EFFECT OF STRESS CONDITIONS ON STABILITY AND BIOLOGICAL ACTIVITY OF A RECOMBINANT *Escherichia Coli* AGAINST THE COTTON LEAFWORM *SPODOPTERA LITTORALIS* (BOISD)

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(Manuscript received 29 January 2012)

Abstract

The effect of stress conditions on viability and activity of the recombinant *E. coli*, that contains cryIAc gene, which encodes a crystal toxin specific to *Spodoptera littoralis*, was tested. Solar radiation showed the worst effect on both viability and activity against the larvae of *S. littoralis*. The drastic effect also happened when recombinant *E. coli* was exposed to heat treatment at 50^{o}C for more than 5 min. and to high concentrations of sucrose (> 20 % for more than 2 days). Also, the same effect happened when cells were exposed to NaCl > 5 %, even after one day of exposure. The viability of *E. coli* cells after preservation by freezing under glycerol, lyophilization and encapsulation indicated that, freezing was the best method for keeping both viability and activity of cells till 9 months post preparation.

INTRODUCTION

The effect of solar radiation and predacious microorganisms on the survival of *E coli* was studied by McCambridge & McMeekin (1981). They found that, the decline in numbers of *E. coli* cells was found to be greater in the presence of both naturally occurring microbial predators and solar radiation than when each of these factors acting independently. Also, the effect of heat shock on *E. coli* at 35 ^{o}C for 20 min on its viability was studied by Berryl *et. al.,* (1991), they reported that, heat shock caused non culturable bacteria to regain their ability to grow on artificial media. The results were discussed in relation to their implications for the water industry and microbiology in general. Growth responses of *E. coli* 0157:117, as affected by NaCl, the pH value and storage temperature, were studied by Sutherland *et. al.,* (1995). They found that NaCl in the range of 0.5 to 6.5 % was suitable for *E. coli* growth.

The main advantage of lyophilization is minimizing the exposure to osmotic stresses. During drying, by the removing of water, the solutes concentrate and the product to be preserved is exposed to increase stresses over long periods of time. In

lyophilization, the products are first frozen and then the water is removed, to minimize osmotic stress buildup (Pereira *et. al.,* 2002).

Sneath *et. al.,* 1986 prefer to store important cultures of *E. coli* in beef broth containing 10 % glycerol at -80 ^{o}C. Screw-capped vials are used for easy access.

The aim of this work is to study some stress conditions such as, solar radiation, heat treatment, sucrose, NaCl concentrations and different methods of preservation for 9 months, on cell viability and activity, of a recombinant *E. coli*.

MATERIALS AND METHODS

Recombinant Escherichia coli

Recombinant *E.coli*, which contains CryIAc gene, used in the present investigation, was kindly provided from Genetics Dept., Fac. of Agriculture, Cairo University.

Used media

MR medium for cultivation and production of recombinant *E. coli*. (Wang and Lee, 1997).

$(NH_4)_2HPO_4$	3g
KH_2PO_4	2.2g
$MgSO_4.7H_2O$	0.7g
Trace metal solution	5ml
Separately sterilized Glucose	20g
Ampicillin	100 µg/ ml of medium
pH	7.0

Effect of solar radiation

To determine the effect of natural solar radiation on *E. coli* survival, the modified MR medium was inoculated with 1 ml of *E. coli* suspension and incubated to give a final concentration of approximately 10^8 cells per ml. The flasks were exposed to direct sunlight at room temperature. Bacterial survival was determined, daily by plate counting. Control flasks were completely covered in aluminum foil. Three replicates of each treatment were conducted, as described by McCambridge and McMeekin, 1981.

Effect of high temperature

An active culture of recombinant E. coli grown on modified MR broth medium was heated by exposing the samples to heat treatment in water bath at 50oC for 5, 8,

12 and 15 min., then cooled to room temperature. The number of survivor cells was determined by plate counting. The activity against the larvae was also assayed.

Effect of NaCl concentration.

Different concentrations of NaCl, (3, 5, 7, 10 and 12 %), were added to recombinant *E. coli* suspension in modified MR medium, and held for three days then cell viability and the efficacy were determined every day intervals.

Effect of sucrose concentration.

Different concentrations of sucrose (10, 20, 30, 40 and 50 %) were added to bacterial suspension in modified MR medium and held for 4 days. Determinations of survival cells was carried out and the efficacy were performed at 24 hours intervals.

Preservation of recombinant *E. coli* and genetic stability.

Preservation must maintain culture viability and culture characteristics. Numerous problems may appear either during manipulation or during storage (genetic mutation, plasmid loss and loss of activity) until a strain is completely lost. No method is 100 % reliable and some are more or less adapted to certain species (Thiery & Franchon, 1997). Therefore, three trials, namely freezing, lyophilization and granulation, were assayed to select the suitable method for maintaining culture viability and activity.

Freezing under glycerol

Freezing under glycerol for several months can be used as a form of long-term storage for internal laboratory use, as follows: 0.8 ml of cell suspension in broth medium, under optimal growth conditions, were obtained in Eppendorf tubes, and 0.3 ml of a 17 % final concentration of sterile glycerol solution was added, then the tubes were freezed immediately at - 20^{o}C (Thiery & Franchon, 1997).

Lyophilization

It is useful for oligosporogenous strains or for strains with spores which are less time-resistant. Apart from collection and storage, it is also used for bacterial products in order to avoid the problem of hydrophobicity sometimes linked with bacterial powder (Thiery & Franchon, 1997).

The equipment used for lyophilization technique is frequently found in laboratories. It is composed of a vacuum pump with a quick freeze dryer system. The technique steps are as follows:

1- Strains were grown on an agar plate, harvested colonies were homogenized in sterile physiological saline containing 20 % horse serum.

2- Hundred µl of the bacterial suspension were placed into 1-2 ml sterile lyophilization tubes with a pipette, then the tube was closed.

3- The tubes were deeply immersed into a mixture of ethanol (100 %) and solid CO_2.

4- The cotton plug was removed and the tubes were attached to the freeze-dryer.

5- After lyophilization, the tubes were sealed with flame under a vacuum.

6- Each lot of tubes was checked systematically, in order to check viability and activity of the strain (Thiery & Franchon, 1997).

Granular formulation

The recombinant *E. coli* strain was used for preparing alginate granules. Heavy cell suspension was obtained on modified MR medium. Five gram of sodium alginate was mixed together with 15g powdered milk and 0.5 g bacterial cells, then 500 ml of distilled water was added and stirred for 1 hr at 45^{o}C. The whole mixture was added to 500 ml of 0.05M of $CaCl_{2,}$ and then kept at room temperature for 1 hr to obtain gel. The gel was washed with 1L distilled water to remove the calcium chloride. At last, the gel was cut with spatula, chopped, sieved and left to dry overnight. Dunkle & Shasha (1989) and McGuire *et. al.,* (1990).

Viability tests

This study aimed to evaluate the survival of the recombinant *E. coli* cells in different preservation methods. The number of CFU, in each of the previous preservation method, was determined after 3, 6 and 9 months after storage. For this purpose, serial decimal dilutions were prepared from suspensions obtained by grinding the beaded formulation, as for the freezed bacteria with glycerol or the lyophilized bacteria, the bacteria were melted first, and then the dilutions were plated onto modified MR agar medium. The bacterial count was determined by spectrophotometer, and then compared.

Insect bioassay with *δ* – endotoxin.

This study aimed to evaluate the activity of recombinant *E. coli* against the 2nd instars of *S.littoralis*. Five concentrations of each preparation were made, and bioassayed against the larvae. The LC- values were calculated and compared with zero time results.

Cell extracts (1 ml), were brushed on each side of 4-5 cm diameter castor bean leave discs. The discs were dried in air and placed in a glass jar with 10 of 2nd instar larvae. The number of dead larvae was recorded after 2 to 7 days of incubation at room temperature (Chak and Ellar 1987).

RESULTS AND DISCUSSION

Effect of solar radiation

In order to determine the ability of the recombinant bacteria to survive under stress conditions, the cells exposed to solar radiation for five days, then were used for studying the viability and activity against the larvae of *S. littoralis*. Data illustrated by Fig (1) clearly show that, after 48 hr cultures were more resistance to solar radiation than that found after 36 hr or 24 hr cultures. The viable cells of the 48 hr culture decreased from 5 to 3 x 10^8 cfu /ml after 2 days of exposure. After 36 hr CFU decreased from 5 to 1 x 10^8 cfu /ml, while the 24 culture decreased from 5 x 10^8 to 5 x 10^7 cfu /ml. The drastic effect of solar radiation clearly appears after 3 days of exposure for all culture ages. With respect to the cell efficiency, results clearly show that, the percentage of mortality represented the same trend. Data in Fig (2) indicated that, the activity of the strain against the larvae, was seriously affected in the first 2 days of exposure. After the 3rd day, the activity was drastically affected. The mortality after 5 days reached 30- 40 % comparing with 70 % at zero time in the first 2 days of the experiment.

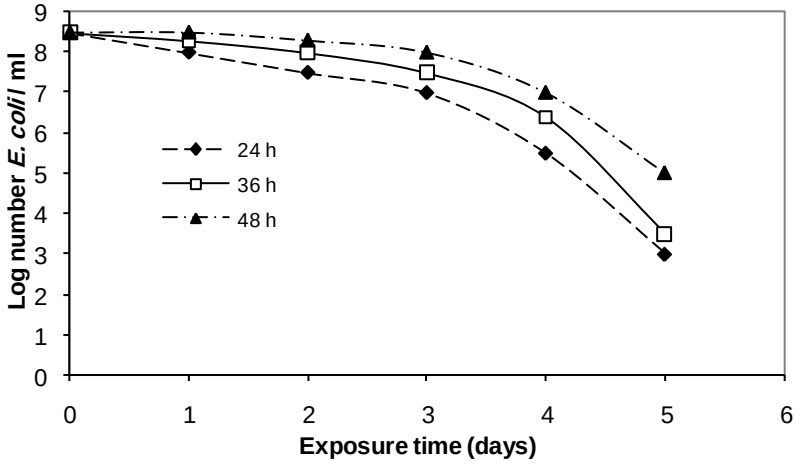

Fig. 1. Effect of solar radiation on the viability of recombinant *E. coli*, during 5 days of treatment.

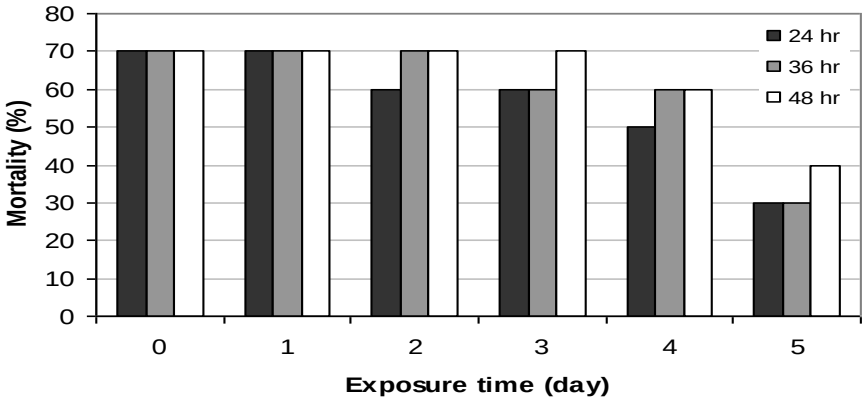

Fig. 2. Effect of solar radiation on the efficacy of recombinant E. coli, during 5 days of treatment.

The same conclusion was noticed by McCambridge & McMeekin (1981), who studied the effect of solar radiation on the survival of *E coli*. They found that, the decline in numbers of *E. coli* cells was found to be higher after 3 days of exposure.

Effect of high temperature

This experiment was carried out to study the effect of high temperature on recombinant *E. coli* and determine the viability and activity of cells after treatment against *S. littoralis*.

Data illustrated in Fig (3) clearly show that, the percentage of cells viability of treated *E. coli* decreased with the function of heating time being 17, 47, 74 and 93 % at 50^{o}C for 5, 8, 12 and 15 min, respectively.

Taking into consideration the activity of recombinant E. coli, it also affected drastically after treatment. The decrease in mortality after 5 min of treatment was 20 % and 43 % after 8 min, till reaching 97 % after 15 min of exposure.

These data are in line with those obtained by Singh, et. al., (1985) who heated the cell suspension of 2 strains of E. coli at 60 or 63oC with holding times ranging from 5 to 30 min. After exposure of strain 0111:84 to 60oC for 10 min, no viable cells were observed on yeast extract agar.

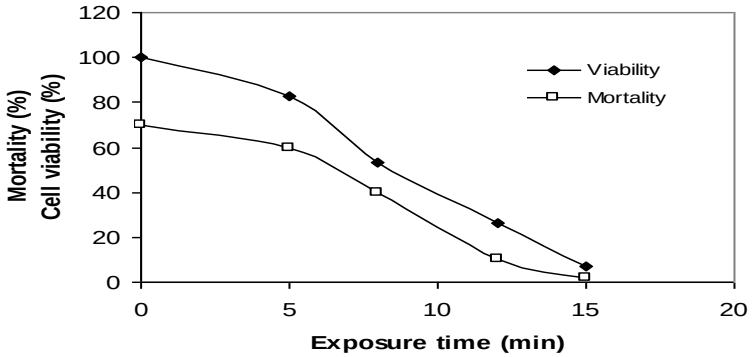

Fig. 3. Effect of heat treatment (50o) on viability and efficacy of recombinant E. coli.

Effect of NaCl concentration

Different concentration of sodium chloride (3, 5, 7, 10 and 12 %) were added to 24 h old culture of recombinant *E. coli*, and kept for 3 days at room temperature. Viable cells and activity were determined daily.

Data illustrated in Fig (4) showed that, the recombinant strain did not resist to high concentrations of NaCl. The percentage of lethality increased with increasing salt concentration and with prolonging time of exposure. The highest percentage of lethality 72 % was obtained when applying the treatment of 12 % salt for three days. With respect to the activity of the *E. coli* against the larvae, it clearly appears from Table (1) that, the treatment has a drastic effect on the efficiency of the cells. The mortality decreased at the first day of exposure, with increasing of NaCl concentration till reaching only 5 % at 7 % NaCl concentration. While, there was no mortality observed at the 2nd and the 3rd day of exposure at all NaCl concentrations.

The same results were noticed by Kitzman (1992) who reported that, NaCl (1.5 and 3.0 %) was an inhibitory agent on *E. coli* cells. They also found that NaCl was a stronger inhibitor than $NaNO_2$, especially at lower pH.

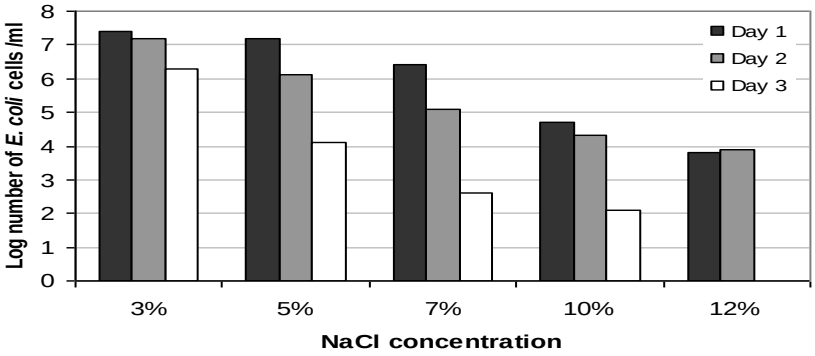

Fig. 4. Effect of NaCl concentration on the viability of recombinant E. coli.

Table 1. Effect of NaCl concentration on activity of recombinant *E. coli*, against 2nd instars of *S. littoralis*, initial concentration (56 x 10^7 cfu/ml)

Time of exposure (day)	NaCl concentration (%)	Total mortality after 7 days (%)
1	3	45
	5	24
	7	5
	10	-
	12	-
2	3	-
	5	-
	7	-
	10	-
	12	-
3	3	4
	5	-
	7	-
	10	-
	12	-

Effect of sucrose concentration

Data illustrated in Fig ($5_{a,b}$) show that *E. coli* treated cells exhibited an increase in viable cell counts when using 10 or 20 % of sucrose during the period of the experiment (4 days). Higher sucrose concentrations showed decrease in viability, reaching (1x10^2 cfu/ml) at 40 % sucrose concentration on the 4th day of exposure. While no viability was observed at 50 % sucrose on the 4th day of exposure.

Concerning the effect of sucrose concentration on activity of the *E. coli* against the larvae, results are showed the same trend with viability. When the viability decreased, the mortality decreased till no mortality was observed on the 3rd and 4th day of treatment at 50 % sucrose concentration. The injurious effect of high sugar concentration on the viability of bacterial cells may be due to increasing the

concentration of intracellular solutes and decreasing water activity, or may be due to osmotic shock.

Such effects may lead to cell wall damage or cell membrane leakage (Mossel *et. al.,* 1995).

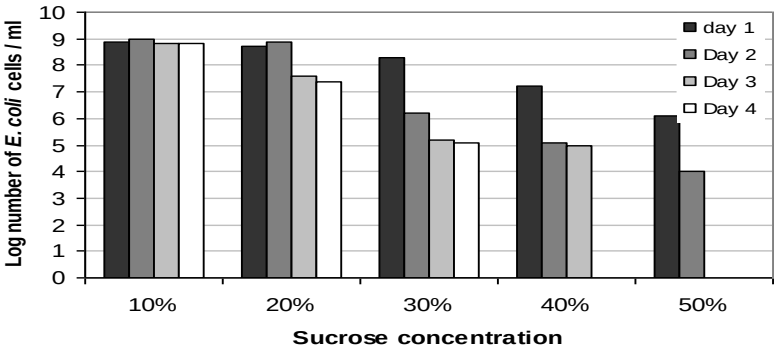

Fig. 5a. Effect of sucrose concentration on the viability of recombinant E. coli.

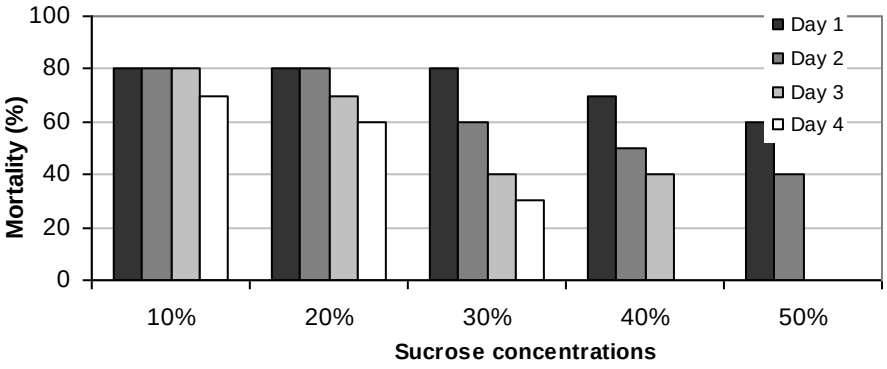

Fig. 5b. Effect of sucrose concentration on the efficacy of recombinant E. coil against 2nd instars of S. littoralis.

Preservation and genetic stability

During 9 months, from preservation procedures, the bacterial cells were counted in modified MR medium, and assayed against the larvae as described previously.

Viability

The effect of different preservation methods on cell viability was tested after 3, 6 and 9 months post preparation, by standard bacteriological plating techniques. Viable cells, based on those counts, indicated the effect of the process of preservation and storage period on the cell viability.

Results in Fig. (6) indicated that, the best preservation method for keeping the viability of recombinant *E.coli* , was the freezing under glycerol while, the freeze-dried preparation was the lowest for keeping the cells survived throughout the test period. The decrease in viability, from zero time to 9 months was as follows:

- The freezing form: the decrease of cell count was 3 % after 3 months for the recombinant *E. coli* strain, and the decrease after 6 months was 6 %. After 9 months, the decrease in viable cell counts was 12 %.

- The lyophilized preparation: the decrease in viability of recombinant *E. coli* cells in lyophilized form was 22 % after the first 3 months, and was 40 % after 6 months. While, after 9 months, the decrease reached 48 % of cell counts.

- Alginate granules: after 3 months of preparation the decrease in viability was 13 %, and after 6 months the decrease was 34 % till reaching 38 % after 9 months.

From the aforementioned results it is clear that, freezing under glycerol was found to be the best method for keeping the viability of cells till 9 month post- preparation. The level of decrease was the lowest in this method indicating its superiority to the other two methods.

Pereira *et. al.,* 2002. proved that *E. coli* was effectively preserved in lyophilized state by 100 mM tryhalose. Also Streeter, 2003 found that, trehalose loading during growth was more effective in protection and survival of cells than when added during desiccation.

Activity test

The efficacy of recombinant *E. coli* was studied in this investigation, in order to test the effect of the preservation methods on the activity of this strain against the larvae of *Spodoptera littoralis*.

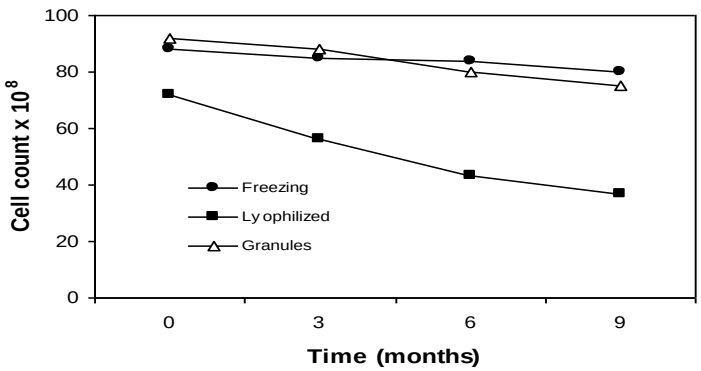

Fig. 6. Effect of preservation method on the viability of recombinant E. coli cells during 9 months post-preparation.

Data illustrated by Fig 7 show the activity of *E. coli* against the larvae, concerning the preservation methods, tabulated data in Table (2) clearly show that, freezing under glycerol showed the highest efficacy against the larvae, followed by alginate granules then the lyophilized form. The LC_{50} values of recombinant *E. coli* were 115.5 x 10^6 cfu/ml, for freezing form, 176.3 x $10^{6\ cfu}$/gm for lyophilized form and 134 x 10^6 cfu/gm for the alginate granules. Also, the LC_{50} values of the strain, after 9 months post preparation were, 1236 x 10^6 cfu/ml, for freezing form, 55266 x 10^7 cfu/gm for lyophilized form and 10785 x 10^6 cfu/gm for the alginate granules.

Table 2. Efficacy of different preservation methods of recombinant *E. coli* against the 2nd instars of *Spodoptera littoralis*.

Preservation method	Time	Fucidal limit			Slope (b)
		LC50 (cfux10^6/unit)	Lower (cfux10^6/unit)	Upper (cfux10^6/unit)	
Freezing form	Zero time	115.5	82.4	498.3	0.314
	6 months	768.5	297	3009	0.27
	9 months	1236	460.5	6581	0.26
Lyophilized form	Zero time	176.3	67.4	407.7	0.314
	6 months	88546	10033	71965000	0.24
	9 months	552660	33158	69947000	0.24
Granules	Zero time	134.0	32.3	355.4	0.264
	6 months	598	154.2	4326.6	0.20

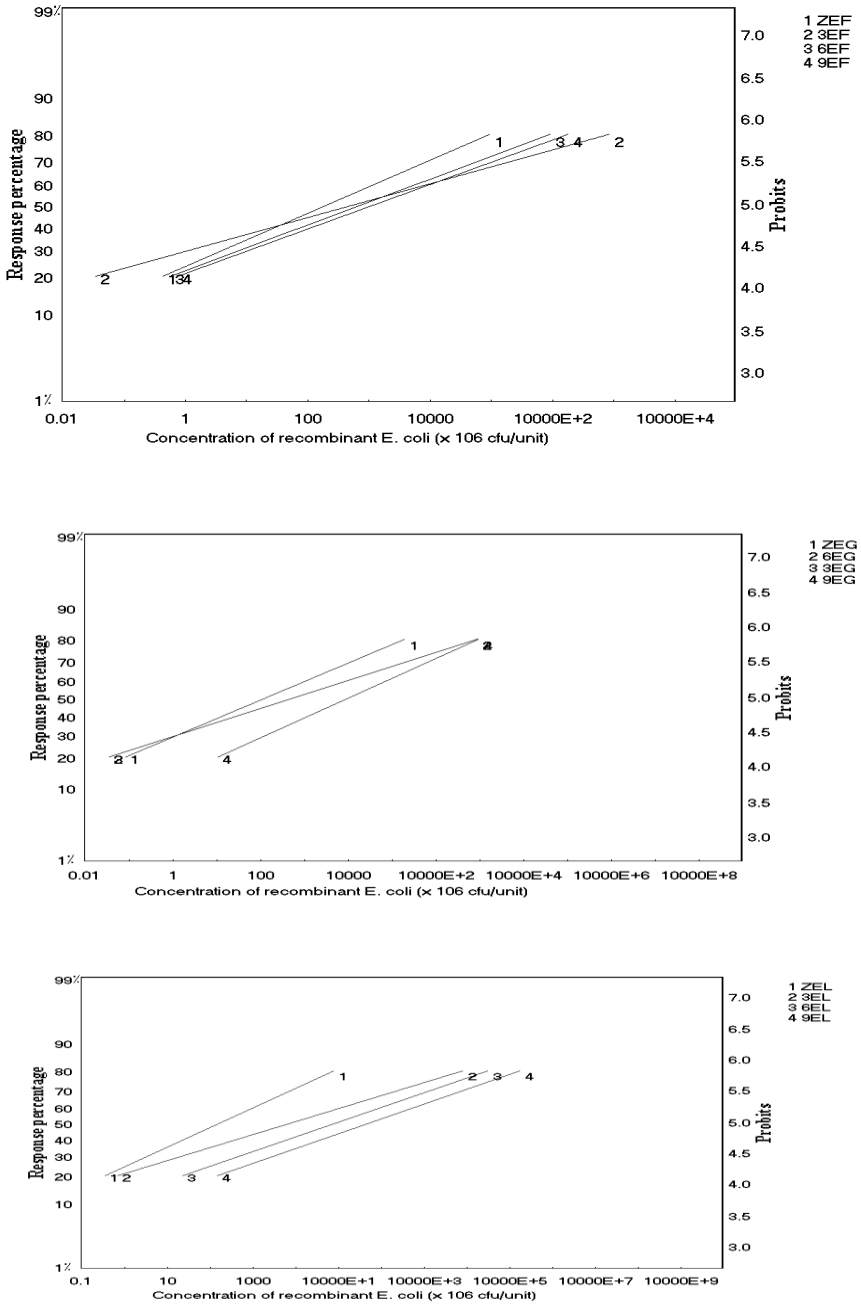

Fig. 7. Efficacy of recombinant *E. coli* against the 2nd instars of *S. littoralis* using different preservation method during 9 months post preparation.

Z: zero time, 3: 3months, 6: 6 months and 9: 9 months.

E: recombinant *E. coli*, F: freezing form, G: granules and L: lyophilized form.

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تأثير ظروف التقسية على الثبات الجيني و النشاط الحيوي لبكتيريا ايشيريشيا كولاي المحولة وراثيا، ضد دودة ورق القطن

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تم دراسة تأثير ظروف التقسية و طرق الحفظ المختلفة على حيوية و فعالية سلالة بكتيرية *E. coli* محولة وراثيا لاحتواء الجين CryI Ac المسئول عن انتاج توكسين كريستالي متخصص لمكافحة يرقات دودة ورق القطن . أثبتت النتائج أن الاشعاع الشمسي له أسوأ تأثير على حيوية و فعالية الخلايا ضد اليرقات . كذلك يحدث تأثير سيئ عند تعريض الخلايا لمعاملة حرارية > 50 °م لأكثر من 5 دقائق أو التعريض لتركيزات من كل من السكروز (> 20% لأكثر من يومين) أو كلوريد الصوديوم (> 5% أكثر من يوم واحد). عند دراسة تأثير طرق الحفظ المختلفة على الخلايا أثبتت النتائج ان التجميد تحت الجلسرول هو أفضل الوسائل المستخدمة في الحفاظ على حيوية و فعالية الخلايا (حتى 9 أشهر) يليه المستحضرات الحبيبية ثم التجفيد. حيث ان الانخفاض في الحيوية عند استخدام التجميد تحت الجلسرول كان 12% فقط أما عند استخدام المستحضرات الحبيبية كان 38 % و 48 % عند استخدام التجفيد.