Al-Azhar J. Agric. Sci. Sector Res.,
Vol. 9: pp 15-37 (December) 2010

PRODUCTION OF SULFATED POLYSACCHARIDE FROM *ARTHROBACTER* FUSANTS AS ANTIVIRAL AND ANTIOXIDANT

Sayed, A. I. H. [1]; EL-Morsy, S. I. [1]; Abd-El-Aal, S. K., [2]; Asker, M. M. S. [3]; El-Shaer, H. F. A. [1]

(1) Genetics, Dept. of Agric., Botany Fac. of Agric. Al-Azhar University, Cairo
(2) Microbial Genetics Dept., National Research Center, Giza, EGYPT
(3) Microbial Biotechnology Dept., National Research Center, Giza, EGYPT

ABSTRACT: Six of higher sulfated polysaccharide producers *Arthrobacter* strains were used in this work, One identified strain, *Arthrobacter viscous* ATC 19584 and five local strains; *Arthrobacter* sp1, *Arthrobacter* sp2, *Arthrobacter viscous* G119, *Arthrobacter* sp1 SA30 and *Arthrobacter* sp1 G140. These strains were used as parents to test and improve their potentialities as sulfated polysaccharide producers. Five experiments of protoplast fusion were done between these six parents. Twenty two genetically sTable fusants were obtained after one hour incubation period. They were higher sulfate polysaccharide production than their parents. The fusants number of each trial was; 8, 3, 4, 4 and 3 fusants for the first, second, third, fourth and fifth protoplast trials; respectively. The first *Arthrobacter viscous* ATCC 19584 (P1) x *Arthrobacter* sp1 SA30 (P5) trial was the best where all obtained fusants showed higher sulfate polysaccharide production ranged from 2 to 3 times than the average of their two parents. All the twenty two obtained recombinants (fusants) were tested for the SDS-PAGE protein banding patterns and compared those of their parental strains. Biological measurements such as, antiviral and antioxidant effect of sulfated polysaccharide were studied.

INTRODUCTION

Coryneform bacteria (species of *Corynebacterium, Brevibacterium* and *Arthrobacter)* have been used extensively in the fermentation industry for production of amino acids, enzymes and steroid drugs (Show and Hartley, 1988). Traditionally, strain improvement of these industrial microorganisms aimed to increasing their products yield has involved intensive programs of mutagenesis, followed by selection of strains with new characteristics (Kikuchi, 1980).

Protoplast fusion is a useful technique for obtaining hybrids or recombinants of different microorganisms. It has proved to be a valuable tool for improving industrial microorganisms with dominant

characteristics such as increasing product yield (Fukaya, 1989).

Lopalco *et al.* (1994) suggested that sulfated polysaccharides are potent and selective *in vitro* inhibitors of human immunodeficiency virus type 1 (HIV-1); however, their therapeutic application is limited by their anticoagulant activity. The most pronounced anti-HIV activity was observed with RO-H, Suc30-H (standard heparin, 30% succinylated), and Suc100-LMW-H (low molecular weight heparin, 100% succinylated); the latter retained only 5% of the anticoagulant activity of standard heparin, whereas RO-H and Suc30-H retained approximately 35% of the anticoagulant activity of standard heparin. Huheihel *et al.* (2002) reported that the cell wall-sulfated polysaccharide exhibited impressive antiviral activity against herpes simplex type 1 and 2 (HSV-1 and HSV-2).

Asker *et al.*, (2009) was identified that the gram-positive strain isolated from soil as non-pathogenic *Microbacterium terregens*. The exopolysaccharide (CPS) produced from *M. terregens* was evaluated antioxidant activity *in-vitro* by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay (RSA). The effect of molecular weight of the polysaccharide on the improvement of the antioxidant potential seems to be significant.

The main objective of this work is proving the *Arthrobacter* strains to producing more of sulfated polysaccharide with highly rate and a good quality. Some biological measurements; antiviral activity and antioxidations; were studied also through the protoplast fusion technique.

MATERIALS AND METHODS

1- Microbial strains:-

1.1- Bacterial strains:

Six of higher sulfated polysaccharide producers *Arthrobacter* strains were used in this work. One industrial strain, *Arthrobacter viscous* ATC 19584 and five local strains; *Arthrobacter* sp1, *Arthrobacter* sp2, *Arthrobacter viscous* G119, *Arthrobacter* sp1 SA30 and *Arthrobacter* sp1 G140 all strains were kindly obtained from A. I. H. sayed Prof. of Genetics, Department of Agriculture Botany Faculty of Agriculture, Cairo Al-Azhar University.

1.2- Viruses strain for antiviral activity:

Infectious Bovine Rhinotrahcities (IBR) Bovine Herpes virus laborations spain and Parainfluenza.3 (PI3) virus, SF4 supplied by HIPRA. Were used as a virus model for testing the possible antiviral

activity of sulfated polysaccharide extracts these viruses were taken from M. H. Abd-ELbaky prof. head of central laboratory for evaluation of veterinang biologics.

2- Media:

2.1- Production of sulfated polysaccharide medium (MI) (Linker and jones, 1966)

2.2- Yeast Malt Extract Medium (YM) (Roseiro *et al.,* 1992).

2.3- Luria broth Medium (LB) (Davis *et al*., 1980).

3- Sodium Dodecylsulphate (SDS-PAGE) methods:

All buffers and solutions were utilized for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) studies, according to Sheri *et. al.* (2000).

4- Antibiotics:

Seven antibiotics were used in this study, their sources and concentrations are listed in Table (1).

Table (1): Sources and concentrations of antibiotics

No	Antibiotic	Conc.(μg/ml)	Sources
1	Chloramphinocol	20	Applichem (Germany)
2	Streptomycin	100	Bioshop (Canada)
3	Ampicillin	300	Bioshop (Canada)
4	Rifampicin	25	Bioshop (Canada)
5	Kanamycine	10	Applichem (Germany)
6	Tetracycline	2	Applichem (Germany)
7	Neomycin	4	Applichem (Germany)

5- Isolation and determination of strains exopolysaccharide and sulfate groups: were detected by using methods of Cheng *et al.,* (2009).

6- Protoplast material and method: were according to (Liu *et al*., 1996)

7- Statistical Analysis:

Data were scored on bases of the presence or absence of bands by using (SPSS) computer program and the phylogenetic relationships were calculated.

8- Biological measurements (Bioeffect):

7.1- Radical scavenging activity (RSA) of sulfated polysaccharide (Antioxidations) was under taken as Shimada *et al.* (1992) method.

7.2- Evalution of the cytotoxic effect and evalution of *in-vitro*

antiviral effect were following as Cox *et al,* (1996) role.

RESULTS AND DISCUSSION

The present study was planned to improve the efficiency of *Arthrobacter* for producing sulfate polysaccharide by genetic approach as protoplast fusion technique.

Exopolysaccharide and sulfate groups production from *Arthrobacter* strains.

In the present work, exopolysaccharide (EPS) and sulfate groups production from *Arthrobacter* were estimated for the six strains; *Arthrobacter viscous* ATC 19584 (P1), *Arthrobacter* sp1 (P2), *Arthrobacter* sp2 (P3), *Arthrobacter viscous* G119 (P4), *Arthrobacter* sp1 SA30 (P5) and *Arthrobacter* sp1 G140 (P6). The results are presented in Table (2).

Results in Table (2) clearly showed a great variation of both total exopolysaccharide and sulfated groups concentration in all used strains. The maximum values were obtained by the local strain (P4), where sulfate groups were 7.838 mg/ml and polysaccharide was 26.5 g/L. On the contrary, the minimum value was obtained by the local strain (P3) (sulfate groups 2.413 mg/ml and polysaccharide 5.7 g/L). Production of total exopolysaccharide and sulfated groups concentration of the rest strains were; (P1) 7.1 g/L and 3.701 mg/ml, (P2) 8.2 g/L and 3.427 mg/ml, (P5) 8.7g/L and 5.756 mg/ml and (P6) 27 g/L and 2.687 mg/ml.

Antibiotic resistance patterns of *Arthrobacter* strains.

Antibiotic resistance test was done using seven of the more frequently applied antibiotics (Table 1). The *Arthrobacter* local sulfate exopolysaccharide producer strains and the industrial strain (P1) were tested for their resistance to these seven antibiotics.

The industrial strain (P1) showed resistance to three antibiotics (chloramphenicol, ampicilline and rifampicin) and sensitivity to the others. The same results were obtained with two local strains; (P2) and (P6). The other three local *Arthrobacter* strains revealed different patterns. Two local strains, (P3) and (P5), showed resistance to all the antibiotics except two antibiotics. Tetracycline (Tc) and neomycin (Nm) for (P3). Tetracycline (Tc) and rifampicin (Rf) for (P5). The sixth strain (P4) showed resistance to three antibiotics; chloramphenicol (Cm), ampicilline (Am) and kanamycin (Km) and sensitivity to the others. Brief

of results are presented in Table (2).

In order to determine the efficiency of protoplast induction and number of regenerated protoplasts for each parental strain (Liu *et al.* 1996).

Table (2): Production of total exopolysaccharide, sulfated groups concentration and antibiotic resistance test of the six *Arthrobacter* strains.

Strains	Genetic markers	Mean of (EPS) g/L	Mean of sulfate (mg/ml)	Code
Arthrobacter viscous ATCC 19584	Sm^s kn^s Nm^s Rf^r	7.1	3.701	P1
Arthrobacter sp1	Sm^s Rf^r	8.2	3.427	P2
Arthrobacter sp2	Nm^s Rf^r	5.7	2.413	P3
Arthrobacter viscous G119	Kn^r Rf^s	26.5	7.838	P4
Arthrobacter sp1 SA30	Sm^r Kn^r Nm^r Rf^s	8.7	5.756	P5
Arthrobacter sp1 G140	kn^s Rf^r	27	2.687	P6

r- Resistance s- Sensitive

Two equal samples of protoplast suspension were used. The first sample was spread on to YM medium after osmotic shock to allow the somatically sTable, non-protoplasted cells which were not converted to protoplast 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 untreated cells. 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. Data obtained were illustrated in Table (3), the protoplast percentage and it's regenerated/ml was calculated.

Table (3): Protoplast percentage and regeneration / ml for the six *Arthrobacter strains.*

Strains	Total count / ml	No. of protoplasts / ml	Protoplast %	No. of regenerated cells / ml
P1	263.66 x10^{6}	115.66 x10^{6}	43.86%	٢٦٠,٣٣ x10^{6}
P2	٣٠٠,٠٠ x10^{6}	140.66 x10^{6}	46.88%	٢٢٧,٠٠ x10^{6}
P3	٢٣٦,٦٦ x10^{6}	148.00 x10^{6}	62.53%	١٤٨,٦٦ x10^{6}
P4	٢٦٥,٠٠ x10^{6}	149.00 x10^{6}	56.35%	٢٠٠,٠٠ x10^{6}
P5	٣٢٦,٣٣ x10^{6}	193.00 x10^{6}	59.14%	٣٠٥,٦٦ x10^{6}
P6	٣٤٩,٦٦ x10^{6}	185.00 x10^{6}	52.29%	٢٢٦,٦٦ x10^{6}

Results in Table (3) showed that the protoplast percentages were 43.86%, 46.88%, 62.53%, 56.35%, 59.14% and 52.29% for the six examined strains (P1), (P2), (P3), (P4), (P5) and (P6); respectively. These results are consistent with those obtained by Piagac *et al.* (1981) who got frequencies of protoplasting range from 56 to 75% depending upon the genome of mutants organized from a slant of *Streptomyces rimosus.*

Protoplast fusion experiments

Protoplast was prepared from the six parents; P1, P2, P3, P4, P5 and P6. They are different in their sulfate exopolysaccharide productivity and also in their antibiotic markers (Table3). Five attempts to obtain *Arthrobacter* fusants were carried out using protoplast fusion technique between induced protoplasts of the parental strains for one hour incubation period as follows;

Industrial strain (P1) x local strain (P5);

Protoplasts were prepared from the parental strains; *Arthrobacter viscous* ATCC 19584 (P1) and local strain (P5). Several dilutions have been made to get equal numbers of protoplasts per volume from each strain.

Then aliquot of 0.5ml of each protoplast mixture was taken for fusion in the presence of 400% PEG 6000 (Liu *et al.* 1996). Sulfate exopolysaccharide productivity of the parental strain *Arthrobacter viscous* was (3.701 mg/l sulfate and 7.1 g/L exopolysaccharide) and it resists the antibiotic rifampicin. Another parental strains (P5) produces (5.756 mg/l and 8.7 g/L) sulfate groups and exopolysaccharide, respectively. This parental strain resists antibiotics; streptomycin (Sm), kanamycin (Km) and neomycin (Nm). Eight regenerated fusants with resistance to the four antibiotics were appeared on selective medium and tested for sulfate exopolysaccharide productivity.

Table (3): Protoplast percentage and regeneration / ml for the six *Arthrobacter strains.*

Strains	Total count / ml	No. of protoplasts / ml	Protoplast %	No. of regenerated cells / ml
P1	263.66 $x10^6$	115.66 $x10^6$	43.86%	٢٦٠,٣٣ $x10^6$
P2	٣٠٠,٠٠ $x10^6$	140.66 $x10^6$	46.88%	٢٢٧,٠٠ $x10^6$
P3	٢٣٦,٦٦ $x10^6$	148.00 $x10^6$	62.53%	١٤٨,٦٦ $x10^6$
P4	٢٦٥,٠٠ $x10^6$	149.00 $x10^6$	56.35%	٢٠٠,٠٠ $x10^6$
P5	٣٢٦,٣٣ $x10^6$	193.00 $x10^6$	59.14%	٣٠٥,٦٦ $x10^6$
P6	٣٤٩,٦٦ $x10^6$	185.00 $x10^6$	52.29%	٢٢٦,٦٦ $x10^6$

Table (4) showed characters of the parental strains and their eight fusants obtained after incubation period and designated in serial numbers from F1 to F8. All of these fusants showed increase in exopolysaccharide productivity comparing to both parental strains. There production ranged from 25 g/L (fusant F2) to 14 g/L (fusant F6). The higher production (25, 19.5, 17 and 15 g/L) were obtained by the fusants F2, F1, F7, and F8; respectively.

Results in Table (4) showed that all tested fusants showed decrease in sulfate productivity comparing to both parental strains. Sulfate productivity of fusant F2 (3.775 mg/ml) and fusant F3 (3.728

mg/ml) were nearly equal the lowest parent productivity P1 (3.701 mg/ml). The other fusants produced sulfate ranged from 3.317 mg/ml for fusant F8 to 1.947 mg/ml for fusant F7.

Table (4): Exopolysaccharide (EPS), sulfate groups productivity, genetic markers of the two parental strains of the fusion P1 x P5 and eight selected fusants.

Strains	Genetic markers	(EPS) g/L	Sulfate groups (mg/ml)
P1	Sm^s kn^s Nm^s Rf^r	7.1	3.701
P5	Sm^r Kn^r Nm^r Rf^s	8.7	5.756
F1	$Sm^r Kn^r Nm^r$ Rf^r	19.5	2.943
F2	Sm^r Kn^r Nm^r Rf^r	25	3.775
F3	Sm^r Kn^r Nm^r Rf^r	15	3.728
F4	Sm^r Kn^r Nm^r Rf^r	15	2.358
F5	Sm^r Kn^r Nm^r Rf^r	15.5	2.715
F6	Kn^r Nm^r $Rf^r Sm^r$	14	2.660
F7	Sm^r Kn^r Nm^r Rf^r	17	1.947
F8	Sm^r Kn^r Nm^r Rf^r	15	3.317

SDS-PAGE protein banding patterns of the two parents (P1 x P5) and their eight fusants are illustrated in Fig (1). The maximum number of bands in this pattern was 14 bands for fusant F2. The minimum numbers of bands were 9 bands for the fusant F3. No specific bands were found. The molecular weight for these bands ranged from 4 to 212 KDs

Analysis of these banding patterns showed 7 common bands were present in all of the ten strains (two parents and eight fusants) with molecular weights; 190, 97.5, 65, 50, 30, 14 and 8 KDs.

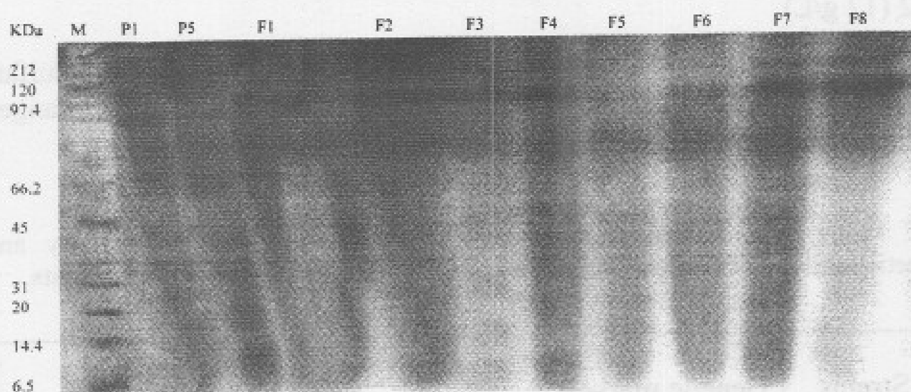

Fig. (1): SDS-PAGE protein banding patterns of the parents P1 x P5 and their eight fusants, from F1 to F8 (M= molecular weight marker).

On the other hand, there were few observable differences in the protein banding pattern among all tested strains. The parental strain A0 and Fusants F5, and F6 have 10 bands. Fusants; F1, F7 and F8 have 11 bands. At the end, the parental strain and fusant F4 have 12 bands. These differences could be attributed to the differences in the new genetic recombination that occur in every diploid fusion before regeneration.

Local strains P3 x P5:

The parental strains (P3) and local strain (P5) were used in this trial. The first parent (P3) showed sulfate exopolysaccharide productivity of (2.413 mg/ml sulfate and 5.7 g/L exopolysaccharide), resistance to rifampicin (Rf) and sensitivity to neomycin (Nm) antibiotic. While, the other parental strain (P5) showed sulfate exopolysaccharide productivity of (5.756 mg/ml sulfate and 8.7 g/L exopolysaccharide), resistance to neomycin (Nm) and sensitivity to rifampicin (Rf). Three regenerated fusants with resistance to both antibiotics (neomycin and rifampicin) were appeared on selective medium and tested for sulfate exopolysaccharide productivity.

Table (5) presents the characters of the parental strains and their three fusants. These fusants were designated F11, F12 and F13 they showed increase in exopolysaccharide productivity comparing to both parental strains. Fusant F13 has the highest efficiency of exopolysaccharide productivity (20 g/L) followed by F11 (13 g/L) and

F12 (12 g/L).

Results in Table (5) showed that all fusants produced less sulfate productivity comparing to both parental strains. This decrease ranged from 1.975 mg/ml in fusant F11 to 1.646 mg/ml for fusant F13.

Table (5): Exopolysaccharide (EPS), sulfate groups productivity and genetic markers of the two parental strains P3 x P5 and three selected fusants.

Strains	Genetic markers	(EPS) g/L	Sulfate (mg/ml)
P3	$Nm^s Rf^r$	5.7	2.413
P5	$Nm^r Rf^s$	8.7	5.756
F11	$Nm^r Rf^r$	13	1.975
F12	$Nm^r Rf^r$	12	1.728
F13	$Nm^r Rf^r$	20	1.646

SDS-PAGE protein banding patterns of the second protoplast fusion attempt between the two parents P3 x P5 and their three fusants are illustrated in Fig. (2). The maximum number of bands in this pattern was 13 bands for the parental strain P5 and the fusant F12. The minimum number of bands was 11 bands for the parent P3 and fusant F11. One specific band was found at molecular weigh 104 KDs for the parental strain P5. There were ten common bands at molecular weights; 190, 130, 113, 75, 66, 41, 29, 18, 14 and 7.5 KDs. The molecular weight for these bands ranged from 6.5 to 190 KDs.

Analysis of these banding patterns showed nearly few observable differences among all the tested strains. Each of the two fusants; F11 and F13 has 12 bands, while the fusant F12 has 13 bands. The first parent P3 showed 13 bands, while the second parent (P5) showed 11 bands.

The previous results showed few differences in protein banding patterns among all tested strains. These differences may be attributed to the differences in the new genetic recombination.

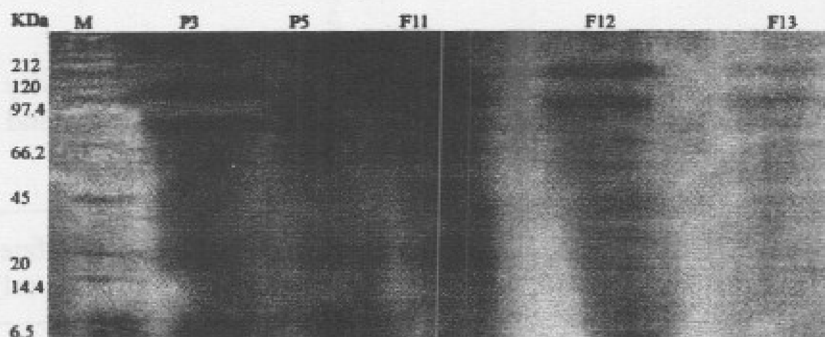

Fig. (2): SDS-PAGE protein banding patterns of the two parents P3 x P5 and their three fusants; F11, F12 and F13 (M= molecular weight marker).

Local strains P4 x P6:

The parental strains P4 and P6 were used in this trial. The first parent P4 showed sulfate exopolysaccharide productivity of (7.838 mg/ml sulfate and 26.5 g/L exopolysaccharide), resistance to kanamycin (Km) and sensitivity to rifampicin (Rf) antibiotic. While the other parental strain (P6) showed sulfate exopolysaccharide productivity of (2.687 mg/ml sulfate and 27 g/L exopolysaccharide), resistance to rifampicin (Rf) and sensitivity to kanamycin (Km). Four regenerated fusants with resistance to both antibiotics (rifampicin and kanamycin) were appeared on selective medium and tested for sulfate exopolysaccharide productivity.

Table (6) showed the characters of the parental strains and their four fusants. These fusants were designated F21, F22, F23 and F24, they showed decrease in exopolysaccharide productivity comparing to both parental strains. Fusant F23 has the lowest efficiency of exopolysaccharide productivity (21 g/L) followed by F21 (22 g/L), F22 (23.5 g/L) and F24 (25.5 g/L).

Results in Table (6) showed that all fusants produce less sulfate productivity comparing to parent strain P4. Three fusant F22, F23 and F24 were nearly equal with parent strains P6.

Table (6): Exopolysaccharide (EPS), sulfate groups productivity and genetic markers of the two parental strains P4 x P6 and four selected fusants.

Strains	Genetic markers	(EPS) g/L	Sulfate (mg/ml)
P4	$Kn^r Rf^s$	26.5	7.838
P6	$kn^s Rf^r$	27	2.687
F21	$Kn^r Rf^r$	22	1.701
F22	$Kn^r Rf^r$	23.5	2.139
F23	$Kn^r Rf^r$	21	2.249
F24	$Kn^r Rf^r$	25.5	2.715

SDS-PAGE protein banding patterns of the parents of the third protoplast fusion P4 x P6 and their four fusants are illustrated in Fig. (3). The maximum number of bands in this pattern was 17 bands for fusant F22. The minimum number of bands was 14 bands for the parental strain P6. The molecular weight for these bands ranged from 5.2 to190.3 KDs.

Analysis of these banding patterns showed 10 common bands present in all of the six strains (two parents and four fusants) at the molecular weights; 190.3, 110.9, 78.3, 67.8, 49.5, 30.2, 21.7, 15.4, 11.2 and 6.3 KDs. Five specific bands were found in five strains (one parental strain and four fusants), one specific band for each strain. The first was 72.8 for the parental strain P6, while the other four specific bands were; 18.6, 132.7, 39.1 and 23.2 KDs for the fusants; F21, F22, F23 and F24, respectively.

On the other hand, there were few observable differences in the protein banding pattern among all tested strains. Fusants F21 and F24 have 15 bands. Each of the parental strain P4 and fusant F23 has 16 bands. These differences could be attributed to the differences in the new genetic recombination that occur in every diploid fusion before regeneration.

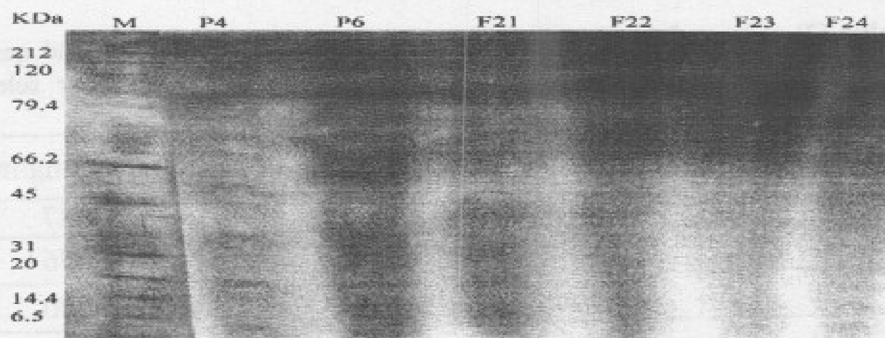

Fig. (3): SDS-PAGE protein banding patterns of the two parents P4 x P6 and their four fusants, from F21 to F24 (M= molecular weight marker).

Local strains P5 x P6:

The parental strains P5 and P6 were used in this trial. The first parent P6 showed sulfate exopolysaccharide productivity of (2.687 mg/ml sulfate and 27 g/L exopolysaccharide), resistance to rifampicin (Rf) and sensitivity to streptomycin (Sm) antibiotic. While the another parental strain P5 showed sulfate exopolysaccharide productivity of (5.756 mg/ml sulfate and 8.7 g/L exopolysaccharide), resistance to streptomycin (Sm) and sensitivity to rifampicin (Rf). Four regenerated fusants with resistance to both antibiotics (rifampicin and kanamycin) were appeared on selective medium and tested for sulfate exopolysaccharide productivity.

Data in Table (7) presents the characters of the parental strains and their four fusants. Fusants were designated F31, F32, F33 and F34, they showed decrease production exopolysaccharide productivity comparing to the parent P6 and showed increase in exopolysaccharide productivity comparing to the other parental strain lowest productivity P5. The polysaccharide production (14.5, 17.5, 21.5 and 24.5 g/L) were obtained by the fusants F31, F32, F33 and F30; respectively.
Results in Table (7) showed that all fusants produced less sulfate

productivity comparing to both parental strains. Only two fusants, F30 and F31 have sulfate productivity nearly equal the parent (P6).

Table (7): Exopolysaccharide (EPS), sulfate groups productivity and genetic markers of the two parental strains P6 x P5 and four selected fusants.

Strains	Genetic markers	(EPS) g/L	Sulfate (mg/ml)
P6	Sm^s Rf^r	27	2.687
P5	Sm^r Rf^s	8.7	5.756
F30	Sm^r Rf^r	24.5	2.720
F31	Sm^r Rf^r	14.5	2.578
F32	Sm^r Rf^r	21.5	2.419
F33	Sm^r Rf^r	17.5	1.536

SDS-PAGE protein banding patterns of the parents of the fourth protoplast fusion between, P6 x P5 and their four fusants are illustrated in Fig. (4). The maximum number of bands in this pattern was 14 bands for F31. The minimum numbers of bands were 10 bands for the parental strain P6 and two fusants; F30 and F33. The molecular weight for these bands ranged from 6.7 to 212 KDs.

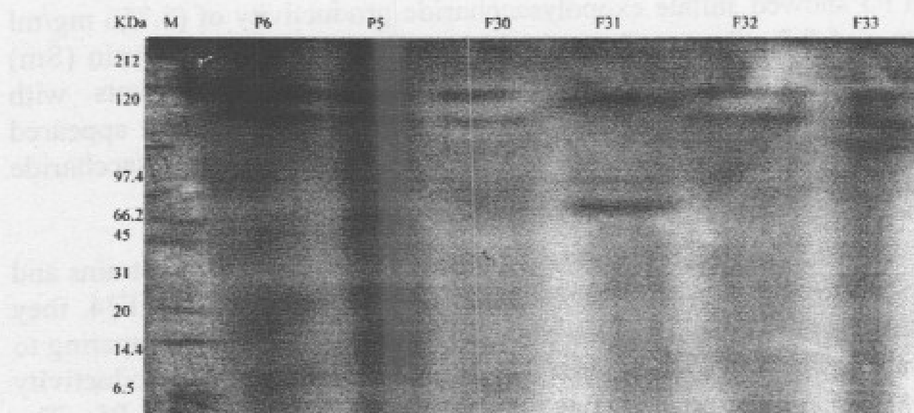

Fig. (4): SDS-PAGE protein banding patterns of the two parents P6 x P5 and their four fusants; F30, F31, F32 and F33 (M= molecular weight marker).

Analysis of these banding patterns showed 8 common bands present in all of the six strains (two parents and four fusants) at the molecular weights; 212, 185.9, 111.5, 79.2, 39, 21.4, 15.8 and 7 KDs.

One specific band was found to be 122.2 KDs. Of the fusant F33 and two specific bands were found; 58 and 74.8 KDs for fusant F31. The parental strain P5 and fusant F32 have 13 bands.

On the other hand, there were few observable differences in the protein banding pattern among all tested strains. These differences could be attributed to the differences in the new genetic recombination that occur in every diploid fusion before regeneration.

Local strains P2 x P5:

The parental strains (P2) and (P5) were used in this trial. The first parent (P2) showed sulfate exopolysaccharide productivity of 3.482 mg/ml and 8.2 g/L exopolysaccharide, resistance to rifampicin (Rf) and sensitivity to streptomycin (Sm) antibiotics. While the other parental strain (P5) showed sulfate exopolysaccharide productivity of 5.756 mg/ml and 8.7 g/L exopolysaccharide), resistance to streptomycin (Sm) and sensitivity to rifampicin (Rf). Three regenerated fusants with resistance to both antibiotics (streptomycin and rifampicin) were appeared on selective medium and tested for sulfate exopolysaccharide productivity.

Data in Table (8) presented the characters of the parental strains and their three fusants. Fusants were designated F41, F42, and F43, they showed increase in exopolysaccharide productivity comparing to both parental strains. Fusant F42 has the highest efficiency of exopolysaccharide productivity (25.5 g/L) followed by F43 (22.5 g/L) and F41 (19.5 g/L).

Results in Table (8) showed that all these fusants gave a different sulfate productivity comparing to both parental strains. Fusant F41 was the highest efficiency of sulfate productivity (7.7564 mg/ml) followed by F42 (3.263 mg/ml) and F43 (1.783 mg/ml).

SDS-PAGE protein banding patterns of the parents of the fifth protoplast fusion attempt P2 x P5 and their three fusants are illustrated in Fig (5). The maximum number of bands in this pattern was 16 bands for the parental strain P5. The minimum number of bands was 12 bands for the parental strain P2 and fusant F43. The molecular weight for these bands ranged from 5.7 to 210 KDs.

Table (8): Exopolysaccharide (EPS), sulfate groups productivity and genetic markers of the two parental strains P2 x P5 and three selected fusants.

Strains	Genetic markers	(EPS) g/L	Sulfate (mg/ml)
P2	$Sm^s\ Rf^r$	8.2	3.482
P5	$Sm^r\ Rf^s$	8.7	5.756
F41	$Sm^r\ Rf^r$	19.5	7.564
F42	$Sm^r\ Rf^r$	25.5	3.263
F43	$Sm^r\ Rf^r$	22.5	1.783

Analysis of these banding patterns showed 9 common bands present in all of the five strains (two parents and three fusants) at the molecular weights; 140.6, 115.4, 90.9, 61.7, 40.2, 31.3, 22.5, 13.8 and 5.7 KDs. Also, the analysis of these banding patterns showed one specific band in all five strains. The parental strain P2 has one specific band at molecular weigh 86.7 KDs. The other parental strain P5 has one specific band at molecular weigh 125.6 KDs. Each of the three fusants; F41, F42 and F43 has one specific band at molecular weigh; 16, 7.9 and 34.4 KDs., respectively. Each of the two fusants F41 and F42 has 13 and 14 bands; respectively.

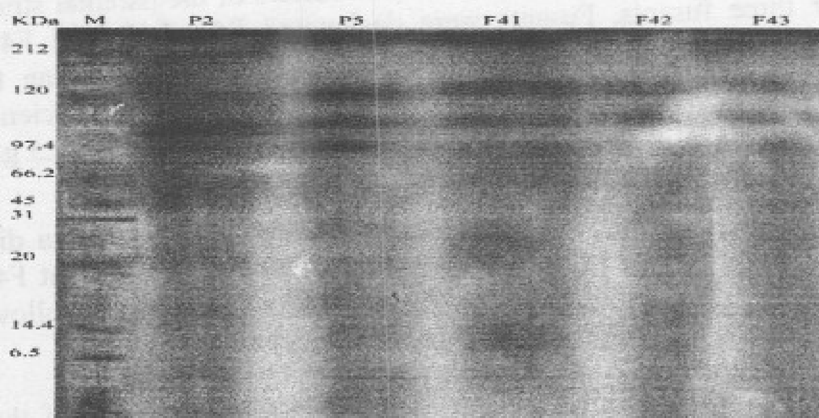

Fig. (5): SDS-PAGE protein banding patterns of the two parents P2 x P5 and their three fusants, F41, F42 and F43 (M= molecular weight marker).

There were few observable differences in the protein banding pattern among all tested strains. These differences could be attributed to the differences in the new genetic recombination that occur in every diploid fusion before regeneration.

In conclusion, in this study twenty two products (fusants) were obtained after five protoplast fusion attempts between six parental strains. All the twenty two obtained recombinants (fusants) were tested for their SDS-PAGE protein banding patterns and compared with protein banding pattern of their parental strains.

The variation in polysaccharide productivity by the different fusants can be attributed to one or more of the following, the number of copies of the corresponding gene(s) exist; the occurrence of different genes affecting the productivity; from the gene pools, or to genes location in the same chromosome (Papabianni *et al.*, 2001).

Biological measurements of sulfated exopolysaccharide

Biological measurements; antioxidations and antiviral activity of sulfated exopolysaccharide of the six parents and some of their fusants were studied as follow:-

Redical-scavening activities of the different sulfate exopolysaccharide from *Arthrobacter* (Anti oxidations).

Some sulfated polysaccharides have exhibited strong antioxidant capability. In these studies different sulfated polysaccharides from *Arthrobacter* were used as antioxidant (Table 9 and Fig. 6). The redical-scavening activities for different sulfate exopolysaccharide examined with seven samples; two local strains (P4 and P5) and five fusants (F2, F13, F24, F31 and F42) comparing with DPPH solution for 30 and 60 min. The results indicated that the highest radical-scavening activities were 40.04% at 30 min and 61.45% at 60 min of strain (P4), 29.96% at 30 min and 37.50% at 60 min of fusant (F13), 27.77% at 30 min and 41.18% at 60 min of fusant (F24), 15.25% at 30 min and 25.75% at 60 min of strain (P5), 10.53% at 30 min and 11.38% at 60 min of fusant (F2), 9.951% for 30 min and 10.08% for 60 min of fusant (F31) and 7.70% for 30 min and 8.891% for 60 min of fusant (F42).

These results indicate that sulfated polysaccharide from *Arthrobacter* have strong antioxidant activates and should be explore as novel potential antioxidants. These results agree with (Yang *et al.*, 2006).

Table (9): DPPH rimming of different exopolysaccharide and sulfate groups from *Arthrobacter* and fusants after (30 & 60 min).

Source of (EPS)	30 min.		60 min.	
	Mean	DPPH rimming %	Mean	DPPH rimming%
DPPH 2.0ml (cont.)	1.035	0.0	1.001	0.0
P4	0.739	40.04	0.620	61.45
P5	0.898	15.25	0.796	25.75
F2	0.926	10.53	0.887	11.38
F13	o.798	29.96	0.728	37.50
F24	0.810	27.77	0.709	41.18
F31	0.932	9.951	0.900	10.08
F42	0.961	7.70	0.943	8.891

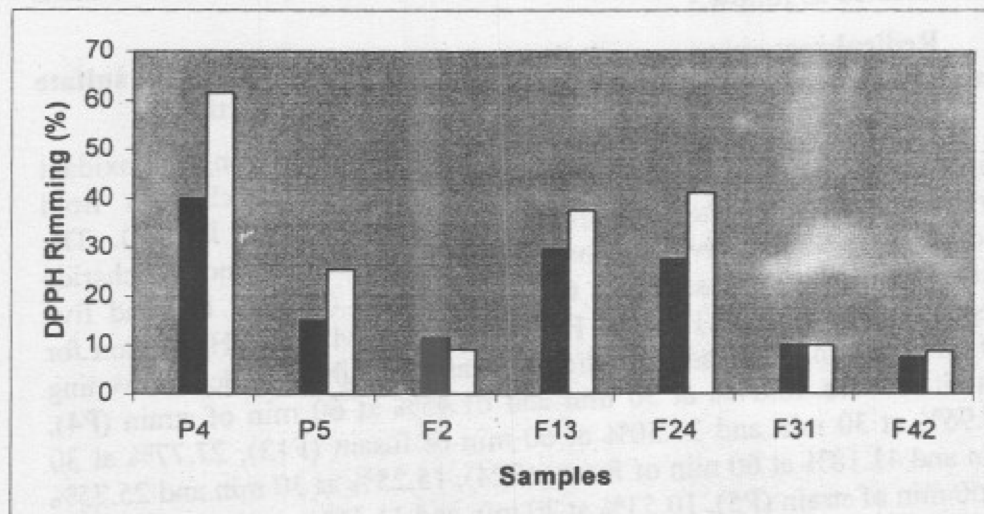

Fig (6): DPPH rimming of different exopolysaccharide sulfate groups of the two *Arthrobacter* parent strains *and* five fusants after two times; 30 & 60 min.

Antiviral activity:

Cytotoxicity of sulfated polysaccharide extracts on MDBK cell culture

The mean cytotoxic concentration 50 (CC_{50}) of the seven samples (P4, P5, F2, F13, F24, F31, F42) preparation of sulfated polysaccharide extracts were 2mg (2000 µg/ml) medium. Cell toxicity might render to high alkaling, alkalinity of the extracts at high concentrations. The other used (tested) concentrations of extracts were safe for MDBK cell cultures based on their morphological features and potentiality for growing and maintenance (Table 10).

Table (10): Cytotoxity effect of polysaccharide and sulfate groups extracts on MBDK cell culture.

MBDK	Concentrations of sulfated polysaccharide extracts				
	4mg	2mg	1mg	0.5mg	0.025mg
Plates	Toxic	Toxic	Safe	Safe	Safe
Flasks	Toxic	Toxic	Safe	Safe	Safe

Antiviral activity of polysaccharide and sulfate group extracts:

The obtained results showed no virus replication inhibitory effect on the IBR and PI3 viruses in MDBK cell cultures using the most higher available concentration of the seven (P4, P5, F2, F13, F24, F31, F42) extracts 1000 µg, 500 µg and 250 µg/ml (Fig. 7).

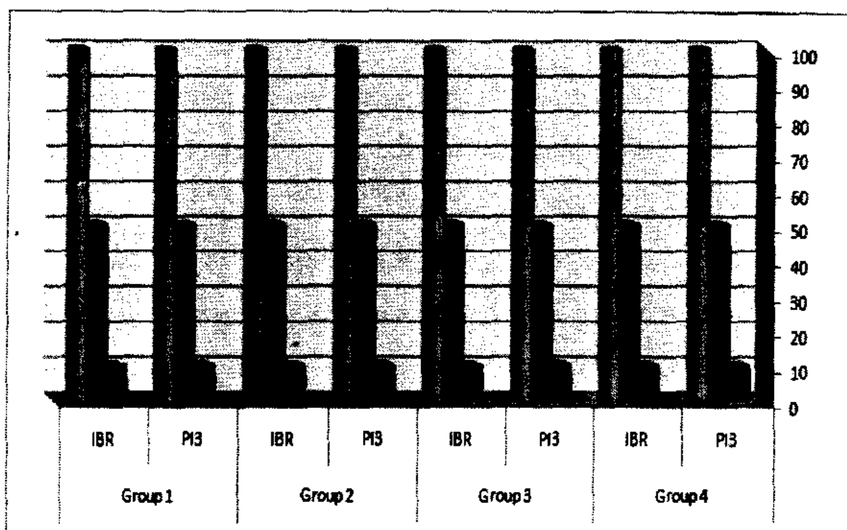

Fig (7): Virus inhibitory effect of polysaccharide and sulfate group extract on IBR and PI3 viruses on MDBK.

Group 1: IBR + Cell culture or PI3 + Cell culture
Group 2: IBR + Cell culture or PI3 + Cell culture + 1mg sulfate polysaccharide
Group 3: IBR + Cell culture or PI3 + Cell culture + 500ug sulfate polysaccharide
Group 4: IBR + Cell culture or PI3 + Cell culture + 250ug sulfate polysaccharide

REFERANCES

Asker, S. M. M.; Ahmed, M. Y. and Ramadan, F. M. (2009). Chemical characteristics and antioxidant activity of exopolysaccharide fractions from *Microbacterium terregens*. Carbohydrate polmers, 77: 563-567.

Cheng, J. J.; Huang, K. N.; Lur, S. H.; Kuo, I. C. and Lu, K. M. (2009). Characterization and biological functions of sulfated polysaccharides from sulfated-salt treatment of *Antrodia cinnamomea*. Process Biochemistry, 44: 453-459.

Cox, S.; Buontempo, p. J.; Wright-Minogue, J.; De Martio, J. L.;

Skelton, A. M.; Ferrari, E.; Schwartz, J.; Rozhen, E. J.; Linn, G. C.; Giri Javallobhan, V. and O,Connell, F. J. (1996). Antipicornavirus activity of SCH 47802 and analogs: *in-vitro* and *in-vivo* studies Antiviral Research, 32:71-79.

Davis, R. W.; Botstein, D. and Rotho, J. R. (1980). A Manual For Genetic Engineering Advanced Bacterial Genetic, Cold Spring Harbor, New york.

Fukaya, M.; Takami, H.; Tayama, K.; Okumurar, H.; Kawamura, Y and Beppu, T. (1989). Spheroplast fusion of *Acetobacter aceti* and its application to the breeding of strain for vinegar production. Agriculture Biological Chemistry, 53: 2435-2440.

Huheihel, M.; Ishanu, V.; Tal, J. and Arad, S. M. (2002). Activity of *Porphyridium* sp. Polysaccharide against hetpes simplex viruse *in-vitro* and *in-vivo*. Jurnal Biophys Methods, 50: 189-200.

Kikuchi, M. (1980). Application of genetics for strain improvement in industrial microorganisms. Biotechnol Bioeng 22 (Suppl 1): 195-208.

Linker, A. and Jones, S. R. (1966). A new polysaccharide resebling alginic acid isolated from *Pseudomonas*. Journal Biology Chemestry, 241: 3845.

Liu, W. H.; Chow, L. W.; and Lo, C. K. (1996). Strain improvement of *Arthrobacter* simplex by protoplast fusion. Journal of industrial, 16: 257- 260.

Lopalco, L.; Ciccomascolo, F.; Lanza, P.; Zoppetti, G.; Caramazza, I.; Leoni, F.; Beretta, A. and Siccardi, A. G. (1994). Anti-HIV type 1 properties of chemically modified heparins with diminished anticoagulant activity. AIDS Research and Human Retroviruses, 10(7): 787-793.

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

Piagac, J.; Hranueli, D.; Smokvina, T. and Alacevic, M. (1981). Optimal cultural and physiological conditions for handling *Streptomyces rimosus* protoplast. Applied and Environmental Microbiolgy, 44: 1178-1186.

Roseiro, J.C.; Esgalhado, M. E.; Amaral Collago, M. T. and Emery, A. M. (1992). Medium development for Xanthan production process Biochemistry, 27: 167-175.

Show, P. C. and Hartley, B. S. (1988). A host-vector system for an *Arthrobacter* species. J Gen Microbiol 134: 903-911.

Sheri, L.H.; Nicholas, E.S.; Michae, T.K. and Joanna, B.G. (2000). Comparison of proteins expressed by *Pseudomonas aeruginosa* strains representing initial and chronic isolate from a cys fibrosis patient: an analysis by 2-D gel electrophoresis and capillary column liquid chromatography tandem mass spectrometry. Microbiology, 146: 2495-2508.

Shimada, K.; Fujikawa, K.; Yahara, K. and Nakamura, T. (1992). Antioxidative properties of xanthin on autoxidation of soybean oil in cyclodextrin emulsion. Journal of Agricultural and Food Chemistry, 40: 945-948.

Yang, B.; Wang, J.; Zhao, M.; Liu, Y,; Wang, W. and Jiang, Y. (2006). Identification of polysaccharides from pericarptissues of litchi (Litchi chinensis Sonn.) fruit in relation to their antioxidant activities. Carbohydrate Resarch, 341: 634-638.

الملخص العربي

إنتاج السكريات العديدة الكبريتية من هجن البروتوبلاست في الإريثروباكتر كمضادات للأكسدة والفيروسات

أ.د/ عبد الهادي إبراهيم حسن سيد[١] ، أ.د/ شفيق إبراهيم المرسى[١] ، أ.د/ سمير خلف عبد٢ العال ، د/ محسن محمد سليم عسكر[٣] ، حسام فوزي أحمد الشاعر[١].

١- قسم النبات الزراعي – كلية الزراعة بالقاهرة - جامعة الأزهر
٢- قسم الوراثة الميكروبية - المركز القومي للبحوث – الدقي
٣- قسم التكنولوجيا الحيوية الميكروبية - المركز القومي للبحوث - الدقي

أستخدمت في هذة الدراسة ست سلالات من بكتريا الإرثروباكتر والتي لها القدرة علي إنتاج بعض السكريات العديدة الكبريتية والمفيدة من الناحية الطبية حيث أجريت لها تجارب دمج البروتوبلاست في خمس محاولات وتم عزل (٢٢) هجين قدر بها السكريات العديدة الكبريتية وتم مقارنتها بالأباء وكانت افضل النتائج في المحاولة الأولي بين السلالة رقم (P1) والسلالة رقم (p5) حيث أنتجت الهجن ثلاث أضعاف السكريات العديدة الكبريتية للأباء. تمت المقارنة بين الأباء وهجن البروتوبلاست الناتجة عن طريق التفريد الكهربي للبروتين. أختبرت السكريات العديدة الكبريتية لبعض الأباء وهجن البروتوبلاست كمضادات للأكسدة والفيروسات ولم تعطي نتيجة كمضادات للفيروسات بينما أعطت نتيجة جيدة كمضادات للأكسدة.