THE PRESERVATIVE EFFECT OF TREHALOSE ON BACTERIA

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ABSTRACT

Two isolates *Staphylococcus aureus* and *Escherichia coli* were used in this study, Results showed that bacteria treated with 100 mM Trehalose was able to preserved *S.aureus* from drying for one month in room temperature, while no effect was observed in the Trehalose treated *E.coli*. Results also indicated that Trehalose preservative effects were for month. The survival percentage of *S.aureus* treated with Trehalose after 1 month was 67% compared with the control 30%, while the results of *E.coli* was 66% compared with the control 32%. Results also indicated that Trehalose can protect the lyophilized bacteria stored in room Temperature for 4 months. The percentage of the viable cells of bacteria was 27% compared with the control 2%. Results also revealed that the protective effects of Trehalose reduced the lethal effects of UV Irradiation. Results indicated that *S.aureus* treated with Trehalose was more resistance to Tetracycline, Gentamycin, Erythromycin and Norfoxacin, Antibiotics in comparison with the control treatment, while no effect was indicated in the trehalose treated *E.coli* resistance ability to these antibiotics.

Key words: Trehalose, Preservation, Freezing, Bacteria, UV Irradiation

التأثيرات الحافظة لسكر التريهالوزعلى البكتريا

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الخلاصة

أُستعملت عزلتان من البكتريا إحدهما تعود إلى جنس المكورات الذهبية الأخرى لبكتريا القولون. أظهرت النتائج بأن معاملة البكتريا بـ 100 ملي مولار تريهالوز له القدرة على حفظ بكتريا المكورات العنقودية من الجفاف ولمدة شهر في درجة حرارة الغرفة الاعتيادية، في حين لم يلاحظ أي تأثير في بكتريا القولون المعاملة بنفس النسبة من التريهالوز. بينت النتائج التأثير الحافظ لسكر التريهالوز في حالة خزن البكتريا في ظروف الانجماد و لمدة أربعة أسابيع وكانت نسبة بقائية بكتريا المكورات الذهبية 67% مقارنة مع السيطرة بنسبة 30% بينما كانت النتائج في بكتريا القولون 66% والسيطرة 32%. وأشارت النتائج أيضاً إلى قدرة الحماية التي يوفرها السكر للبكتريا المجفدة المخزونة في درجة حرارة الغرفة ولمدة 4 أشهر، حيث كانت نسبة البقائية للبكتريا المعاملة بالتريهالوز 27% والسيطرة 2%. أظهر التريهالوز تأثيرات الحماية للبكتريا من الأشعة فوق البنفسجية. بينت النتائج المعاملة بالتريهالوز ان بكتريا المكورات العنقودية الذهبيةأصبحت أكثر مقاومة لمضادات Gentamycin, Norfloxacin, Erythromycin, Tetracycline في حين بقت بكتريا السيطرة حساسة لها، ولم يظهر أي تأثير للتريهالوز في مقاومة بكتريا القولون لمضادات الحيوية.

INTRODUCTION

Trehalose, a naturally occurring, non reducing disaccharide consist of two glucose units,(1) is commonly found in bacteria, yeast, algae, insects, plants animals and human organs(2). Trehalose is known to help certain animals, plants and Microorganisms to survive desiccation, high osmolarity, and damage by both freezing and heat. It used to preserve biological materials (3,4) and as a stabilizer for unstable proteins, including dehydrated and frozen enzymes, diagnostoc reagents, pharmaceuticals, and cosmetics (5). Trehalose possesses several unique properties including high hydrophilicity, chemical stability, non hygroscopic glass formation. The combination of these features explains the principal role of trehalose as a stress metabolite (6). The particular properties of Trehalose have given rise to surprisingly wide range of applications of this disaccharide in technology including the stabilization of proteins, membranes, Liposome, and Vaccines(6,7). While the detailed picture for the molecular mechanism responsible for membrane stabilization is still lacking (8). Trehalose found in high concentration 7% in materials named (Manna) used by bidwen peoples in north of Iraq Desert as coffee sweeting. The Manna could be one of secretion of some insects.

Trehalose is also found in number of different bacteria, including *Streptomyces hyroscopicus* and other of *Streptomyces spp* (9,10). Different mycobacteria including *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* (11). In many of these organisms the function of trehalose is still not clear. Several of the Organisms listed appear to have rather unusual biosynthetic pathways for synthesizing trehalose (12).The aim of the present study was to determined the preservative effects of Trehalose treated bacteria towrdes several stress conditions such as drying, freezing, lyophilization, UV irradiation and Antibiotics sensitivity.

MATERIALS AND METHODS

Bacterial strains

Two isolates *Staphylococcus aureus* an *Escherichia coli* were identified and used According to (13).

Protection from dehydration

To evaluate the effect of Trehalose on dehydration of bacteria a novel method was used. The two bacterial isolates were each individually inoculated into standard culture media in a 96 well microtiter dish. And incubated overnight at 37°C. In the following day an equal volume of preservation media, with10mM of divalent cation $CaCl_2$ Containing Trehalose was add to the growing cultures giving a final concentration of Trehalose 100 mM. The cultures were gently rocked over a 96 hour period and the contents allowed drying. The dish was then covered and placed at room temperature. After one week the individual culture were rehydrated with sterile water, prewarmed to 37°C.

Added to nutrient media to test for viability. After1month cultures were rehydrated with sterile water, prewarmed to 37°C. Added to nutrient broth media to test the viability.

Effect of Trehalose against Freezing

The two isolates were incubated in nutrient broth medium for 18hr. After the incubation period a 100 mM Trehalose was then added to the culture, while the control treatment left without any addition. All the tubes were then seald and stored in -20°C for (40) days a sample were then lifted each 10 days, recultured on Nutrient agar medium to determine Its viability.

Effect of Trehalose after bacterial freeze drying (Lyophilization)

Two Flasks containing 50ml of nutrient broth culture medium were prepared, and inoculated with *S. aureus* 18 hr at 37°C. A100mM trehalose was added to the culture while the control treatment left without any addition. The two flasks then transfer to sterilize lyophilizers flask and then lyophilized and the flasks sealed and stored for 4 months. A sample each month was then diluted and cultured in Brain Heart Agar to determine the viable cells by incubation for 18 hrs at 37°C.

Protection from UV Irradiation

Trehalose protection from UV light damage was studied using the procedure of (14) by culturing *S.aurens* in Brain Heart medium with 100mM trehalose and without (control) until the mid exponential phase. The bacterium was then pelleted from 5ml by centrifugation at 4000 rpm for 10min, washed twice and resuspended in the same volume of phosphate buffer (ph.7.0).

The UV Source was: UV –Transilluminator – cross Linker(FLX-20-M,Vibler Lourmat- France) The tray size for irradiation was approximately 15 X 25 cm. Then the samples were exposed on Petri dishes, multi-well plates, membrane, etc, to direct irradiation from (15 watts) 254nm bulbs. A UV photoelectric cell detects actual intensity. The dose rate of UV irradiation was 2-5 $J/m^2/s$.

The distance between UV Source and irradiated suspension was11cm. Then a sample (3ml) of bacterial suspension in phosphate buffer contain Trehalose and the control was irradiated in sterile Petri dish for the following doses, (0,10,20,30,40 and 50) J/m^2. Two samples of each treatment were taken and as follows: 0.1 ml sample diluted properly and spread on BHA, then incubated at 37°C for 24 hr. To determine the total viable count (survival Fraction).

Antibiotic Sensitivity Test

Disk Diffusion Test

This test was done According to (15,16,17). Two test tubes contain 10 ml of nutrient broth medium one with 100 mM of trehalose, and the other without (control) inoculated with the bacterial isolates *S.aureus* , *E.coli*, the cultures were incubated at 37°C to mid log phase giving $1x10^8$ bacterium /ml.

Then 0.1 ml of inoculated broth was transferred to BHA. A sterile cotton swab was used to streak the inoculums on the plate approximately 60 each time to obtain an even distribution of the inoculum).The inoculated plates were then placed at room temperature for 10 minutes to allow absorption of excess moisture. A selected antibiotic disc was then placed using a sterile forceps. The inoculated plates were then placed on the Inoculated plates and incubated at 37°C for 18 hr. in an inverted position. After incubation, the diameter of inhibition zone were measured by a ruler in mm, results were determined. According to the NCCLS (16).

Effect of Trehalose on Antibactorial Activity

Antibacterial Agents Test

Inhibitory effect was determined by the agar diffusion method described by (15). Using the bacterial inoculation technique. Commercially available 60 mM Petri dishes containing BHA were inoculated with clinical isolate (*S.aureus* with 100 mM trehalose and without trehalose as control). By using sterile swabs. Two evenly spaced holes 6mm, in diameter were made in the agar of each plate with sterile cork borer, To identify the intrinsic antibacterial activity of the diluents, wells were filled with *S.epidermidis.* An equal volume (200 ml) of each agents was expressed into each well Test plates were then incubated at 37°C for 24hr andZones of inhibition were measured with millimeters. A clear inhibition zones indicates that the agent had retained its antibacterial activity.

RESULTS AND DISCUSSION

Protection From dehydration

The results showed that the *S.aureus* Isolate had a higher level of viability after one month when preserved in the presence of Trehalose and without. The two primary stresses that are proposed to destabilize lipid bilayers during dehydration are fusion and lipid phase transitions. Studies by Laser Light Scattering or other techniques demonstrate that Trehalose and other sugars inhibit fusion between the vesicles during drying, but the inhibition of fusion alone is not sufficient to preserve the dry vesicles. Thus Trehalose is also necessary to prevent phase transition (18).

The evidence suggests that Trehalose depresses the phase transition temperature of the dry lipids which maintains them in the liquid crystalline phase in the absences of water (19) while the result of *E.coli* isolate shown no effect of Trehalose in preservation in dry state. The disaccharide Trehalose acts as a carbon source in *E.coli* at high Osmolarity of the growth medium. The modes of Trehalose utilization are different under different conditions and have to be well regulated (20).

Protection from Freezing Condition

Two isolates of bacteria S.aureus and *E.coli* were stored by using the Freezing technique and the results appears in Figure(1). The bacterial isolate were stored at 20°C in addition of 100mM Trehalose and without (control). There was increasing capability of viability of bacterial isolate S.aureus when 100mM of Trehalose was added before freezing compared with the control treatment. The S.aureus isolate keeps it viability after 4 weeks of freeze preservation while the control lost most of its viability after 4 weeks Figure(2). Showed that the preservation of *E.coli* in Trehalose had the same effect on compared with control. The plasma membrane is a key component of the cell and must be maintained during freezing conditions if the cell is to be kept alive (21). It has been demonstrated with artificial membranes, such as unilamellar vesicles, that damage measured by intermixing and fusion can be reduced by a series of cryoprotectents, with Trehalose and sucrose being more protective than glycerol, thus, these sugars probably play a key role in preventing deleterious alteration to the membrane during reduced-Water States (21,22).

Protection from UV light irradiation

The survival curve of S.aureus after exposed to different doses of UV irradiation is shown in Figure(3). The result indicated that S.aureus lost It's viability exponentially while addition of Trehalose make the bacterial isolate more resistance to UV. The survival curve of *S.aureus* without addition of Trehalose (control). Showed increases of lethal percentage exponentially with the increase of UV doses and the highest lethal percentage or the less survival fraction was 1.1×10^{14} when the bacteria exposed to 40 J/m^2 of UV irradiation. Our results agreed with (23) who mentioned that the protective effect increases with the increasing amount of Trehalose. Other study of lethal effect of UV light on *S.aureus* showed that this bacterium was sensitive to higher Doses of UV (14). Results indicated that *S.aureus* without addition of Trehalose was sensitive to mutation induced by UV, while the addition of Trehalose protect the bacterium from harmful of UV irradiation (23).

Antibiotic Sensitivity

Two isolates *S.aureus* and *E.coli* were tested using different antibiotic disks. The resistance and sensitive values were taken in comparison of the diameter of inhibition zone with that of standard value of NCCLS. Results shown in table(1)indicate that resistance to antibiotics was widely increased among isolate of *S.aureus* treated with Trehalose than the control. It was resistant to Norfloxacin, Erythromycin, Streptomycin and Gentamycin while the control was sensitive to these antibiotics.

While Results shown in table (2) indicate that Trehalose had no effect on antibiotics resistance in E.coli because of the Trehalose enzyme. The disaccharides Trehalose acts as a carbon source in *E.coli*. At high osmolarity of the growth medium, however, they can also degrade Trehalose as the sole source of carbon under both high and low osmolarity growth conditions. The modes of Trehalose utilization are different under condition and have to be well regulated (20).

Figure(1) :The effects of freezing at 20̇C on the viability of *S.aureus* with and without Trehalose

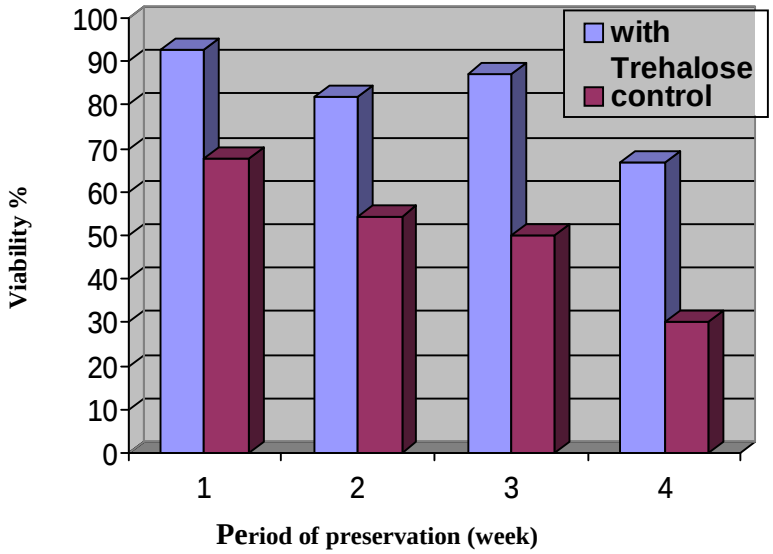

Figure (2) : The effects of freezing at 20̇C on the viability of *E.coli* with and without Trehalose

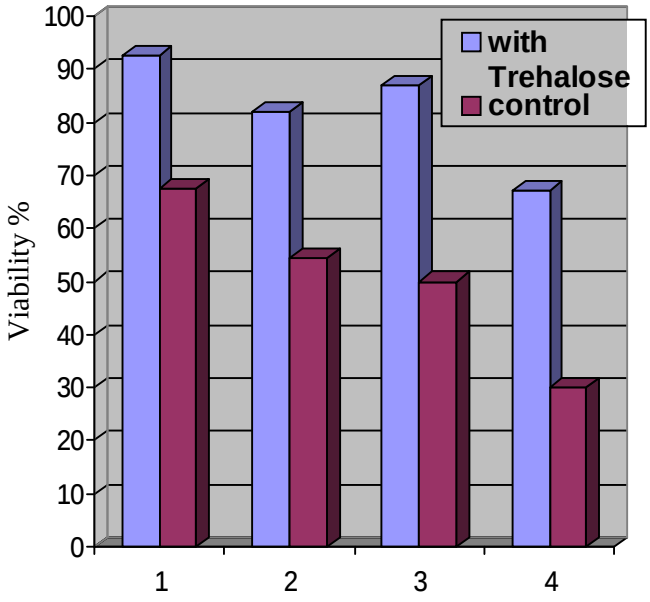

Figure (3):The survival curve of S.aureus after exposed to different doses of UV irradiation

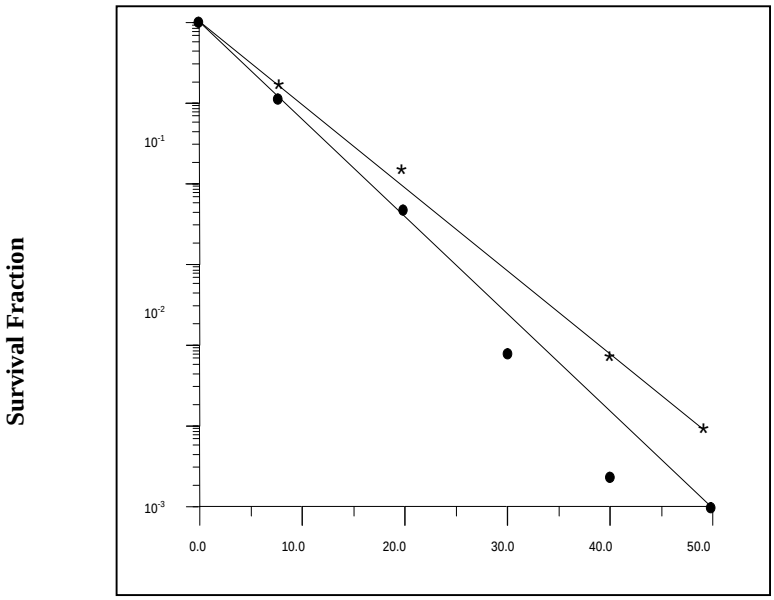

Table (1): Result of antibiotics disc test against *S.aureus*

Bacterial isolates	Antibiotics					
	Nor 10Mg	E 15 Mg	S 10Mg	TE 30Mg	GM 10Mg	VA 30Mg
S.aureus treated with Trehalose	R	R	S	R	R	S
S.aureus without Trehalose control	S	S	S	S	S	S

***Nor 10Mg , E 15 Mg , S10Mg , TE 30 Mg , GM 10Mg , VA30 Mg**

Table (2): Result of antibiotics disc test against *E.coli*

Bacterial isolates	Antibiotics					
	Nor 10Mg	E 15 Mg	S 10Mg	TE 30Mg	GM 10Mg	VA 30Mg
E.coli treated with Trehalose	S	S	R	R	S	R
E.coli without Trehalose control	S	S	R	R	S	R

Nor = Norfoxacin GM=Gentamycin
E = Erythromycin VA= Vancomycin
S = Sytriptomycine
TE = Tetracycline

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