International Journal of ChemTech Research
CODEN(USA): IJCRGG ISSN : 0974-4290
Vol.6, No.1, pp 64-79, Jan-March 2014

Improvement The Efficiency Of *Sphingomonas Paucimobilis* To Produce Gellan Gum By Genetically Approach

A. G.Attallah[1]*, S. K. Abd-El-Aal[1], S. A. Ibrahim[2] and M.A. El-Sayd[1]

[1]Microbial Genetics Department, National Research Centre, Cairo, Egypt
[2]Genetics Dept., Faculty of Agriculture, Ain Shams Univ., Cairo, Egypt.

***Corres.author: ashrafgamil2000@yahoo.com**

Abstracts: Protoplast fusion experiment was performed between *Sphingomonas paucimobilis* ATCC 31461 (*S.p.*ATCC 31461) and the two local strains; *S.p.*Q6 and *S.p.* G1. Seventeen genetically stable fusants were isolated after the fusion (*S.p.*ATCC 31461:: *S.p.*G1) and only three genetically stable fusants were obtained after protoplast fusion (*S.p.*Q6 :: *S.p.*G1). High level of gellan gum production (16.82 g/ liter; F13; and 17.16 g/ liter; F22) was obtained by fusion of *S.p.*ATCC 31461:: *S.p.*G1 and *S.p.*Q6:: *S.p.*G1 , respectively. All the 20 recombinants (fusants) were tested for detection of *S. paucimobilis* specific genes using specific primer. The *pgmG* gene was cloned based on the PCR amplification of a DNA fragment of 670 bp from *S. paucimobilis* ATCC 31461 genomic DNA using degenerate primers from conserved regions of the phosphohexosemutase protein sequences in databases: the synthetic oligonucleotides *PGM*1 (sense) (5`-ACCGSCAGCCABAAYCCG-3`) and *PGM*2 (antisense) (5`-BSCGCTCATYTCGCC-3`) . The results indicated that a fragment of the expected size of 670 bp for *pgmG* gene and 897 for *rmlA* gene were detected in all fusants and their parents. The SDS-PAGE protein banding pattern of these fusants was performed and compared with protein banding pattern of their parental strains. Furthermore determination of the polysaccharide produced by the three selected strains *S.p.*ATCC 31461, F13 and F22 which have high polysaccharide productivity was carried out using HPLC chromatography technique. The hydrolysis of the gellan gum was resulted in consists of three mono sugars; glucose, glucuronic acid and rhamanose at ratio 2:1:1.

Key Words: *Sphingomonas paucimobilis*, gellan gum, protoplast fusion, PCR and SDS-PAGE.

Introduction

The genus *Sphingomonas* was defined as groups of Gram-negative[1], rod-shaped, chemoheterotrophic, strictly aerobic bacteria produce yellow-pigmented colonies[2]**.** *Sphingomonas* bacteria are known to produce extracellular biopolymers, including certain gellan-related exopolysaccharides[3]**.** *S. paucimobilis* produce a gellan-related polysaccharide that forms a gel if deacetylated[4]**.** Gellan-related polysaccharides synthesized by strains of *Sphingomonas* are collectively referred to as "sphingans"[5]**.** They share the same carbohydrate backbone structure (–X-glucose-glucuronic acid-glucose-X–) in which X is either L-rhamnose or L-mannose. The yield, molecular structure, and properties of sphingans are significantly affected by growth medium composition.[6]

Sphingomonas paucimobilis ATCC 31461 synthesizes the exopolysaccharides (EPS) gellan, a gelling agent with applications in the food, pharmaceutical, and other industries[6]. This EPS is composed of a repeating linear tetrasaccharide unit consisting of D-glucose (Glc), D-glucuronic acid (GlcA), and L-rhamnose (Rha) in a 2:1:1 ratio, respectively, with glycerate and acetate substituents.[7]

Sphingomonas will likely an important source of organisms for research and development of novel exopolysaccharides. Protoplast fusion technology is considered a good tool for having new genetic recombination. In this process, the two genetically distinct strains are mixed and protoplast together with the use of lysozyme and then fused in the presence of polyethylene glycol. The fused protoplasts are then allowed to grow into vegetative cells. Recombination occurs within the transient diploid protoplasts, which is followed by segregation and haploidization. It appears to be a useful method to bring about recombination among bacteria in which other mechanisms of genetic exchange are not yet known[8]**.**

Bacterial exopolysaccharides (EPS) are biotechnology products that are of great interest owing to their rheological properties. This is the case for gellan gum, a multifunctional gelling agent that is produced in high yield by the nonpathogenic bacterium *Sphingomonas elodea* ATCC 31461. It has approval in the US and EU for food use as a gelling, stabilizing and suspending agent, either alone or in combination with other hydrocolloids. In its native form, gellan is a linear high-molecular-weight anionic EPS based on a tetrasaccharide repeat unit composed of two molecules of d-glucose, one of l-rhamnose and one of d-glucuronic acid. The native gellan is partially esterified with acyl substituents (1 mol glycerate and 0.5 mol acetate) per repeat unit [9]. Gellan and gellan-like polymers have unique characteristics and have found many applications, particularly in the food, pharmaceutical and biomedical fields.[10]

Recently, several biotechnological strategies have been developed to construct strains of *Sphingomonas* that enhance highly exopolysacharide productivity. Protoplast fusion technology improves the genetic traits of Gram-positive[11] and Gram-negative bacteria[12,13]as well as nodulation efficiencies [14] where some of the intra-specific fusion products were one to five folds.

The *pgmG1* gene is the first gene of *pgmG* 4-gene another cluster; *pgmG1, pgmG2, pgmG3* and *pgmG4* required for gellan biosynthesis by *Sphingomonas paucimobilis* ATCC. It encodes a 50,059-Da polypeptide that has phosphoglucomutase (PGM) and phosphomannomutase (PMM) activities and is 37 to 59% identical to other bifunctional proteins with PGM and PMM activities from Gram-negative species, including *Sphingomonas paucimobilis. PgmG* protein can convert mannose-6-phosphate into mannose-1-phosphate in the initial steps of alginate biosynthesis and, together with other results, suggests that *PgmG* may convert glucose-6-phosphate into G1P in the gellan pathway[15]**.**

The *pgmG* gene was cloned based on the PCR amplification of a DNA fragment of 670 bp from *S. paucimobilis* ATCC 31461 genomic DNA using degenerate primers from conserved regions of the phosphohexosemutase protein sequences in databases: the synthetic oligonucleotides PGM1 (sense) (5`-ACCGSCAGCCABAAYCCG-3`) and PGM2 (antisense) (5`-BSCGCTCATYTCGCC-3`), The PCR product was resolved on 0.8 % agarose gel in 1X Tris acetate buffer. The gel was stained with ethidium bromide[16]**.**

The main objective of this study was Genetics improvement of gellan gum productivity via protoplast fusion technique between different gellan producer strains.

Materials and Methods

-Bacterial Strains: The strains used in fusion experiments were constructed as described in Table (1). [17]

Table (1): Characterization of the three parental *S. paucimobilis* strains used in protoplast fusion experiments.

Strain	Phynotypic changes
S. paucimobilis ATCC* 314 6 1	Km^s Rif^s Sm^r Nm^r
S. paucimobilis Q6	Km^r Rif^r Sm^s Nm^r
S. paucimobilis G1	Km^r Rif^r Sm^r Nm^s

Microorganism and cultivation. The strains used in this study were maintained on YPG slants containing (liter^{-1}) 30 g glucose, 5 g peptone, 3 g yeast extract, 5 g NaCl, and 20 g agar (pH 7.0). The slants were incubated at 30°C for 24 h, and the fully grown slants were stored at 4°C. The production medium; S media; contained (liter^{-1}) 1 g Na_2HPO_4, 10 g K_2SO_4, 3 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, and 1 g yeast extract (pH 7.0). For fermentation, the appropriate culture was inoculated into 100 ml of the production medium in 500-ml Erlenmeyer flasks. The inoculated flasks were kept on a rotary shaker at 200 rpm at 30°C.

-Antibiotics resistance test: this test was carried out according to Moazed and Noller(1987) and Four antibiotics were used with final concentrations as follows: Rifampicin (Rif) 100 µg/ml, streptomycin (Sm) 200 µg/ml, kanamycin (km) 40 µg/ml, neomycin (Nm) 40 µg/ml . the Kirby-Bauer disc diffusion method for antimicrobial susceptibility test was used 18,19.

Protoplast Fusion: The protoplasts were collected by centrifugation at 3000 rpm for 10 min.The precipitate was washed with Tris-HCl buffer with an osmotic stabilizer and the resulting precipitate was re-suspended in the same buffer to allow all protoplasts to regenerate. Protoplast suspension was diluted and overlaid on the LB medium solidified by adding 2% agar and the colonies were counted after 2-5 days. Aliquots (1.0 ml each) of the two parental protoplasts were mixed in the presence of 25% PEG 6000 and 100 mM $CaCl_2$ (Hopwood *et al.*, 1985) and incubated at 30°C. Aliquots of 100 µl from the mixture were taken every 10 min and added to selective agar medium (solidified LB medium + selectable antibiotics) and overlaid on the same selective basal medium to screen the fusants.[14]

-Extraction and determination of polysaccharide.[20]

- Detection of some gellan genes by Polymerase Chain Reaction (PCR): Two sets of oligonucleotide primers were used for PCR amplification with genomic DNA isolated from *S. paucimobilis*. The *pgmG* primer PGM1 (sense) (5`ACCGSCAGCC ABAAYCCG-3`) and PGM2 (antisense) (5`- BSCGCTCATYTCGCC 3`) and the other for *rmlA* gene (5`-GCAGCTGCTTCCCGTCTATGA-3`) and (5`CGAGCCGGGTGTG TGGAGGTC-3`), purchased from Pharmacia (Uppsala, Sweden)[21]**.**

-SDS-PAGE electrophoresis technique: will be done by the method of Laemmli [22] and modified by **Sambrook** [23] for the three parent strains and their 20 fusants.

Determination of sugars by HPLC. Hydrolysis was carried out according to the method of Evince and Linker [24].

Results and Discussion

1-Genetic improvement of Gellan gum productivity

Protocol was applied in this study for improving *Sphingomonas paucimobilis* productivity of the exo-polysaccharide (EPS) gellan gum was protoplast fusion technique as a tool for producing new genetic recombinants.

1.1-Selection of strains with appropriate genetic markers

Detection of *S. paucimobilis* genetically stable strains was necessary to facilitate tracing and manipulating of desired strains through genetic experiments.

In present study the three parental strains; *S. paucimobilis* ATCC 31461 and the local strains *S. paucimobilis*.Q6 and *S. paucimobilis* G1 were tested for viscosity values and exo-polysaccharid production. Four antibiotics; Kanamycine (Km), rifampicin (Rif), streptomycin (Sm) and neomycin (Nm), were used as genetic markers of the most appropriate strains for protoplast fusion experiments are the three parental strains [17]. The important characteristics of the selected strains are summarized in Table (2).

Table (2): The important characteristics of the selected strains for genetic improvement of (Exopolysaccharide (EPS) productivity and Viscosity (cP).

Strain	Viscosity (cP)*	EPS (g/l)	Antibiotic resistance
S. paucimobilis ATCC 31461	3920	6.07	Km^s Rif^s Sm^r Nm^r
S. paucimobilis Q6	2630	2.15	Km^r Rif^r Sm^s Nm^r
S. paucimobilis G1	2650	2.22	Km^r Rif^r Sm^r Nm^s

*cP. A centimeter-gram-second unit of dynamic viscosity equal to one hundredth (10^{-2}) of a poise.

Table (2) revealed that the three strains ;(*S. paucimobilis ATCC* 31461 and the other two local strains; *S. paucimobilis* Q6 and *S. paucimobilis* G1) showed different resistance patterns for the four antibiotics and also for viscosity value and polysaccharide productivity. These results and also genetic markers required for selection of fusants; accordingly these selected strains were used as parental strains in protoplast fusion experiments.

1.2- Induction of genetic recombination using protoplast fusion.

Based on the main focus of the present study, which is the enhancement of polysaccharide production by the selected strains, two weak strains (*S.p*.Q6 and *S.p* G1) and one efficient strain (*S.p* ATCC31461) were selected for protoplast fusion. *S.p* Q6 and *S.p* G1 produced the lowest productivity of exopolysaccharide and viscosity (2.22 and 2.15 g/l, respectively) as shown in Table 2, therefore, they were selected to investigate the efficiency of the tested technique (protoplast fusion).On the other hand *S.p* ATCC31461 produced relatively high productivity of polysaccharide and viscosity (6.07g/l and3920 cP) so it was Selected to investigate the ability of the tested technique to enhance this capacity. In addition *S.p* ATCC, *S.p* Q6 and *S.p* G1 exhibited multiple resistances of the tested antibiotics respectively,

Protoplast fusion was carried out according to the method described above[14]**.** The *Sphingomonas* cells were treated with protoplast buffer up to 3 h. The protoplast induction was monitored at 10 min intervals where induction efficiency has been estimated by comparing the colony count when the protoplast preparation was exposed to an osmotic shock, i.e., by dilution in water, before plating. Protoplast formation was tested by phase contrast microscopic detection and by OD reading at 600 nm after ten times dilution in distilled water. The protoplasts of *S.p* ATCC31461 and *S.p* G1 (1 ml each) were mixed in the presence of 25% PEG 6000 and incubated at 30 ^{o}C. An aliquot of 100 µl from the fusion mixture was taken regularly at 10 min time intervals and diluted 10 times in protoplast buffer. A sample of 100 µl of the fusion mixtures of *S.p* ATCC31461 and *S.p* G1 were plated directly into selective plates containing selective antibiotic to counter-select the parents and only the hybrid between parents will grow. Plating the protoplast directly onto selective medium will tend to underestimate the total frequency of fusion events related to the nature of complementation and segregation after fusion [25]**.** Protoplast fusion was also performed between *S.p*.Q6 and *S.p*.G1. The parental protoplast mixture were mixed in the presence of 25% PEG 6000 and incubated at 30^{o}C. They were then plated on the same selective medium containing the proper antibiotic to counter-select the parents.

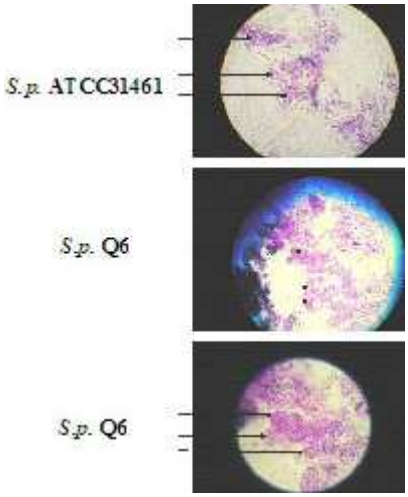

Fig. (1): Light microscope plates reveal protoplast formation for the three parental strains, *S.p.* ATCC31461, *S.p.* Q6, *S.p.* G1.

In order to determine the efficiency of protoplast induction and number of regenerated protoplasts for each parental strain, two equal samples of protoplast suspension, were used. The first sample was spread onto YM medium after osmotic shock to allow the osmotically stable, non-protoplast cells which were not transformed to protoplast form to grow. The ratio of protoplasting was calculated after subtracting the number of colonies appeared on YM medium after osmotic shock from the total number of cells before protoplasting treatments. The second sample was added to the top layer medium then overplayed on the regeneration medium and grown cells were also counted. The regeneration ratio was calculated after subtracting the number of remainder cells after protoplasting treatment from the number of the colonies appeared on the regeneration medium. These results are illustrated in Table (3).

The protoplasting percentages were 55.7%, 73.7% and 70.2% for the three examined strains *S.p.* ATCC31461, *S.p.* Q6, *S.p.* G1, respectively. These results are consistent with those obtained by Piagac [26] who got frequencies of protoplasting range from 56 to 75% depending upon the genome of mutants organized from a slant of *Streptomyces rimosus.*

As shown in Table (3) strain *S.p.*Q6 gave the highest regeneration ratio (92.3%) followed by strain *S.p.*G1 (86.5%). The industrial strain *S.p.*ATCC31461 showed 74.8% regeneration ratio.

Table (3): Total protoplast and regeneration ratio of the three *Sphingomonas paucimobilis* parental strains.

Strains /Parameter	*S.p.*ATCC 31461	*S.p.*Q6	*S.p.*G1
Total count /ml	863 x10^6	810x10^6	941 x10^6
No. of non-protoplasted cells /ml	382 x10^6	213 x10^6	280 x10^6
No. of protoplasts /ml	481 x10^6	597 x10^6	661 x10^6
Protoplasting %	55.7	73.7	70.2
No. of colonies on regeneration medium /ml	742 x10^6	764 x10^6	852 x10^6
Regenerated protoplasts /ml	360 x10^6	551 x10^6	572 x10^6
Regeneration %	74.8%	92.3%	86.5%

1.3- Effect of protoplasting and regeneration on polysaccharide productivity

To ensure that any expected change in the polysaccharide productivity of the fusants comparing to their corresponding parental strains, is a result of the genetic recombination's of these fusants, it is of importance to examine the effect of protoplasting and regeneration on polysaccharide productivity of the three parental strains.

Three regenerated colonies were randomly selected and tested for their polysaccharide productivity and compared to the productivity of the corresponding parental strains. Results are presented in Table (4).

Table (4): Effect of protoplasting and regeneration on polysaccharide productivity of the three *Sphingomonas paucimobilis*. parents.

Original Strains	EPS (g/L)	Regenerated colony	EPS g/L	Mean
S. paucimobilis ATCC 31461	6.07	*S.p.* ATCC 31461R1	5.95	6.09
		S.p. ATCC 31461R2	6.30	
		S.p. ATCC 31461R3	6.01	
S. paucimobilis Q6	2.15	*S.p.* Q6R1	2	2.15
		S.p. Q6R2	2.15	
		S.p. Q6R3	2.3	
S. paucimobilis G1	2.22	*S.p.* G1R1	2.12	2.4
		S.p. G1R2	3	
		S.p. G1R3	2.08	

In general, no variation was observed, indicating no effect of the exposure of the cells to protoplasting and regeneration on the polysaccharide productivity. For example, the mean of the productivity of the three regenerated colonies recorded 6.09 and 2.4 g/L for the parental strains *S. p* ATCC 31461 and *S. p* G1 which recorded 6.07 and 2.22 g/L, respectively. On the other hand the mean of the productivity of the three regenerated colonies of the third parental strain *S. p* Q6 recorded the same polysaccharide productivity (2.15 g/L) of their parental strain.

1.3- Protoplast fusion between (*S.p.* ATCC 31461:: *S.p.*G1).

Polysaccharide productivity of the parental strain ATCC 31461 is 6.07 g/l and it resists the antibiotic neomycin (Nm), while the other parental strain *S.p.*G1 produces 2.22 g/l polysaccharides and resists both antibiotics rifampicin (Rif) and kanamycin (Km). Seventeen regenerated fusants with resistance to the three antibiotics were appeared on selective medium and tested for polysaccharide productivity.

Table (5) showed the characters of the parental strains and their seventeen genetically stable fusants obtained after one hour of incubation period and designated from F1 to F17. All these fusants showed increase in polysaccharide productivity comparing to both parental strains. Only three fusants; F13, F9 and F17 showed three times a highest productivity of polysaccharide 16.82, 16.3 and 15.3 g/l respectively than the parents.

1.4- Protoplast fusion between (*S.p.*Q6 :: *S.p.*G1):

The parental strains *S.p.*Q6 and *S.p.*G1 used in this trial. The parent (*S.p.*Q6) showed polysaccharide productivity of 2.15 g/l, resistance to neomycin (Nm) and sensitivity to streptomycin (Sm) antibiotic. While the other parental strain (*S.p.*G1) showed polysaccharide productivity of 2.22 g/l, resistance to streptomycin (Sm) and sensitivity to neomycin (Nm). Three regenerated fusants with resistance to both antibiotics (Nm and Sm) were appeared on selective medium and tested for polysaccharide productivity.

Also, Table (6) showed the result of fusion between (*S.p.*Q6:: *S.p.*G1). Three genetically stable fusants were obtained. All these fusants which were designated F20, F21 and F22, showed increasing in polysaccharide productivity comparing to both parental strains. Fusants F22 has the highest efficiency of polysaccharide productivity (17.16 g/l) followed by F20 (12.7 g/l) and F21 (10.86 g/l).

Table (5): Polysaccharide productivity and genetic markers of the two parental strains of the fusion (*S. p.* ATCC 31461 :: *S. p.* G1) and their seventeen selected fusants.

Strain	Genetic markers	EPS(g/l)
S. p. ATCC 31461	Rf^s Kn^s Nm^r	6.07
S. p. G1	Rf^r Kn^r Nm^s	2.22
F1	Rf^r Kn^r Nm^r	12.78
F2	Rf^r Kn^r Nm^r	8.02
F3	Rf^r Kn^r Nm^r	13.6
F4	Rf^r Kn^r Nm^r	12.08
F5	Rf^r Kn^r Nm^r	8.8
F6	Rf^r Kn^r Nm^r	10.14
F7	Rf^r Kn^r Nm^r	9.86
F8	Rf^r Kn^r Nm^r	6.34
F9	Rf^r Kn^r Nm^r	16.3
F10	Rf^r Kn^r Nm^r	7.42
F11	Rf^r Kn^r Nm^r	12.06
F12	Rf^r Kn^r Nm^r	12.08
F13	Rf^r Kn^r Nm^r	16.82
F14	Rf^r Kn^r Nm^r	10.1
F15	Rf^r Kn^r Nm^r	12.92
F16	Rf^r Kn^r Nm^r	11.44
F17	Rf^r Kn^r Nm^r	15.3

Table (6): Polysaccharide productivity and genetic markers of the two parental strains of the fusion (*S.p.*Q6 :: *S.p.*G1) and three their fusants.

Strain	Genetic markers	EPS (g/l)
S. paucimobilis Q6	Sm^s Nm^r	2.15
S. paucimobilis G1	Sm^r Nm^s	2.22
F20	Sm^r Nm^r	12.7
F21	Sm^r Nm^r	10.86
F22	Sm^r Nm^r	17.16

On the other hand, the variation in polysaccharide productivity by the different fusants can be attributed to the number of copies of the gene(s) introduced, probably the introduction of different genes from the gene pools and location in which genes had been integrated in the same chromosome[27]**.** A problem that may impede the functionality of some strains constructed using DNA from diverse origins is a lack of gene expression. Although many foreign genes are expressed in frequently used hosts, a number of exceptions are known[28]**.**

The main objective of this part is to use protoplast fusion for inducing more genetic variations through its ability to induce new genetic recombination. Protoplast fusion has been used for the transfer of both plasmid and chromosomal genes between genetically marked strains and the intergeneric transfer of a plasmid from one strain to another. Protoplast fusion technique used to obtain fusants (new strains) harboring desirable economical characters Protoplast fusion is one of the most effective techniques since it facilitates the genetic material exchange more than other mating methods, because the genetic exchange between the intact chromosomes of two couplet parental genomes in every fused protoplast is possible[25]**.**

In conclusion, a high level of gellan gum production (16.82 g/ liter and 17.16 g/ liter) was obtained by fusion of *S. p* ATCC 31461, *S.p*. Q6 and *S.P*. G1, respectively.

2-Biochemical and molecular genetics investigations of the protoplast fusion products

After fusion, genetic rearrangement can occur to give rise to new gene combinations, Gene's arrangements able to regenerate, a vegetative cell can be recovered from the fusion and the desirable recombinants can be selected.

In this work, 20 obtained recombinants (fusants) were tested for detection of *S. paucimobilis* specific genes using specific primer and the SDS-PAGE protein banding pattern of these fusants was performed and compared with protein banding pattern of their parental strains. Furthermore Determination of the polysaccharide produced by selected strains was carried out using HPLC chromatography technique.

2.1- Detection of *S. paucimobilis* specific genes using specific primer.

In this experiment, PCR technique was carried out using the two gellan gum genes *pgmG* and *rmlA*. primers were chosen based on published sequence data [29,16] as mentioned in materials and methods. The PCR product was analyzed by electrophoresis on 0.8% agarose. A fragment of the expected size of 670 bp for *pgmG* gene was detected in the PCR product of the seventeen fusants (F1–F17) (Fig. 2a) as well as in the three fusants (F20-F22) and their parents*; S.p.*Q6 and *S.p.*G1 (Fig.3a). Two genes, *rmlA* and *pgmG* were cloned based on the PCR amplification of a DNA fragment.

The gene (*rmlA*) is first gene of the *S. p* ATCC 31461 of four- *rml*A gene cluster; *rmlA, rmlB, rmlC* and *rmlD* present in the 18-gene (in four clusters) required for gellan biosynthesis (synthesis of L-rhamnose). Based on sequence homology, the putative *rml* operon is presumably involved in the biosynthesis of dTD Prhamnose, the sugar necessary for the incorporation of rhamnose in the gellan repeating unit· **[20].** To identify the *rmlA* gene from *S. paucimobilis* ATCC 31461 genomic DNA, it sequence was cloned based on the PCR amplification of a PCR product or DNA fragment of (876 bp) using degenerate primers[30]**.**

The *pgmG1* gene is the first gene of *pgmG* 4-gene another cluster; *pgmG1, pgmG2, pgmG3* and *pgmG4* required for gellan biosynthesis by *Sphingomonas paucimobilis* ATCC. It encodes a 50,059-Da polypeptide that has phosphoglucomutase (PGM) and phosphomannomutase (PMM) activities and is 37 to 59% identical to other bifunctional proteins with PGM and PMM activities from gram-negative species, including *Sphingomonas paucimobilis. PgmG* protein can convert mannose-6-phosphate into M1P in the initial steps of alginate biosynthesis and, together with other results, suggests that *PgmG* may convert glucose-6-phosphate into G1P in the gellan pathway[15]**.**

The *pgmG* gene was cloned based on the PCR amplification of a DNA fragment of 670 bp from *S. paucimobilis* ATCC 31461 genomic DNA using degenerate primers from conserved regions of the phosphohexosemutase protein sequences in databases: the synthetic oligonucleotides PGM1 (sense) (59-ACCGSCAGCCABAAYCCG-39) and PGM2 (antisense) (59- BSCGCTCATYTCGCC-39), The PCR product was resolved on 0.8 % agarose gel in 1X Tris acetate buffer. The gel was stained with ethidium bromide [31,16]**.**

On the other hand a fragment of the expected size of 897 bp for *rmlA* gene was detected in the PCR product of the seventeen fusants (F1–F17) and their parents; *S.p.* ATCC 31461 and *S.p.*G1 (Fig. 2b) as well as in the three fusants (F20-F22) and their parents*; S.p.*Q6 and *S.p.*G1 (Fig. 3b).

.

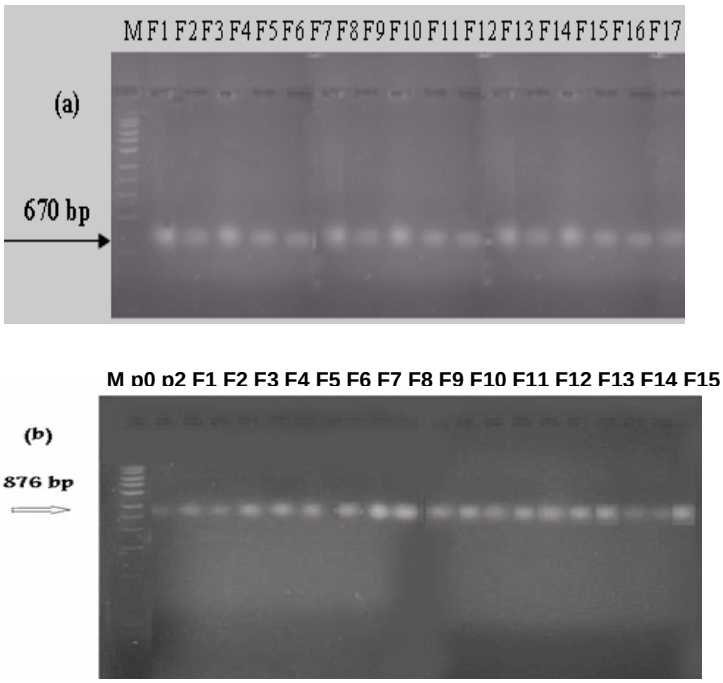

Fig. (2): Agarose gel electrophoresis of PCR products representing (a) *pgmG* gene and (b) *rmlA* gene, amplified from genomic DNA of the seventeen fusants and their parents (*S.p.* ATCC 31461 and *S.p.*G1) with specific primers, showed fragments of the expected size; 670 bp for *pgmG* gene and 876 bp for *rmlA* gene**.**

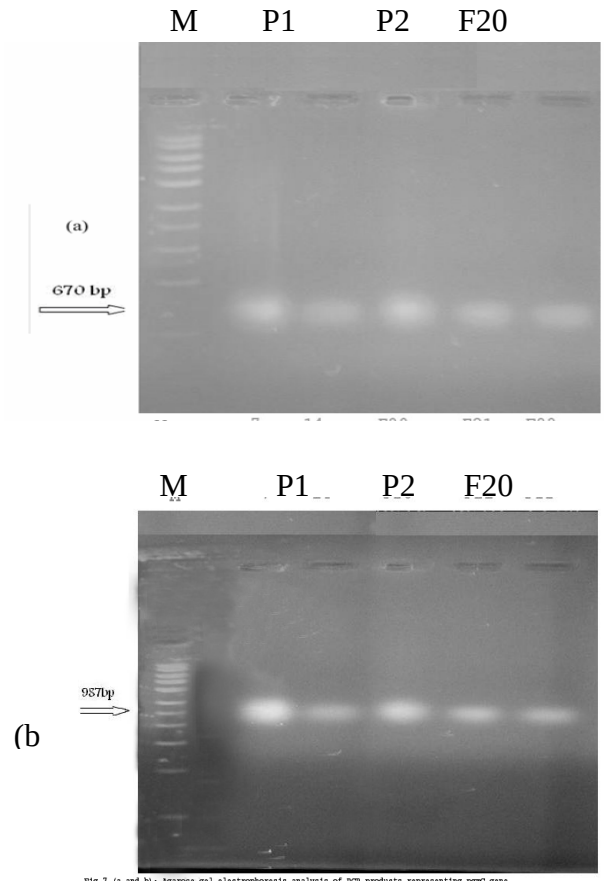

Fig.(3): Agarose gel electrophoresis of PCR products representing (a) *pgmG* gene and (b) *rmlA* gene, amplified from genomic DNA of the three fusants and their parents (*S.p.*Q6 and *S.p.*G1) with specific primers, showed fragments of the expected size; 670 bp for *pgmG* gene and 876 bp gene.

2.2- SDS-Polyacrylamid gel electrophoresis banding pattern.

SDS-PAGE protein banding patterns of the parents *S. p.* ATCC 31461:: *S.p.*G1 and their 17 fusants are illustrated in Fig. (4) and table (7). The maximum number of bands in this pattern was 17 bands for F8. The minimum number of bands were 10 bands for three fusants, F6, F16 and F17. No specific bands were found. The molecular weight for these bands ranged from 8.7 to105 kDs**.**

Analysis of these banding patterns showed 11 common bands present in all of the nineteen strains (two parents and seventeen fusants) at the molecular weights; 94, 82, 67, 55, 50, 40, 5, 27, 23, 12 and 8 kDs.

On the other hand, there were few observable differences in the protein banding pattern among all tested strains. Fusants F6, F9, F10, F14, F15, F16 and F17 have 11 bands. Fusants F1 and F4 have 12 bands; Fusants F2, F3, F11, F12 and parental strain *S.p.*G1 have 13 bands. Fusants F5, F7 and F13 have 15 bands. The parental strain *S.p.* ATCC31461 has 14 bands. Fusants F7 and F13 have 15 bands also, fusant F8 has 17 bands. These differences could be attributed to the differences in the new genetic recombination that occur in every diploid fusion before regeneration.

Results showed in Fig. (4) revealed that two common bands with specific importance were observed at two molecular weights; 50 and 31 kDa. These molecular weights refer to the products of the two genes *pgmG* and *rmlA*, respectively. These genes are known to be involved in the biosynthetic pathways of gellan gum production. These results confirm the presence and expression of each of the *pgmG and rmlA* genes in the seventeen fusants and their parents.

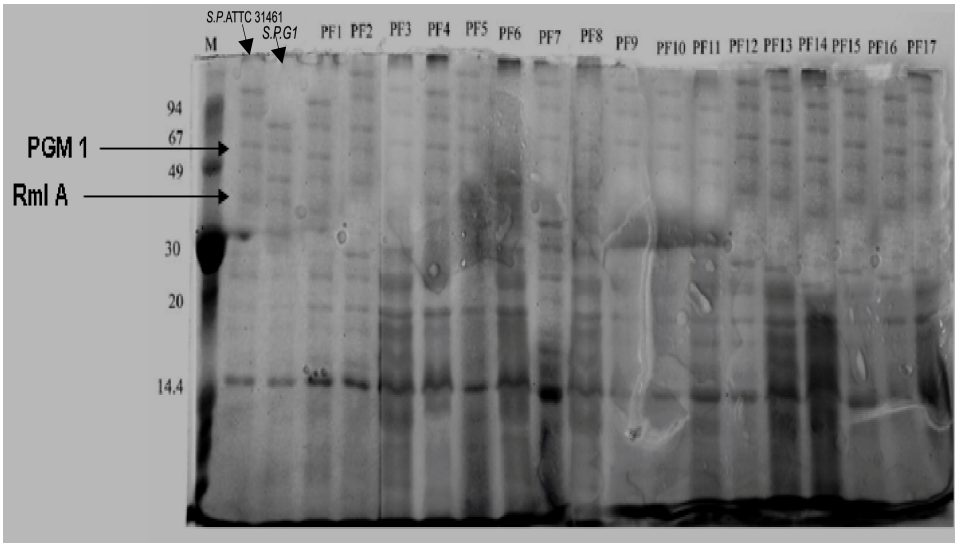

Fig. (4): SDS-PAGE protein banding patterns of the parents of the first protoplast fusion *S. p.* ATCC 31461:: *S.p.*G1 and its 17 fusants.

Table (7): Summary of densitometric analysis for total protein profiles of *S. p.* ATCC 31461:: *S.p.*G1 and its seventeen fusants.

Band No	Strains																		
	S.P. IS	*S.P.* G1	F 1	F 2	F 3	F 4	F 5	F 6	F 7	F 8	F 9	F 10	F 11	F 12	F 13	F 14	F 15	F 16	F 17
1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
2	1	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0	0	1
3	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	0	1	1	0
4	1	0	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
5	0	1	1	1	1	1	1	0	0	0	0	0	1	0	0	1	0	0	0
6	0	0	0	0	0	0	1	1	0	0	0	0	0	1	1	0	1	0	1
7	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	0	1	0
8	1	1	0	1	1	0	1	0	1	0	0	0	1	1	0	1	0	0	0
9	0	0	1	0	0	1	0	0	0	1	1	1	0	0	1	0	1	0	1
10	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0
11	1	1	1	0	0	1	1	0	1	1	0	0	1	1	1	1	1	1	1
12	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	0	0	1
13	0	1	1	1	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0
14	1	1	1	1	0	1	1	0	1	0	0	0	1	1	1	0	1	0	0
15	0	0	0	0	1	0	0	0	1	1	1	1	1	1	1	1	0	1	1
16	1	1	1	0	0	0	0	1	1	1	0	0	0	0	0	0	1	0	1
17	0	0	0	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	0
18	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
19	1	1	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0
20	0	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	0	0	0
21	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	0	0	1
22	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	1	1	0
23	0	1	0	0	1	1	0	1	0	0	0	0	0	0	1	0	0	0	0
24	1	0	0	0	0	0	1	1	1	1	0	0	1	1	0	0	1	1	0
25	0	0	0	0	1	0	1	0	0	1	0	0	0	0	1	0	0	0	0
26	0	0	0	0	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0
27	1	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0
28	0	0	0	0	1	0	1	1	0	1	0	0	0	0	1	0	0	0	0

SDS-PAGE protein banding patterns of the parents of the second protoplast fusion *S.p.*Q6:: *S.p.*G1 and its three fusants are illustrated in Fig. (5) And table (8). The maximum number of bands in this pattern was 19 bands for F20 and F21. The minimum number of bands was 12 bands for the parent *S.p.*Q6. No specific bands were found. The molecular weight for these bands ranged from 14.4 to 95 kDs.

Analysis of these banding patterns showed few observable differences among all the tested strains. Fusants F20 and F21 have 19 bands, while the fusants F22 has 14 bands. The first parent *S.p.*Q6 showed 12 bands, while the second parent *S.p.*G1 showed 13 bands.

It is also observed that the two bands with approximate molecular weights of 50 and 31 kDa appeared in the five protein banding patterns. These results confirm the presence and expression of each of the *pgmG and rmlA* genes in the five tested strains.

Table (8): Summary of densitometry analysis for total protein profiles of *S. p.*Q6 :: *S.p.*G1 and its three fusants.

Band No	Strains				
	*S.P.*Q6	*S.P.*G1	F20	F 21	F 22
1	0	0	1	1	0
2	1	0	0	0	0
3	0	0	0	1	1
4	1	0	1	0	0
5	0	1	1	1	1
6	0	0	1	1	0
7	0	0	1	0	0
8	1	1	0	1	1
9	0	0	1	0	0
10	1	1	1	1	1
11	0	1	1	0	0
12	1	1	1	1	0
13	0	1	1	1	1
14	1	1	1	1	0
15	0	0	0	0	1
16	1	1	1	0	0
17	0	0	0	1	1
18	1	1	1	1	1
19	1	1	0	1	1
20	0	1	1	1	0
21	1	1	1	1	1
22	0	0	0	0	0
23	0	1	0	0	1
24	1	0	1	1	0
25	0	0	1	1	1
26	0	0	1	1	1
27	1	0	1	1	0
28	0	0	0	1	1

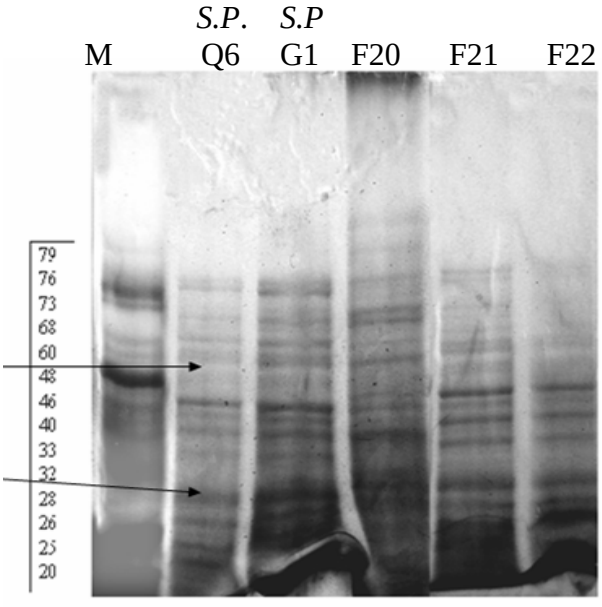

Fig. (5): SDS-PAGE protein banding patterns of the parents of the second protoplast fusion *S. p.* Q6 :: *S.p.*G1 and its three fusants.

2.3- Determination of the polysaccharide by HPLC.

Gellan gum is water-soluble polysaccharide of three mono-sugars; glucose, glucuronic acid, and rhamnose at the ratio 2:1:1 [32]. The natural gum consists of repeating tetrasaccharide units composed of 1,3, B-D-glucose, 1,4,B-D-glucuronic acid, 1,4,B-D-glucose and 1,4, α-L-rhamnose[33] with glycerate and acetate substitutions [7].

HPLC chromatography technique was used to determine the ratio between the three mono-sugars of different producer strains of gellan gum. The highest gellan gum producer fusants strain of each protoplast fusion experiments (F13 and F22) was chosen in addition to the industrial strain *S. p.* ATCC 31461.

A polysaccharide could be de-polymerized by various procedures to break linkages. This is usually performed by different concentrations of acids. The linkage of (1--6) are more resistant to mild acid hydrolysis than (1-4) linkage. Also, uronic acid reside are not readily hydrolyzed from the chain to which they are glycosidically bond. The stability of the glycosidic bond to acid hydrolysis leads to extensive destruction of the uronic and other sugar units, if forced hydrolysis attempts of the polysaccharide are carried out**.** [34,35]**.**

HPLC technique followed by complete acid hydrolysis (using formic acid) for three gellan gum producer strains were done to determine the molar ratio of gellan gum monosugars. Data presented in table (9) and Fig (6) revealed that the molar ratio of gellan gum monosugars; glucose, glucuronic acid, and rhamnose of the extracted gellan gum from three producer strains. This ratio, nearly, was 2:1:1 for the all extracted gum from the three chosen producer strains; *S. p.* ATCC 31461, F13 and F22. This result is in agreement with the obtained data from (Pollock *et al*., 1993 and Ashtputre and Shah ,1995)[36,37]. They reported that the gellan gun consists of three monosugars; glucose, glucuronic acid, and rhamnose at ratio 2:1:1.

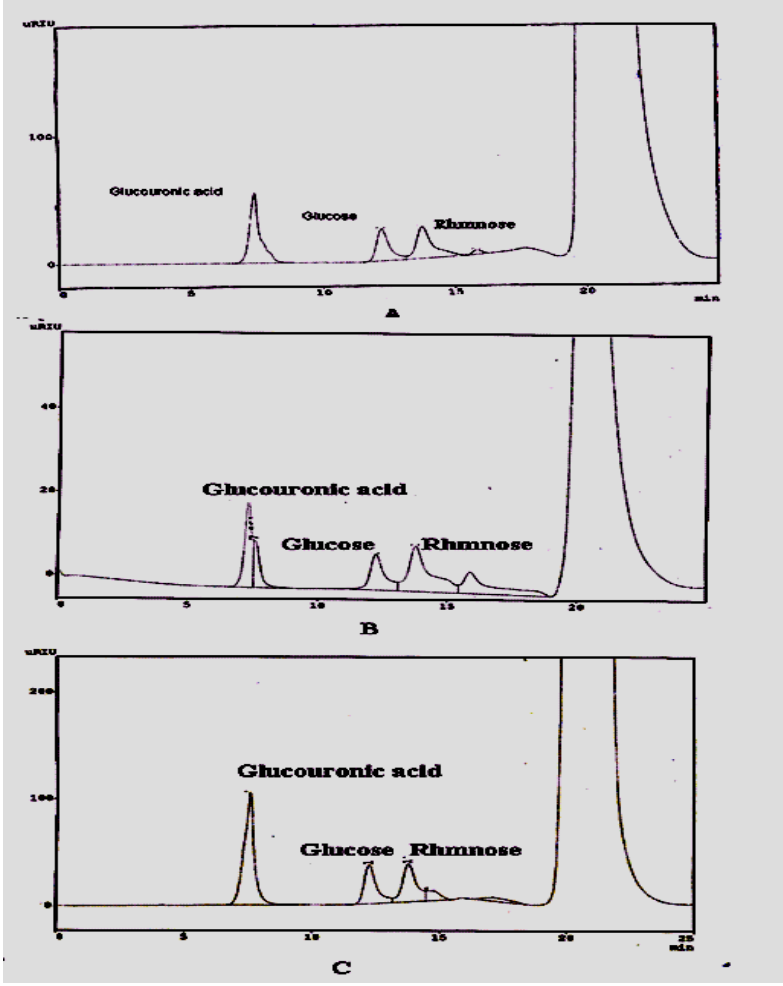

Fig. (6): Three histograms; a, b and c for three strains; P0, F13 and F22, respectively. Each histogram showed three peaks of the three monosugars; glucouronic acid, glucose and rhamnose.

Table (9): Molar ratio and yield of exopolysaccharides for three chosen gellan gum producer strains

Strain	Monosugars	Monosugar (µg/ml)	Molar ratio* (MR)	EPS g/l
S.p. ATCC 31461	Glucouronic aid	1.27	1	6.07
	Glucose	2.15	2	
	Rhmnose	1.17	1	
F13	Glucouronic aid	0.050	1	16.82
	Glucose	0.091	2	
	Rhmnose	0.061	1	
F22	Glucouronic aid	0.153	1	17.16
	Glucose	0.324	2	
	Rhmnose	0.144	1	

* The ratio between the three monosugars.

References

1- Yabuuchi, E., Yano,I., Oyaizu,H., Hashimoto,Y., Ezaki,T. and Yamamoto, H.(1990). Proposals of *Sphingomonas paucimobilis* gen. nov. and comb. nov., *Sphingomonas parapaucimobilis* sp. nov., *Sphingomonas yanoikuyae* sp. nov., *Sphingomonas adhaesiva* sp. nov., *Sphingomonas capsulate* comb. nov, and two genospecies of the genus *Sphingomonas*. Microbiol. Immunol. 34:99–119.

2- Shin, S., C., Ahn D. H. , Lee, J. K., Kim, S. J., Hong, S. G., Kim, E. H. and Parka, P. (2012). Genome sequence of *Sphingomonas* sp. strain PAMC 26605, isolated from arctic lichen (*Ochrolechia* sp.). Journal of Bacteriology p. 1607.

3- Hashimoto, W., and Murata, K. (1998). Alpha-L-rhamnosidase of *Sphingomonas sp. R1* producing an unusual exopolysaccharide of sphingan Biosci. Biotechnol. Biochem. 62:1068–1074.

4- Anson, A., Fisher,P. J. A., Kennedy,F. D. and Sutherland, I. W. (1987).A bacterium yielding polysaccharide with unusual properties.J.Appl. Bacteriol. 62:147-150

5- Banik, R. M. and Santhiagu, A. (2006).Improvement in production and quality of gellan gum by Sphingomonas paucimobilis under high dissolved oxygen tension levels. Biotechnol Lett 28:1347–1350.

6- Fialho, A. M., Marttins,L. O., Donval,M. L., Leitao,J. H., Ridout,M. J., Jay,A. J., Morris, V. J. and Sa-Correia, I. (1999). Structure and properties of gellan polymers produced by *Sphingomonas paucimobilis* ATCC 31461 from lactose compared with those produced from glucose and from cheese whey. Appl. Environ. Microbiol. 65:2485-2491.

7- Kuo,M. S. and Mort, A. J.(1986).Identification and location of L-glycerate an unusual acyl subsistent in gellan gum. Carb. Res. 156:173.

8- Videira, P. A., Fialho,A. M., Geremia,R. A., Breton,C. and Sa´-Correia,I. (2001). Biochemical characterization of the _-1,4-glucuronosyltransferase GelK in gellan gum producing strain *Sphingomonas paucimobilis* ATCC 31461. Biochem. J. 358:457–464.

9- Jay, A. J., Colquhoun, I. J., Ridout, M. J., Brownsey, G. J., Morris, V. V., Fialho, A. M., Leitao, J. H. and Sá-Correia, I. (1998). *Carbohydr. Polym.*35, 179–188.

10- Fialho, Arsenio M. Leonilde M. Moreira, Ana Teresa Granja, Alma O. Popescu, Karen Hoffmann, Isabel Sá-Correia (2008).Occurrence, production, and applications of gellan: current state and perspectives. Applied Microbiology and Biotechnology Volume 79, Issue 6, pp 889-900

11- Hotchkiss, R.D. and Gabor, M.H. (1980) Biparental products of bacterial protoplast fusion showing unequal parental chromosome expression. Proc Natl Acad Sci USA 77:3553–3557. doi:10.1073/pnas.77.6.3553

12- Chrungu, B., Verma, N. and Fredrich, A. (1999) Production and characterization of interspecific hybrids between brassica maurorum and crop brassicas. Theor Appl Genet 98(3–4):608–613. doi: 10.1007/s001220051111

13- Attallah,A.G. and Abd-El-Aal, SK. (2007).Interspecific protoplast fusion between Streptococcus lactis rennin producing strains. Res J Agric Biol Sci 3(6):562–576

14- El-Gaali, E., Kazuhiko,M. and Mori, N. (1995). Enhanced Nitrogen Fixation Capability of Soybean Rhizobia by Inter–and Intra–specific Cell fusion. Japanese J. of Crop Sci. 64: 273-280.

15- Harding, N. E., Patel,Y. N. and Coleman, R. J. (2004). Organization of genes required for gellan polysaccharide biosynthesis in *Sphingomonas elodea* ATCC 31461. J. Ind. Microbiol. Biotechnol. 31:70–82.

16- Videira , P. A., Cortes,L. L., Fialho,A. M. and Correia, S. A. (2000).Identification of the *pgmG* gene, encoding a bifunctional protein with phosphoglucomutase and Phosphoma-nnomutase activities, in the gellan gum-producing strain *Sphingomonas paucimobilis* ATCC 31461. Applied and Environ. Microbiol. 66 (5): 2252-2258.

17- Ibrahim, S. A., Abd-El-Aal, S. Kh., Fatma, M.I., Badawy., Attallah, A. G. and El-Sayd, M. A. (2011).Isolation and Molecular genetic Identification of some *Sphingomonas paucimobilis* local strains. Austrailan Journal of Basic and Applied science, 5(12): 2847-2854.

18- Moazed, D. and Noller, H. F.(1987). Interaction of antibiotics with functional sites in 16s ribosomal RNA. Nature 327: 389-394.

19- Hynes, M.F., Quandt, J., O'Connell,M. P. and Pohler, A (1989). Direct selection for curing and deletion of *Rhizobium* plasmids using transposons carrying the *Bacillus subtilis sac B* gene. Gene. 78: 111 - 120.

20- Hebbar, K. P., Gueniot,A., Heyraud,P., Colin-Morel, T., Heulin, B .J. and Rinaudo, M. (1992). Characterization of exopolysaccharides produced by rhizobacteria Appl Microbiol Biotechnol.38:248-253.

21- Silva, E., Marques,A. R., Fialho,A. M., Granja,A. T. Sa´-Correia, and I.(2005).Proteins encoded by *Sphingomonas elodea* ATCC 31461 *rmlA* and *ugpG* genes, Involved in gellan gum biosynthesis, exhibit both dTDP- and UDP-Glucose pyrophosphorylase activities. Appl. and Environ. Microbiol. 71 (8): 4703-4712.

22- Laemmli,U.K.(1970).Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227:680-685.

23- Sambrook, J., Fritsch,E. F. and Maniatis, T.(1989).Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y

24- Evince, L. R. and Linker, A. (1973). Production and characterization of the slime polysaccharide of *Pseudomonas aeruginosa*. J. of Bacteriol., 116: 915-924.

25- Hopwood, D. A. (1981). Genetic studies with bacterial protoplasts. Ann. Rev. Microbiol. 25: 237-272.

26- Piagac,J., Hranueli,D., Smokvina,T. and Alacevic, M.(1981).Optimal cultural and physiological conditions for handling *Streptomyces rimosus* protoplast. Appl. Environ. Microbiol. 44 : 1178-1186.

27- Papabianni, M., Psomas,S. L., Batsilas,S. V., Paras, D.A.and Liakopoulou, K.M.(2001). Xanthan production by *Xanthomonas campestris* in batch cultures..proccess- Biochem. 37 (1):73-80.

28- Li. Y., J. Tang, J. Feng, D. Cha, Q. Ma, Y. Li, J. Tang, J. Feng, D. Cha, Q. S, Lima (1999). Sequencing analysis of "1.9" kb EcoRI DNA fragment related to biosynthesis of xanthan gum. J. of Guangxi Agric. and Biol. Sci., 18 (1):6-9.

29- Willlimas, J. G. K., Kubelik,A. R., Rafalski,J. A. and Tingey, S.V.(1990).DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nuc. Aci. Res. 18: 6531- 6535.

30- Marques,A. R.,Ferreira,P. B.,Sa´-Correia,I. and Fialho,A. M.(2003).Characterization of the *ugpG* gene encoding a UDP-glucose pyrophosphorylase from the gellan gum producer *Sphingomonas paucimobilis* ATCC 31461. Mol. Genet. Genomics 268:816–824.

31- Paula, A., Videira,L. L., Cortes,A. M., Fialho,M. and Isabel, S. (2000).Identification of the *pgmG* gene, encoding a bifunctional protein with phosphoglucomutase and Phosphoma-nnomutase activities, in the gellan gum-producing strain *Sphingomonas paucimobilis* ATCC 31461. Applied and Environ. Microbiol. 66 (5): 2252-2258.

32- Ashtaputre, A. and Shah, A. (1995).Studies on a viscous, gel-forming exopolysaccharide from *Sphungomonas paucimobilis* GS1. Appl Environ Microbiol. 61:1159-1162.

33- Jansson,F. E. and Lindberg, B.(1983).Structure studies on gellan gum and extracellular polysaccharide elaborated by *Pseudomonas elodea*. Carb. Res. 124-135.

34- Thorne, L., Mikolajczak, M. J., Armentrout, R. W.and Pollock, T.J. (2000). Increasing the yield and viscosity of exopolysaccharides secreted augmentation of chromosomal genes with multiple copies of cloned biosynthetic genes. J Ind Microbiol Biotechnol 25:49–57.

35- Giavasis, I., Harvey, L. M. and McNeil, B. (2006). The effect of agitation and aeration on the synthesis and molecular weight of gellan in batch cultures of Sphingomonas paucimobilis. Enzyme Microb Technol 38:101–108

36- Pollock, T. J. (1993).Gellan-related polysaccharides and the genus *Sphingomonas*. J. Gen. Microbiol. 139:1939–1945.

37- Ashtaputre, A.A., Shah, A.K. (1995): Studies on a viscous, gel-formingexopolysaccharide from Sphingomonas paucimobilis GS1.Appl. Environ. Microbiol. 61, 1159–1161.

*****