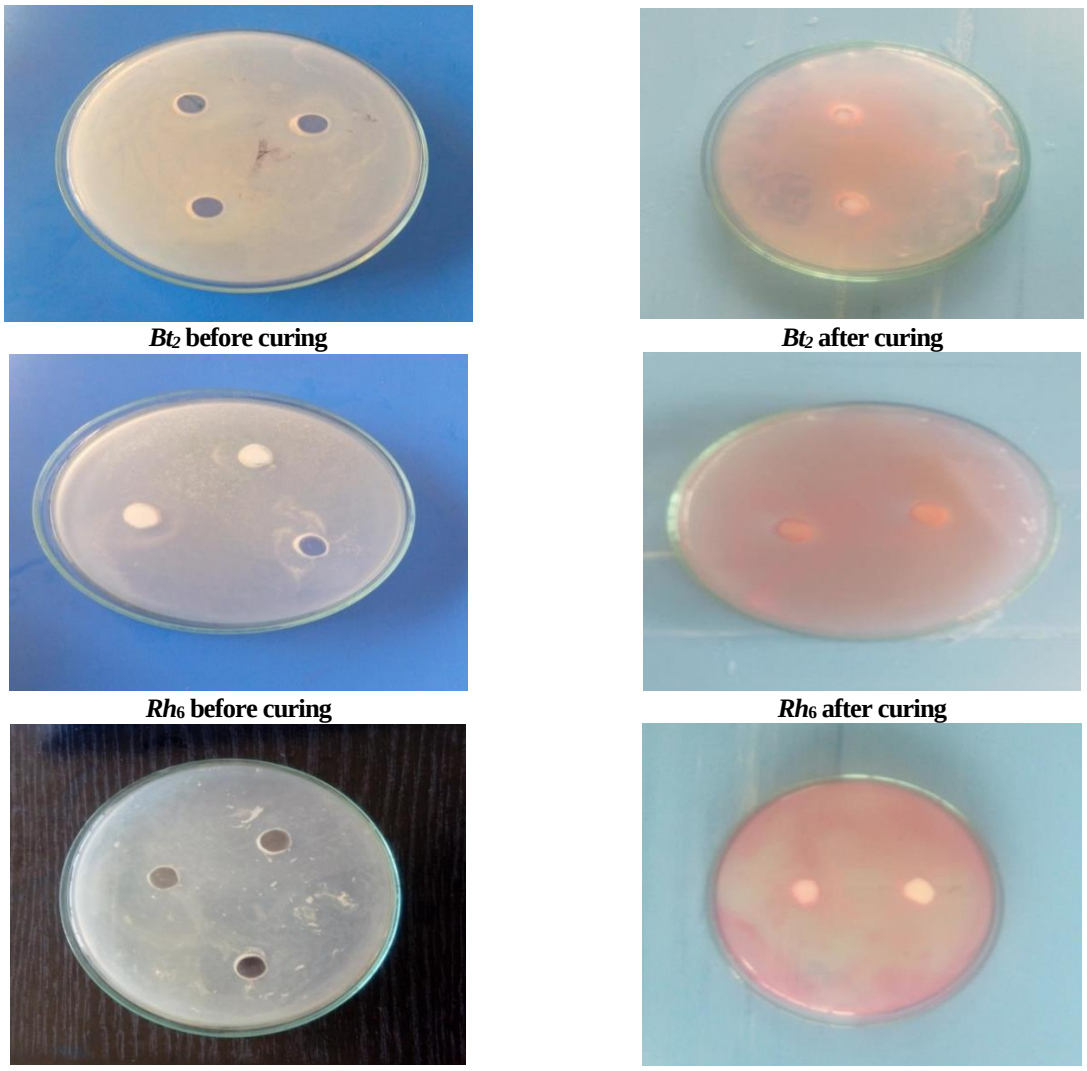
Tr_4 before curing



Tr_4 after curing

Figure 1. Cellulose biodegradation by cured mutants of *Rhizobium* recombinants resulted from the mating between Bt_1 x Rh_{11}.

The data summarized in Table 5 and Figure 2 illustrated that all transconjucants resulted from the mating between *Bt2* x *Rh6* and *Bt2* x *Rh11* appeared insignificant differences between different genotypes in cellulose biodegradation before curing and after curing. This indicated that genome shortening do not affect on the activity of cellulase genes. However, the parental strains (*Rh6* and *Rh11*) expressed significant cellulose biodegradation after curing. Furthermore, all transconjugants resulted from both matings appeared insignificant differences in cellulose biodegradation between wild type strains and cured mutants. This result was agreed with Morales *et al*. (1984), who showed that curing of pSym did not affect on cellulase production from *R. leguminosarum bv. trifolii* .



***Bt*2 before curing** ***Bt*2 after curing**

***Rh*6 before curing** ***Rh*6 after curing**

Tr8 **Tr8**

Figure 2. Cellulose biodegradation by cured mutants of *Rhizobium* recombinants resulted from the mating between *Bt*2 x *Rh*6.

Table 4. Effect of plasmid curing in *Rhizobium* recombinants resulted from the mating between Bt1 x Rh6 and Bt1 x Rh11 on cellulose biodegradation.

Strains	Cellulose biodegradation				L.S.D	
	Diameter of clear zones before curing(cm)	Diameter of clear zones after curing (A)	Diameter of clear zones after curing (B)	F-test	0.05	0.01
*Bt*1	1.13	1.35	1.99	**	0.262	0.396
Rh 6	1.25	1.63	1.71	**	0.234	0.354
Mid-parent	1.19	1.49	1.85			
Tr1	1.25	1.63	1.58	IS		
Tr2	1.15	1.73	1.42	IS		
Tr3	1.35	1.63	1.42	IS		
*Bt*1	1.13	1.35	1.99	**	0.262	0.396
Rh 11	1.10	1.74	1.68	*	0.410	0.622
Mid-parent	1.12	1.53	1.83			
Tr4	1.05	1.35	1.57	*	0.325	0.492
Tr5	1.10	1.55	1.55	*	0.383	0.579
Tr6	1.00	1.38	1.43	*	0.331	0.501
F-test	**	IS	IS			
LSD 0.05	3.64					
LSD 0.01	4.97					

***,**: Significant differences at 0.05 and 0.01 levels of probability.**
IS: Insignificant differences.
A: Cellulose added to agar spot.
B: Cellulose added and disrubted in the medium.

Table 5. Effect of plasmid curing in *Rhizobium* recombinants resulted from the mating between Bt2 X Rh6 and Bt2 x Rh11 on cellulose biodegradation.

Strain	Cellulose biodegradation				L.S.D	
	Diameter of clear zones before curing (cm)	Diameter of clear zones after curing (A)	Diameter of clear zones after curing (B)	F-test	0.05	0.01
*Bt*2	1.20	1.48	1.83	*	0.422	0.640
Rh 6	1.25	1.63	1.71	**	0.234	0.354
Mid-parent	1.22	1.55	1.77			
Tr7	1.25	1.38	1.83	IS		
Tr8	1.20	2.10	1.58	IS		
Tr9	1.20	2.00	1.74	IS		
*Bt*2	1.20	1.48	1.83	*	0.422	0.640
Rh 11	1.10	1.74	1.68	*	0.410	0.622
Mid-parent	1.15	1.61	1.75			
Tr10	1.25	1.35	1.30	IS		
Tr11	1.25	1.47	1.40	IS		
Tr12	1.30	1.38	1.30	IS		
F-test	**	**	IS			
LSD 0.05	6.81	0.36				
LSD 0.01	9.35	0.49				

***,**: Significant differences at 0.05 and 0.01 levels of probability.**
IS: Insignificant differences. A: Cellulose added to agar spot.
B: Cellulose added and disrubted in the medium.

The data summarized in Table 6 and Figure 3 illustrated that all transconjucants resulted from the mating

between Sm x Rh_6 and Sm x Rh_{15} appeared significant differences in cellulose biodegradation between wild type strains and their cured mutants. On the other hand, all parental strains (*Sm* , *Rh_6* and *Rh_{15}*) expressed significant cellulose biodegradation after curing. However all transconjugants Tr_{13}, Tr_{14} and Tr_{15} resulted from the mating between Sm and Rh_6 appeared significant degradation of cellulose after curing. Furthermore, all transconjugants Tr_{16}, Tr_{17} and Tr_{18} resulted from the mating between *Sm and Rh_{15}* appeared the same trend of cellulose biodegradation after curing. This agreed with Mateos *et al*. (1992), who reported that cellulolytic enzymes are produced commonly by different rhizobial species.

The results obtained herein agreed with Medie *et al*. (2012), who reported that most bacteria that encode a cellulase harboring only one cellulase gene in their genotypes which were located in all phyla analysed. However, bacteria harboring two or three cellulases genes were present in all phyla except for synergistetes. Therefore, some of the bacteria harboring more than three cellulases which are found in the phyla actinobacteria, bacteroidetes, thermotogae and in the class clostridia. In addition, bacteria degraded plant cell walls often encode various cellulases together with hemicellulolytic and pectiolytic enzymes. Thus, the cooccurrence of genes encoding cellulases and those encoding hemicellulolytic and/ or pectinolytic enzymes accompanies a true saprophytic lifestyle. Various bacteria produced cellulase using the enzymes encoded by the bacterial cellulose synthesis (*bcs*) operon. The *bcs* operon also encoded a cellulose of the GH8 family. Thus, the bacteria encoding cellulases are likely to be involved in cellulose synthesis.

Table 6. Effect of plasmid curing in *Rhizobium* recombinants resulted from Sm x Rh_6 and Sm x Rh_{15} on cellulose biodegradation.

Strain	Cellulose biodegradation				L.S.D	
	Diameter of clear zones before curing (cm)	Diameter of clear zones after curing(A)	Diameter of clear zones after curing (B)	F-test	0.05	0.01
Sm	1.38	1.39	2.19	*	0.521	0.788
Rh_6	1.25	1.63	1.71	**	0.234	0.354
Mid-parent	1.31	1.51	1.95			
Tr_{13}	1.20	1.83	1.97	**	0.205	0.310
Tr_{14}	1.50	1.80	2.08	*	0.357	0.541
Tr_{15}	1.35	1.55	2.03		0.186	0.282
Sm	1.38	1.39	2.19	*	0.521	0.788
Rh_{15}	1.05	1.50	1.84	**	0.231	0.351
Mid-parent	1.21	1.44	2.01			
Tr_{16}	1.05	1.50	1.52	**	0.328	0.497
Tr_{17}	1.00	1.50	1.52	**	0.260	0.394
Tr_{18}	1.15	1.20	1.53	**	0.201	0.484
F-test	**	**	*			
LSD 0.05	5.73	0.201	0.452			
LSD 0.01	7.82	0.275	0.619			

***,**: Significant differences at 0.05 and 0.01 levels of probability.**
A: Cellulose added to agar spot.
B: Cellulose added and distributed in the medium.

On the other hand, Menendez *et al*. (2019) decided that cellulase is a symbiotic endoglucanase originally described in *Rhizobium leguminosarum biovar trifolii* that specially nodulates white clover. Furthermore, rhizobial cellulases plays a significant role for the localized penetration of compatible rhizobia into root hairs of specific legume hosts (Menedez *et al*.2019). When the bacteria were bound to the root hair tip, cellulase effects seem to be more localized than the addition of purified enzyme, indicating that it is cell – boun (Jimenez- Zurdo *et al*. 1996). Changes in the production of these signals can hamper early steps of symbiosis development. *Rhizobium leguminosarum bv. trifolii* cellulase has a strong substrate specificity for noncrystalline cellulose located in the root hair tips of its specific host. The substrate specificity is the key to the success of the canonical primary infection in *Rhizobium trifolium* symbiosis. Moreover, cellulase led to alterations in the primary infection points confirming the importance of cellulase in the symbiotic process. Furthermore, Doi and Kosugi (2004) decided that the complete digestion of cellulose is largely restricted to a specific group of cellulolytic microorganisms that produce combinations of cellulases, hemicellulases and pectinases which act synergistically to break down cellulose and associated cell wall components. In addition, Buttner and Bonas (2010) reported that plant pathogens produce several types of cell wall – degrading enzymes as cellulases, pectinases, xylanases and proteases. These enzymes play a crucial roles in pathogenicity because they break down the components of the host plant cell walls.

The importance of cellulases in pathogenicity and symbiotically performance has studied through mutational analyses of cellulase- encoding genes in both bacterial and fungal pathogens (Van Vu *et al*.2012). Morever, Medie *et al*. (2012) reported that 40% of bacterial plant pathogens genome encode at least one cellulase gene. Cellulase- lacking mutants of *Xanthomonas oryzae* causing bacterial leaf blight in rice showed attenuated virulence in the host plants (Xia *et al*. 2016).

The fungal rice blast pathogen *Magnaporthe oryzae* has two cellulase genes that play significant roles in its virulence (Van Vu *et al.* 2012). The genetic analysis of *Clavibacter michiganensis* reveled 130- kb chromosomal pathogenicity island that carries various virulence genes (Gartemann *et al*. 2003). Besides the pathogenicity, two extrachromosomal plasmids are known to be involved in the pathogenicity (Gartemann *et al*. 2008). These plasmids were important in the pathogenicity as confirmed by virulence assayes using strains naturally lacked one or more plasmids or mutants that lost one or both plasmids (Thapa *et al*. 2017). The *cel*A gene plasmids located encode a cellulase and this gene was critical for causing disease in tomato (Eichenlaub and Gartemann 2011). The *cel* A gene was importance in pathogenicity through infection (Thapa *et al.* 2017).The genome analysis revealed another cellulase gene, *cel*B on its chromosome (Cartemann *et al*. 2008). The expression of *cel*B was up- regulated through infection of tomato plants (Chalupowicz *et al*. 2010)

Cellulases are enzymes that degrade cellulose fibers a major components of plant cell walls. Cellulases are major virulence factors that play important roles in pathogenicity during infection of *Rhizobium*. Thus, the transposon- inserted cellulase mutant lost most of the ability of microbe to cause disease in the host plant indicating that cellulase genes are required for pathogenicity. In addition, mutants carrying a partial deletion in cellulose genes fail to recover its pathogenesis. The biodegradation of cellulose fibers needing

a better contact with cellulase enzymes. The results obtained in this study indicated that cellulose genes are chromosomal and plasmids located which led to focus on the efficiency of cellulases in plasmid cured mutants. The secreted cellulases from plasmid cured mutants may increase than in the wild type strains to overcome the deletion of cellulose genes which extrachromosomal located, because it is very likely a functional cellulase genes that can be more secreted cellulase enzymes in plasmid cured mutants.

In conclusion, this study focus about the effect of plasmid curing on the effective of cellulase genes from *Rhizobium* and *Bt* to be understand the potential biotechnological applications of cellulases to overcome agricultural wastes. Genes encoding cellulase provides more effective in plasmid curing strains than the wild type strains which indicated that the genome shortening strains were more effective in cellulases production than that carried the whole genome. Thus, the cellulase genes might expressed as active enzymes in genome shortening strains because of the problems related to heterologous gene expression between chromosomal and extrachromosomal genes. Therefore, cellulases genes represented at least some of the cellulases operating efficiency in genome shortening strains.

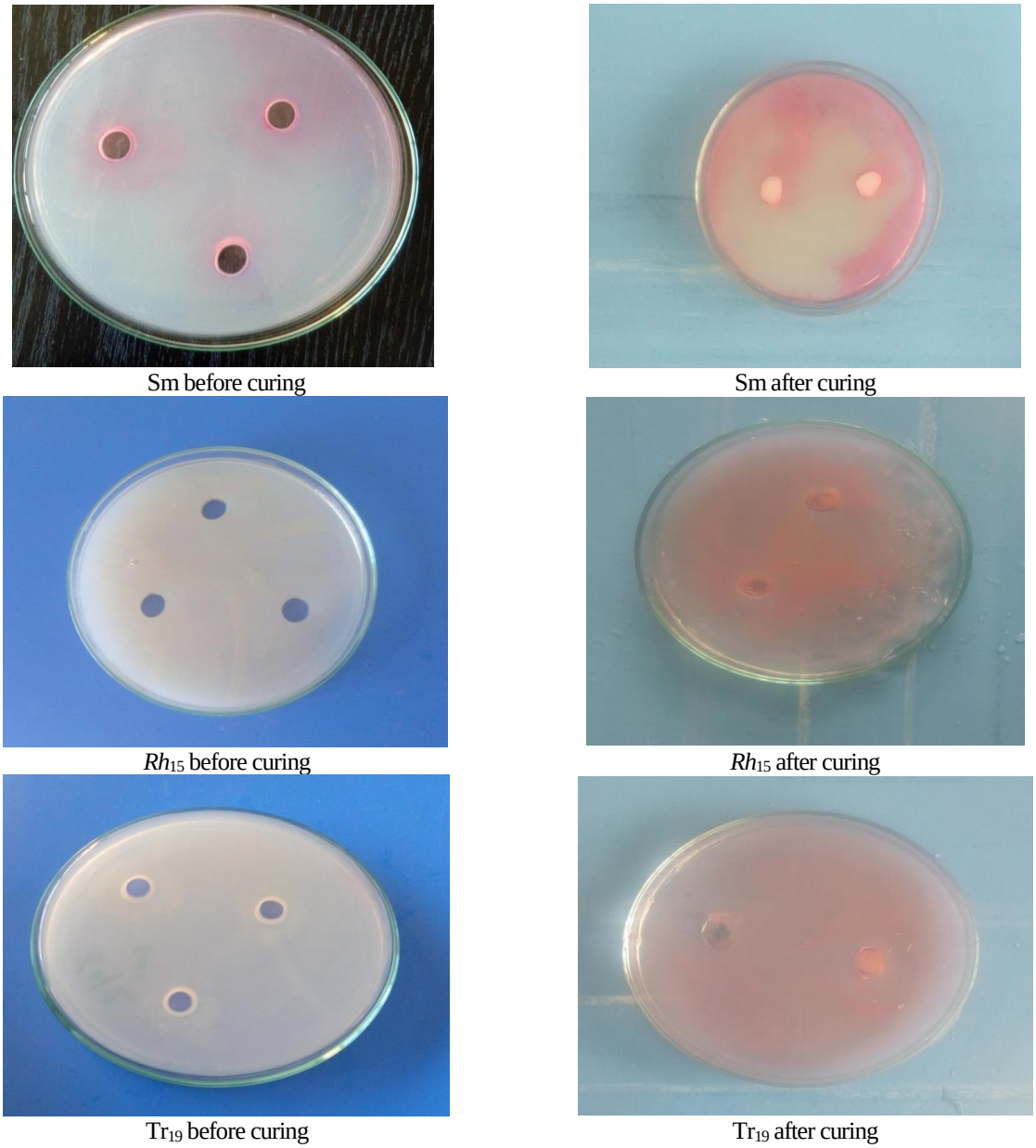


Figure 3. Cellulose biodegradation by *Rhizobium* recombinants resulted from the mating between *Sm* x *Rh*15.

Furthermore, cellulase activity and secretion efficiency are critical in plasmid curing mutants. It will be important to conducted further studies on cellulase genes that are plasmids and chromosomal located to explore their genetic locations in the bacterial genome and their roles in the biodegradation of cellulose fibers.

The outhors declare that they have no conflicts of interest.

REFERENCES

Allen, O. N. 1959. Experimental in soil Bacteriology. Burgess pub. Co. Minn, Minnesota, USA.

Baldani, J. I.; R. W. Weaver; M. F. Hynes and B.O. Eardly .1992. Utilization of carbon substrate, electropherotic enzyme patterns, and symbiotic performance of plasmid- cured clover *Rhizobia*. Appl. Environ. Microbiol; 58:2308-2314.

Bashir, Z.; V.K. Kondapalli; N. Adlakha; A. Sharma; R.K. Bhatnagar; G. Chan and S.S. Yazdani. 2013. Diversity and functional significance of cellulolytic microbes living in termite, pill-bug and stem-borer guts. Scientific Reports, 3:2553-2558.

Buttner, D. and U. Bonas. 2010. Regulation and secretion of Xanthomonas virulence factors. FEMS Microbiol. Rev,; 34:107-133.

Chandra, R.P.; R. Bura; W.E Mabee; A. Berlin; X. Pan and J.N Saddler. 2007. Substrate pretreatment: the key to effective enzymatic hydrolysis of lignocellulosics? Advances in Biochemical Engineering Biotechnology, 108: 67–93.

Chalupowicz, L.; M. Cohen-Kandli; O. Dror; R.Eichenlaub; K. H. Gartemann; G. Sessa; I. Barash and Manulis-Sasson, S. 2010. Sequential expression of bacterial virulence and plant defense genes during infection of tomato with Clavibacter michiganensis subsp. Michiganensis. Phytopathology; 100:252-261.

Collins, C. H. and P.M. Lyne. 1985. Microbiological Methods . 5th Ed. Butterworths, London, Toronto, 167-181.

Demain, A. L.; M. Newcomb and J.H. Wu. 2005. Cellulase, clostridia, and ethanol. Microbiol Mol. Biol. Rev; 69(1):124–154.

Doi, R. H. and K. A. Cellulosomes. 2004. Plant-cell-wall-degrading enzyme complexes. Nature Rev. Microbiol; 2: 541–551 .

Dror, T.W.; E. Morag; A. Rolider; E.A. Bayer; R. Lamed and Y. Shoham. 2003. Regulation of the cellulosomal *cel*S (*cel*48A) gene of *Clostridium thermocellum* is growth rate dependent. J .Bacteriol; 185:3042-3048.

Eichenlaub, R. and K. H. Gartemann. 2011. The Clavibacter michiganensis subspecies: Molecular investigation of gram-positive bacterial plant pathogens. Annu. Rev. Phytopathol;49:445-464.

Gartemann, K. H.; O. Kirchner; J. Engemann; I. Grafen; R. Eichenlaub and A. Burger. 2003. Clavibacter michiganensis subsp. michiganensis: First steps in the understanding of virulence of a gram-positive phytopathogenic bacterium. J. Biotechnol, 106:179-191.

Gartemann, K. H.; B. Abt; T. Bekel; A. Burger; J. Engemann; M. Flugel; L.Gaigalat; A. Goesmann; I. Grafen; J. Kalinowski; O. Kaup; O. Kirchner; L. Krause; B. Linke; A. McHardy; F. Meyer; S. Pohle; C. Ruckert; S. Schneiker; E. M. Zellermann; A. Puhler; R. Eichenlaub; O. Kaiser and D. Bartels. 2008. The genome sequence of the tomato-pathogenic actinomycete *Clavibacter michiganensis subsp. michiganensis* NCPPB382 reveals a large island involved in pathogenicity. J. Bacteriol, 190:2138-2149.

Grinsted, J. and P. M. Bennett. 1990. Methods in Microbiology : Plasmid Technology (2nd edition) Published by Academic Press.

Han, J.S. and J. S. Rowell .1996. Chemical composition of fibers; In Eds. Rowell RM, RowellJ (Paper and composites from agro-based resources).

Jeong, W. S.; D. H. Seo; J. H. Jung; D. H. Jung; D. W. Lee; Y. S. Park and C. Park. 2017. Enzymatic characteristics of a highly ther mostable beta-(1-4)-glucanase from Fervidobacterium islandicum AW-1 (KCTC 4680). J. Microbiol Biotechnol; 27(2):271–276.

Jimenéz-Zurdo, J. I.; P. F. Mateos; F. B. Dazzo, and E. Martinez-Molina, 1996. Cell-bound cellulase and polygalacturonase production by *Rhizobium* and *Bradyrhizobium* species. Soil Biol Biochem 28(7), 917–921 .

Karanja, N . K. and M. Wood . 1988. Selection phaseoli strain for use with beans (*Phaseolus vulgaris* L.) in Kenya :Tolerance of high temperature and antibiotic resistance . plant and soil ;112: 15-22.

Kato, K. and N. Miura. 2008. Effect of matured compost as a bulking and inoculating agent on the microbial community and maturity of cattle manure compost. Bioresour Technol; 99(9):3372–3380.

Kuhad RC.; R. Gupta and .A. Singh. 2011. Microbial Cellulases and their industrial applications. Enzyme Research, Article ID 280696, (doi:10.4061/2011/280696).

Liang ,Y.S.; X.Z Yuan; G.M Zeng; C.L Hu, H. Zhong; D.L Huang; L. Tang and J.J Zhao .2010. Biodelignification of rice straw by Phanerochaete chrysosporium in the presence of dirham nolipid. Biodegradation 21(4):615–624.

Mazza, P. F. Z. and P. Martelli.1992. Studies on the antibiotic resistance of *Bacillus subtilis* strains used in oral bacteriotherapy. Boll Chim Fam, 131(11): 401-408.

Mateos, P.F.; J. I. zurdo; J. C. H. Chen; A. S. Souratini; S. K. Haack; E. M. Molina; D. H. Hubbell. and F.B. Dazzo.1992. Cell-Associated Pectinolytic and Cellulolytic Enzymes in *Rhizobium leguminosarum Biovar trifolii*. Applied and Environmental Microbiology, 58 : 1816-1822.

Mateos, P. F.; D. L. Baker; M. Petersen and E. Velazquez. 2001. Erosion of root epidermal cell walls by *Rhizobium* polysaccharide-degrading enzymes as related to primary host infection in the *Rhizobium–legume* symbiosis. Can. J. Microbiol; 47:475–487.

Melo, I. S.; T. D. Zucchi; R. E. Silva; E. S. Vilela; M.L. Saber; L. H. Rosa and V.H. Pellizari. 2014. Isolation and characterization of cellulolytic bacteria from the Stain house Lake, Antarctica. Folia Microbiol; 59(4):303–306.

Medie, F. M.; G. J. Davies; M. Drancourt and B. Henrissat, 2012. Genome analyses highlight the different biological roles of cellulases. Nat. Rev. Microbiol; 10:227-234.

Menéndez, E.; P. M. Hidalgo; L. R. Silva and E. Velazquez. 2019. Recent advances in the active biomolecules involved in *Rhizobia-Legume* symbiosis. In: Microbes for Legume Improvement; 9: 45–74 .

Morales, V.M.; E. M. Molina and D. H. Hubbell.1984.Cellulase production by *Rhiobium*. Plant and soil, 80: 407-415.

Nataf, Y.; L. Bahari; H. Kahel-Raifer; I. Borovok; R. Lamed; E. A. Bayer; A. L Sonenshein and Y. Shoham. 2010. *Clostridium thermocellum* cellulosomal genes are regulated by extracytoplasmic polysaccharides via alternative sigma factors. Proc. Natl. Acad. Sci. USA , 107:18646-18651.

Oldroyd, G. E. D.; Speak; friend, and enter.2013. Signalling systems that promote beneficial symbiotic associations in plants. Nature Publishing Group, 11: 252–263.

Palleroni, N. J. 1984. Genus *Pseudomonas*. In Krieg and Holt (Editors), Bergey's Manual of Systematic Bacteriology, 1st Ed., 1, The Williams & Wilkins Co., Baltimore. pp. 141–199.

Robledo, M.; J. I. Jiménez-Zurdo; E. Velázquez; M. E. Trujillo; J. L. Zurdo-Piñeiro; M. H. Ramírez-Bahena; B. Ramos; J. M. Díaz-Mínguez; F. Dazzo; E. Martínez-Molina, and P. F. Mateos. 2008. *Rhizobium* cellulase CelC2 is essential for primary symbiotic infection of legume host roots. Proc. Natl. Acad. Sci. USA , 105: 7064–7069.

Robledo, M. R. ; J. I. J. Zurdo ; M. J. Soto ; E. V. Velázquez ; F. D. Dazzo ; E. M. Molina. and P. F. Mateos . 2011. Development of Functional Symbiotic White Clover Root Hairs and Nodules Requires Tightly Regulated Production Rhizobial Cellulase CelC2. The American Phytopathological Society, 24 : 799-907.

Robledo, M.; E. Menendez; J. I. Jiménez-Zurdo; R. Rivas; E. Velázquez; E. Martínez-Molina, and P. F. Mateos. 2018. Heterologous Expression of Rhizobial CelC2 Cellulase Impairs Symbiotic Signaling and Nodulation in *Medicago truncatula*. MPMI, 31: 568–575.

Russell, J. B.; R. E. Muck and P. J. Weimer. 2008. Quantitative analysis of cellulose degradationand growth of cellulolytic bacteria in therumen. FEMS Microbiology Ecology, 67: 183–197.

Schwarz, W. H.; V. V. Zverlov and H. Bahl. 2004. Extracellular glycosyl hydrolases from *clostridia*. Adv Appl Microbiol;56:215-261.

Schwarz, W. H.; O. J. G. Bello; W. De Jong; J. J. Leahy; K. Oyaas; L. Sorum and H. Steinmuller.2011. Strategic energy technology plan. In Scientific Assessment in support of the Materials Roadmap enabling Low Carbon Energy Technologies. Edited by Baxter D, Tzimas E. Joint Research Centre–Institute for Energy and Transport.

Somasegaran, P. and H. J. Hoben. 1985. To collect nodules and isolate *Rhizobium* . In: Methods in legume– Rhizobium Technology . United States Agency for International Development. pp.4-21.

Snedecor , G. W. and W. G. Cochran. 1955. Statistical methods applied to experiments in agriculture and biology. 5th ed. Ames, Iowa: Iowa State University.

Thorne, S.H. and H. D. Williams . 1997. Adaptation to nutrient starvation in *Rhizobium leguminosarum bv. Phaseoli*: analysis of survival, stress resistance, and change in macro- molecular synthesis during entery to and exit from stationary phase . J. Bacteriol, 179:6894-6901.

Thapa, S. P., S. Pattathil; M. G. Hahn; M. A. Jacques; R. L. Gilbertson and G. Coaker. 2017. Genomic analysis of Clavibacter michiganensis reveals insight into virulence strategies and genetic diversity of a grampositive bacterial pathogen. Mol. Plant-Microbe Interact; 30:786-802.

Van Vu, B.; K. Itoh; Q. B. Nguyen; Y. Tosa and H. Nakayashiki, 2012. Cellulases belonging to glycoside hydrolase families 6 and 7 contribute to the virulence of *Magnaporthe oryzae*. Mol. Plant-Microbe Interact, 25:1135-1141.

Vincent, J. M. 1970. A Manual for the practical Study of the Root Nodule Bacteria. IBP15. Blackwell Scientific Publications. Oxford.

Willis, J. D.; C. Oppert and J. L Jurat-Fuentes .2010. Methods for discovery and characterization of cellulolytic enzymes from insects. Insect Science, 17: 184-198.

Xia, T.; Y. Li; D. Sun; T. Zhuo; X. Fan and H. Zou. 2016. Identification of an extracellular endoglucanase that is required for full virulence in *Xanthomonas citri subsp. citri*. PLoS One 11:e0151017.

Zhang, X. Z. and Y. H. P. Zhang .2013. Cellulases characteristics, sources, production, and applications. Bioprocessing Technologies in Biore finery for Sustainable Production of Fuels, Chemicals, and Poly mers. John Wiley & Sons, Inc

التخلص الآمن من السليلوز من خلال تحليله حيوياً بواسطة إتحادات وراثية جديدة من الرايزوبيوم تحمل جينات السليوليز

ميرفت إبراهيم كمال ، خليفه عبد المقصود زايد ، ميرفت جلال شرابيه و أشرف حسين عبد الهادي

قسم الوراثة – كلية الزراعة – جامعة المنصورة – مصر

عادة تحلل المادة النباتية بكفاءة بواسطة العديد من الإنزيمات الميكروبية المختلفة. تم في هذه الدراسة تطوير البكتيريا المحللة للسليلوز وراثياً من خلال النقل البلازميدى وإختزال حجم الجينوم. لتحقيق هذا الهدف تم إجراء ست تزاوجات بين ست آباء تعمل علامات وراثية متفارقة وذلك من خلال إستخدام ثلاث سلالات من الرايزوبيم كمستقبلات للمادة الوراثية بينما إستخدمت سلالات الباسيليس ثيرونجنسز والسراتيا المعبرة وظيفياً عن نشاط إنزيم السليوليز كآباء معطية للمادة الوراثية ومن خلالها تم عزل وتقييم 18 من المتحولات التزاوجية بالنسبة لكفاءتها في تحليل السليلوز. أحدثت السلالات البرية الأبوية من الباسيليس ثيرونجنسز والرايزوبيم تغيير معنوى في تحليل السليلوز بعد إختزال حجم الجينوم. هذا بالإضافة إلى أن كل المتحولات التزاوجية الناتجة عن التهجين بين Bt_1 x Rh_{11} قد عبرت عن نشاط معنوى لإنزيم السليوليز بعد إختزال حجم الجينوم. على النقيض من ذلك ، أظهرت كل المتحولات التزاوجية الناتجة عن التهجين بين Bt_2 x Rh_6 , Bt_2 x Rh_{11} إختلافات غير معنوية في نشاط إنزيم السليوليز بين السلالات المختزل والغير مختزل فيها حجم الجينوم. بينما عبرت السلالات الأبوية Rh_6, Rh_{11} عن نشاط معنوى لإنزيم السليوليز بعد إختزال حجم الجينوم. علاوة على ذلك ، عبرت كل الهجن الناتجة من التزاوج بين Sm x Rh_6 ، Sm x Rh_{15} عن نشاط معنوى لإنزيم السليوليز بعد إختزال حجم الجينوم. هذه الدراسة تعكس كفاءة إختزال حجم الجينوم على كفاءة التعبير الوظيفى في إنتاج إنزيمات السليوليز في الطفرات المختزل فيها حجم الجينوم مقارنة بالسلالات البرية. لذلك فإن عدوى النباتات البقولية يتأثر بشدة بإنزيمات السليوليز التى تنتجها الرايزوبيا والتى تعتبر عامل محدد في عملية تكوين العقد الجذرية.