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EXOPOLYSACCHARIDES AND LIPOPOLYSACCHARIDE PRODUCTION BY SINORHIZOBIOBIUM FREDII Tn*5* MUTANTS INFECTING V*igna radiata* L.

Neeraj[1], Gaurav S. S.[1], S. C.[2], Chatterjee Sachin[4] and Mahesh Chandra[3]
[1]Department of Biotechnology, C.C.S. University, Meerut, U. P., India
[2]Division of Plant Pathology, IARI, New Delhi, India
[3]Division-FLOF, Central Institute of Medicinal and Aromatic Plants, Lucknow, India
[4]Sardar Vallabh Bhai Patel University of Agriculture and Technology, Meerut, (UP), India
E-Mail: neerajtandan@gmail.com

ABSTRACT

Mutant of *S. fredii* R0132 were developed by gene mutation with the help of transposon Tn5 mediated mutagen sixteen hundred mutants were developed. Significantly, influenced production level of lipopolysaccharidesand exopolysaccharides as observed in different *S. fredii* mutants. Maximum mutants showed the increased level of lipopolysaccharides than the wild type. Rhizobial mutants also varied in lipopolysaccharides content ranging from lowest value 72.3 µg/mg (*S. fredii* R0132 (1116): *Tn5*) to highest value 183.3 µg/mg (*S. fredii* R0132 (0123): *Tn5*) of fresh weight of cell wall. About 10-15 percent reduction in production in LPS was observed in all the mutants including parent strain at 32°C and 36°C. The mutants were also differed from wild type *S. fredii* R0132 in their capability to produce exopolysaccharides. Significantly, increased production was noticed in case of mutants R0132 (0521): *Tn5*, R0132 (1106): *Tn5*, R0132 (1112): *Tn5* and R0132 (1421): *Tn5* where 49.1, 61.0, 59.2 and 44.4 percent increase was observed over the parent isolate. In presence of mannitol and sucrose higher EPS production was observed in all mutant strains as well in wild type. Mutant R0132 (1106), showed higher EPS production (156.8 µg/mg of FW of cell wall) while lower was observed in case of R0132 (0923): *Tn5* (96.5 µg/mg FW of cell wall) in the presence of mannitol. On other hand sucrose increased EPS production in R0132 (1106): *Tn5* upto 178.3 µg/mg of FW of cell wall, which was the highest in all the exogenous sugars applied.

Keywords: Rhizobium, Tn5 mutants infecting *Vigna radiata* L. Exopolysaccharides, lipopolysaccharide production.

1. INTRODUCTION

Plant roots contribute to soil organic matter, and consequently to soil aggregate stability, directly through the root material itself (Watt *et al*., 2003), and indirectly through stimulation of microbial activities in the rhizosphere. Among the microbial activities involved in plant interaction, the production of polysaccharides has been shown to be of great importance (Lynch *et al*., 1985). *Rhizobia* are known to contain lipopolysaccharide (LPS) as an abundant component of the outer membrane and produce extracellular polysaccharides on the cell surface known as exopolysaccharides (EPS). In recent years, the possible roles of surface polysaccharides in symbiosis have received some attention. Although the function of these polysaccharides is by no means completely understood, it appears that the roles of these compounds vary between different strains of *rhizobia*. When the rhizosphere is nitrogen-starved, legumes and *rhizobia* (soil bacteria) enter into a symbiosis that enables the fixation of atmospheric dinitrogen.

This implies a complex chemical dialogue between partners and drastic changes on both plant roots and bacteria. Several recent works pointed out the importance of *rhizobial* surface polysaccharides in the establishing of the highly specific symbiosis between symbionts. Exopolysaccharides appear to be essential for the early infection process. Lipopolysaccharides exhibit specific roles in the later stages of the nodulation processes such as the penetration of the infection thread into the cortical cells or the setting up of the nitrogen-fixing phenotype. More generally, even if active at different steps of the establishing of the symbiosis, all the polysaccharide classes seem to be involved in complex processes of plant defense inhibition that allow plant root invasion. Their chemistry is important for structural recognition as well as for physicochemical property (Bradley *et al*., 2005). These cell surface molecules present on the *Rhizobium* cell surface are known to participate during interaction of rhizobia-legume associations. Specificity of the association is dependent on specificity of surface molecules of Rhizobium that participate in the establishment of symbiotic relationship (Dudman, 1977). Furthermore lipopolysaccharide and exo-polysaccharide play role in determining the host specificity and nodulation establishment. In the present investigation, Transposon (Tn *5*) mediated mutagenesis was carried out *Sinorhizobiobium fredii* to develop the strain showing higher or lower level of these surface polysaccharides and their effect on the production of LPS and EPS in Tn *5* mutants of *Sinorhizobiobium fredii*.

2. MATERIALS AND METHODS

E.coli WA 803(*pGS9)* was obtained from IARI New Delhi. Antibiotic were procured form Sigma Corporation for U.S.A and other media and chemicals were procured from this media Private Limited. Therefore to standardize the mating period between WA803 (*pGS9*) and selected *rhizobial* isolate, the patch from the mixture

of their cell on TY agar (Streptomycin 200 µg/mL + Kanamycin 50 µg/mL) surface were to grow for 4, 8 and 16 hr at 28°C.

2.1 Transposon (Tn5) **mutagenesis**

Culture of E. coli WA 803 (pGS9) and Sinorhizobium fredii R0132 were grown to log phase in LB at 30°C, rpm 200. To raise WA 803, Kan_{50} and Clm_{10} was added (subscripts indicate concentration in µg/ml). One ml each culture was spun at 5000 rpm for 5 minutes and re-suspended in1.0 ml 10 mM $MgSO_4$. Equal volumes (200 µl) of two washed suspensions were mixed, centrifuged at 5000 rpm for 5 minutes and resuspended in 100 µl of 10mM, $MgSO_4$. This was spotted for patch-mating on LA surface and incubated for 8 h at 30 °C. The cells were resuspended later in 2.5 ml of 10m M $MgSO_4$, plated on M9G medium with Kanamycin (50 µl/ml) and incubated at 30 °C for 24 hours. Individual R0132::Tn5 exconjugants streaked for analysis of HAM Agar.

2.2 Cell wall isolation

The cells of different S. fredii mutants were in YEM liquid broth were kept in incubator shaker up to 200 rpm at 28°C ± 1°C. The cells harvested during late exponential phase of growth by centrifuging the cultures at 5000 X g for 10 minutes at 4°C. The supernatant discarded and pellet washed three times with tris HCI buffer (pH 7.2). The cells crushed in pre-chilled mortar pestle with glass powder in homogenization buffer (4.0 ml).

Sucrose:	0.25 M
Tris- HCl pH (8.5):	50.0 ml
EDTA:	10.0 mM
Mercaptoethanol:	5.0 mM
MgSO4:	10.0 mM
KCl:	10.0 mM

The obtained slurry centrifuged at 500 rpm for 5 min at 4°C to remove glass particles. The supernatant centrifuged at 6000 rpm for 20 minute at 4°C with 40 percent sucrose cushion and the pellet thus obtained used as crude cell wall fraction. The supernatant concentrated by filtration against polyethylene glycol/ 1.0 M sucrose solution in dialysis bags.

2.3 Estimation of Lipopolysaccharides

LPS of cell walls measured in terms of total heptose and glycoprotein because heptose is major constituent of the LPS. For this, essentially the method of Osborne (1963) using D- glycero monogluco heptose as standard was followed. Wall fraction of rhizobiamade in 0.5 ml of distilled water and cooled in an ice bath 2.25 ml of reagent (conc. H_{24}6.0 volume and 1 volume D.W.) Slowly added and mixed by shaking in the cold. After 3 min tubes were transferred to a water bath for another 10 min. After cooling 0.05 ml of 3 percent solution of freshly prepared cystein HCl was added in to each sample. Heptose produced a purple colour with absorbance maxima at 545 nm. Exactly two hours after adding the cystein HCl, the sample read against the blank at 545 and 505 nm. The difference between two absorbance values is linear function of the heptose concentration. Under these conditions 1.0 M of L-glycero-D mannoheptose gives a value of $E_{545}E_{505}$ to 1.07. The concentration of LPS was expressed µg/mg fresh weight of walls.

2.4 Estimation of Exopolysaccharides

The amount of EPS was determined according to the method followed by Damery and Alexander, (1969). The rhizobia grown in YEM broth at 28°C keep on a gyrorotary shaker at 200 rpm. At the exponential phase of growth (after 96 hours) the supernatant was concentrated at the 60°C, and mixed with two volumes of acetone in the cold and stored for over night at 4°C. The polysaccharide removed from solution by centrifugation at 5000 X g for 30 minutes. The weight of polysaccharide was determined. The amount of EPS production represented as mg/100 ml culture.

Finally one potential mutant of S. fredii R 0132 selected and was designated as R0132 (number of mutant): Tn5 which used for further studies.

3. RESULTS AND DISCUSSIONS

To develop the mutant of S. fredii R0132, gene mutation by the help of transposon, 5, was carried out by the random transposon mutagenesis procedure. The procedure for random transposon mutagenesis given by Selvaraj and Iyer (1983) was modified by (Khanuja, 1987) to suit Rhizobium. The period of mating on agar surface had been found to be significantly affecting the vector transfer recovery of Tn5 mutants Table-1. The result indicated that a minimum of 8h of mating was necessary (low frequency) for recovery of mutants containing Tn5. When mating was carried out for 16 h or overnight improvement in cell titer was observed. Since, objective was to isolate 2 to 3 mutants in experiment; 8 h of incubation period was used for mating. As a result, more than1600 mutants were generated by means of Tn5 mediated mutagenesis where after screening; only 15 mutants were selected and use further studies.

Table-1. Optimization of incubation period for patch mating to transfer pGS9 plasmid into R. leguminosarum.

S.N.	**Incubation time for patch mating** (h)	**Average titer of cells/ml**
1.	0 h	Nil
2.	4 h	Nil
3	8 h	$1.2x10^2$
4	6 h	$6.2x10^3$

3.1 Lipopolysaccharides production

The data presented in the Table-2, reveals that the amount of lipopolysaccharides in the wall of different rhizobial isolates varied from 72.3 to 189.3 µg/mg fresh weight of cell wall and constituted only 7.85 to 18.05 percent of the cell wall weight. Mutant R0132 (1106): Tn5 and R0132 (1112): Tn5 showed the very low level of

lipopolysaccharides which was at least 30 % less than of the wild type S. fredii (R0132). However, maximum mutants showed the increased level of lipopolysaccharides than the wild type.

Table-2. Productions of lipopolysaccharides from wild type and mutant of rhizobial isolates.

#	Isolate	LPS µg/mg FW of cell wall
1.	R0132 (wild type)	102.90 ± 0.33
2.	R0132(0056)::*Tn*5	132.10 ± 0.624
3.	R0132(0072)::*Tn*5	156.00 ±1.73
4.	R0132(0097)::*Tn*5	144.70 ±1.26
5.	R0132(0123)::*Tn*5	183.20 ±0.93
6.	R0132(0263)::*Tn*5	122.30 ±0.60
7.	R0132(0521)::*Tn*5	114.50 ±0.40
8.	R0132(0599)::*Tn*5	156.20 ±1.82
9.	R0132(0923)::*Tn*5	88.40 ±0.67
10.	R1032(1106)::*Tn*5	72.30 ±0.60
11.	R0132(1112)::*Tn*5	189.30 ±2.66
12.	R0132(1421)::*Tn*5	113.00 ±0.88
13.	R0132(1508)::*Tn*5	103.00 ±0.88
14.	R0132(1621)::*Tn*5	102.80 ±0.57
15.	R0132(1821)::*Tn*5	116.30 ±0.58
16.	R0132(1832)::*Tn*5	172.00 ±0.57

3.2 Effect of temperature on the production of lipopolysaccharides

S. fredii 32 mutant also varied in lipopolysaccharides content ranging from lowest value 72.3 µg/mg (S. fredii R0132 (1116): Tn5) to highest value 183.3 µg/mg (S. fredii R0132 (0123): Tn5) of fresh weight of cell wall. About 10-15 percent reduction in production in LPS was observed in all the mutants including parent strain at 32^{o}C and 36^{o}C. However this decrease was prominent and about 40 percent of LPS production was decreased in parent and mutant strains. Surprisingly mutant S. fredii R0132 (0123): Tn5 and S. fredii R0132 (0056): Tn5 showed no or little reduction in production of LPS Figure-2. Tn 5 mutagenesis, significantly influenced production level of lipopolysaccharidesand exopolysaccharides as observed in different S. fredii mutants.

3.3 Exopolysaccharides production

Mutants were differ from wild type S. fredii R0132 in their capability of the production of exopolysaccharides Table-3. Significantly increased production was noticed in case of R0132 (0521): Tn5, R0132 (1106): Tn5, R0132 (1112): Tn5 and R0132 (1421): Tn5 where 49.1, 61.0, 59.2 and 44.4 percent increase was observed over their parent, surprisingly, mutant R0132 (0072): Tn5 showed a slight decrease in production of EPS. The actual function of EPS in symbiosis has not been elucidated but there are several data that indicate a function of EPS as a biologically active signaling molecule. It was also hypothesized that EPS may act as a suppressor of the plant defense system during nodulation of alfalfa by S. meliloti (Niehaus *et al*., 1993). Several observations of R. leguminosarum bv. viciae and bv. trifolii indicated that their mutants were found to be showing increased EPS production are affected in their ability to induce infection threads or in the bacteroid release and development (M. Janczarek, *et al*., 2001). In some mutants (Rolfe, *et al.*, 1996) reported that the infection threads were enlarged, tightly packed with bacteria, with abnormal morphology of infection threads walls indicating plant defense response elicited by mutated rhizoid.

Table-3. Productions of exopolysaccharides from wild type and mutant of rhizobial isolates

#	Isolate	LPS µg/mg FW of cell wall
1.	R0132 (wild type)	91.20±0.52
2.	R0132 (0056)::*Tn*5	113.86±0.31
3.	R0132 (0072)::*Tn*5	82.20±0.57
4.	R0132 (0097)::*Tn*5	102.80±0.61
5.	R0132 (0123)::*Tn*5	94.60±0.37
6.	R0132 (0263)::*Tn*5	102.70±0.78
7.	R0132 (0521)::*Tn*5	136.80±0.32
8.	R0132 (0599)::*Tn*5	91.80±0.32
9.	R0132 (0923)::*Tn*5	96.50±0.31
10.	R1032 (1106)::*Tn*5	146.90±1.16
11.	R0132 (1112)::*Tn*5	145.20±0.63
12.	R0132 (1421)::*Tn*5	131.80±0.56
13.	R0132 (1508)::*Tn*5	131.80±0.39
14.	R0132 (1621)::*Tn*5	116.40±0.60
15.	R0132 (1821)::*Tn*5	123.80±0.6
16.	R0132 (1832)::*Tn*5	117.56±0.57

3.4 Effect of temperature on production of exopolysaccharides

The EPS production by mutants in culture medium by different mutants influence by temperatures is given in Table-4. Only few mutants demonstrated the production of EPS at high temperature (42^{o}C). However, their EPS production level was comparatively lower than by those secreted at 28^{o}C. Different species and strains of rhizobia differ in their resistance or tolerance to high temperature (Karanja and Wood, 1988). Some thermotolerant strains of Rhizobium remain infective even at high temperature (Karanja and Wood, 1988; Kishinevsky *et al.*, 1992). The basis of thermo tolerance

and capacity to nodulate at high temperature remains unknown. This might be due to certain metabolic changes occurring in the bacterial cells under high temperature conditions.

Table-4. Effect of temperature on production of EPS from wild type and mutant of rhizobial isolates.

#	Isolates	EPS µg/mg FW of cell wall			
		28^0C	32^0C	36^0C	42^0C
1.	R0132 (wild type)	91.20±0.63	88.70±0.49	32.00±0.28	32.40±0.69
2.	R0132 (0056)::*Tn*5	113.86±0.52	101.30±0.66	39.48±0.44	59.20±0.16
3.	R0132 (0072)::*Tn*5	82.20±0.40	62.40±0.69	41.30±0.40	36.70±0.41
4.	R0132 (0097)::*Tn*5	102.80±0.78	52.40±0.64	50.20±0.34	48.40±0.91
5.	R0132 (0123)::*Tn*5	94.60±0.54	32.00±0.57	30.60±0.47	37.70±0.51
6.	R0132 (0263)::*Tn*5	102.70±0.81	41.60±0.37	35.30±0.37	-
7.	R0132 (0521)::*Tn*5	136.80±0.72	103.00±0.57	39.40±0.57	-
8.	R0132 (0599)::*Tn*5	91.80±0.11	27.00±0.57	33.60±0.63	47.40±0.43
9.	R0132 (0923)::*Tn*5	96.50±0.63	39.90±0.69	33.30±0.37	33.70±0.72
10.	R0132 (1106)::*Tn*5	146.90±0.11	121.40±0.64	83.80±0.53	61.20±0.34
11.	R0132 (1112)::*Tn*5	145.20±0.63	118.60±0.57	59.40±0.40	-
12.	R0132 (1421)::*Tn*5	131.80±0.54	101.30±0.37	42.60±0.36	42.40±0.43
13.	R0132 (1508)::*Tn*5	131.80±0.54	82.50±0.57	60.30±0.41	49.70±0.50
14.	R0132 (1621)::*Tn*5	116.40±0.69	22.00±0.57	58.65±0.44	-
15.	R0132 (1821)::*Tn*5	123.80±0.69	52.63±0.61	46.85±0.38	58.30±0.37
16.	R0132 (1832)::*Tn*5	117.56±0.49	38.46±0.43	43.75±0.67	-

3.5 Production of exopolysaccharides in presence of different sugars

The exopolysaccharides excreted by wild type R0132 and its mutants in culture medium containing different sugars viz; glucose, mannitol, fructose and sucrose showed higher degree of variability. The amount of produced EPS was ranging 25.6 to 178.3 µg/mg of fresh weight of cell wall. EPS secreted by mutants varied in the range from 44.3 to 101.36 µg/mg and 39.3 to 68.7 µg/mg of fresh weight of cell wall in presence of glucose and fructose, respectively. However in presence of mannitol and sucrose higher EPS production was observed in all mutant and wild type isolate, mutant S. fredii R0132 (1106): Tn5 showed higher EPS production (156.8 µg/mg of FW of cell wall) while lower was observed in case of R0132 (0923): Tn5 (96.5 µg/mg FW of cell wall) in the presence of mannitol .On other hand sucrose increased EPS production in R0132 (1106): Tn5 upto 178.3 which was the highest in all the sugars studied Figure-1.

Previously, Skorupska (1995), described the isolation of several EPS mutants of R. leguminosarum bv. trifolii strain 24.1 obtained by transposon Tn5 mutagenesis. These mutants acquired the mucoid morphology. In our report we described mutants of S. fredii by Tn5 insertions which resulted in to Exo and lipo mutants showing lower (or no)/ higher EPS and LPS production. It seems that insertion mutation could occur in structural or other 'non structural' gene. Further work is in progress, to understand the precise localization of Tn5 insertion sites in these mutants, which could be confirmed by sub cloning and sequencing the adjacent fragments of genomic DNA.

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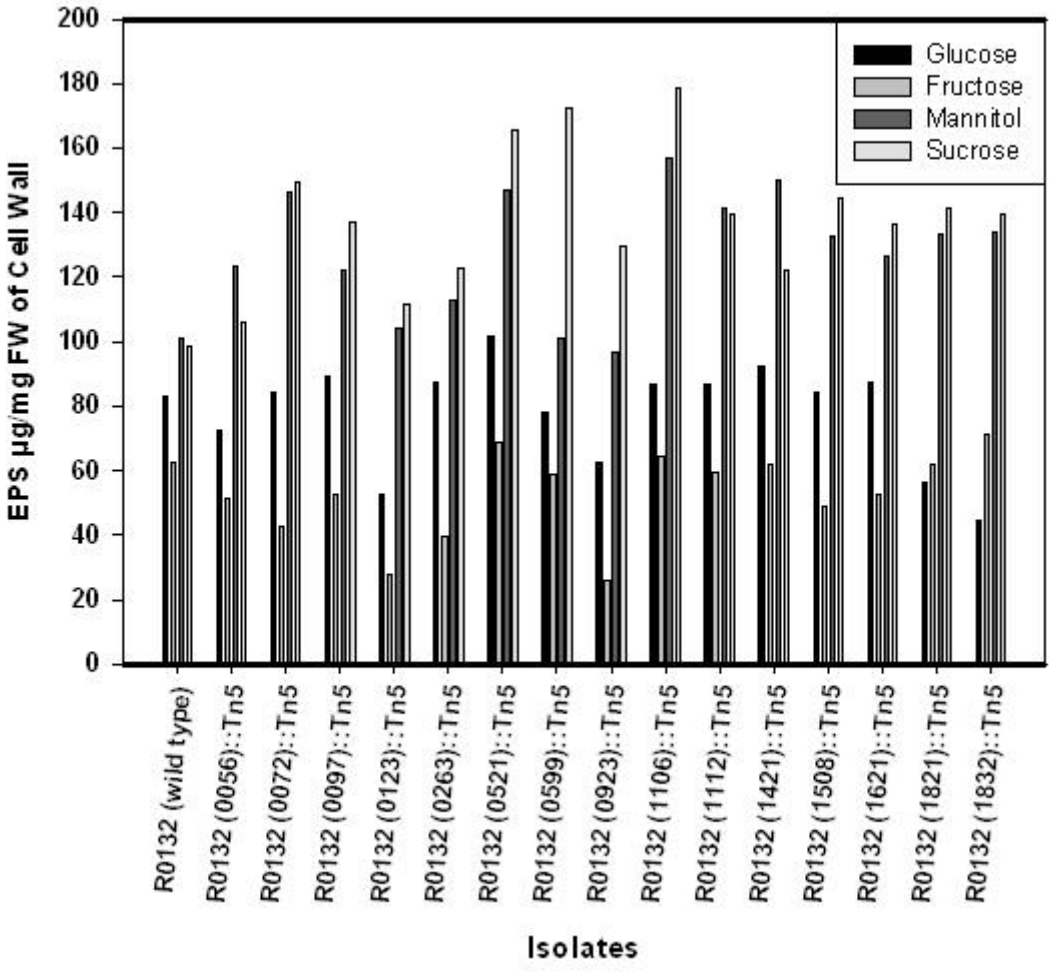

Figure-1. Production of eps in presence of different sugars from wild type and mutant of rhizobial is lates.

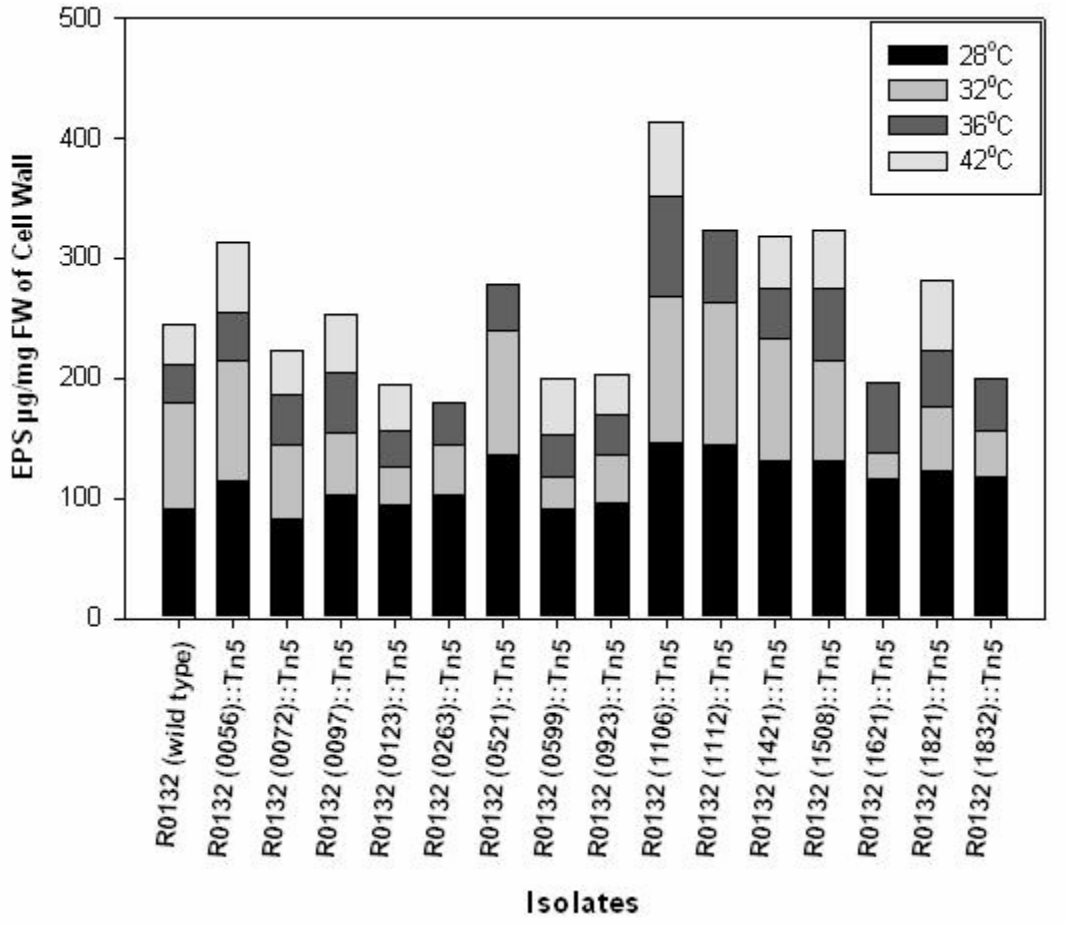

Figure-2. Production of eps in presence of different sugars from wild type and mutant of rhizobial is lates.