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Media Improvement for Detection Freezing Stressed *Escherichia coli*

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Abstract

Escherichia coli was injured by freezing at-18ºC. Surviving cells were recovered on nonselective medium (tryptone glucose extract agar) and selective medium (violet red bile salt agar) supplemented with compounds that degrade hydrogen peroxide or block its formation. Various concentrations of the following compounds were tested: sodium pyruvate, sodium thioglycolate,and n-propyl gallate.Sodium pyruvate and sodium thioglycolate, when added to either medium, significantly increased ($P \leq 0.05$) for recovering of injured cells. Supplementation of violet red bile agar increased recovery cells, but the counts remained considerably lower than the tryptone glucose extract agar counts. The repair detection of freezing stressed *E.coli* was greatly improved by the addition of sodium pyruvate and thioglycolate .We therefore recommend that pyruvate (0.4%) and thioglycolate (0.04%) be used to supplemented media in the repair detection procedure.

Introduction

Microorganisms present in processed food, dairy products, water and the environment may be injured and hence more exacting in their growth requirements. Such organisms may be difficult to detect because they fail to grow on the selective media communally used in their isolation [1]. However, under suitable conditions injured cells can repair cellular damage and recover all their normal properties including virulence. An appreciation of nature of sublethal injury and its repair is therefore important for detecting and enumerating microbes [2,3]. Injured cells may showed an extended lag phase restricted temperature range for growth ,and increased sensitivity to selective agents ,salt, acidity and oxidative stress[4,5,6]. Within any population of microorganisms surviving exposure to preservation treatment or environmental stresses there exist individual cells that are regarded as sublethally injured. Stress treatments that cause injury included heating, refrigeration, freezing, irradiation, higher acid or alkaline, high salt levels, preservative, desiccation, exposure to disinfectants and starvation or nutrient limitation [7,8,9]. Injury may be measured by the difference in counts when stressed cells are simultaneously enumerated on selective and nonselective medium [10]. Only uninjured cells are recovered on selective medium, whereas the nonselective medium is assumed to recover both injured and uninjured cells [11,6]. Baird-Parker and Davenport [12] proposed that pyruvate was the component which was responsible for enhanced growth of stressed cells and that this effect was due to the degradation of hydrogen peroxide (H_2O_2). *James, et al* [14] confirmed the enhancing action of pyruvate in the enumeration of stressed *Staphylococcus aureus* and *E.coli*. The production of H2O2 during aerobic growth and the inability of the cell to destroy this toxic compound result in loss of colony forming ability. While *Martin et al*.[15,16] noted improved recovery of injured *Staphylococcus aureus, Pseudomonas fluorescens, Salmonella typhimurium* and *Escherichia coli* when selected media are supplemented with either catalase or sodium pyruvate.

This study was undertaken to evaluate the modified Trypton Glucose Extract Agar (TGA) and modified Violet Red Bile Agar (VRBA) for their suitability for the recovery of *E. coli* cells injured by freezing and determined the effect on injured cell recovery that either degrade H_2O_2 or block its formation.

Materials and Methods

Test organism. *Escherichia coli* isolate obtained from the department of Food Science and Biotechnology was grown in TGA slants and stored at 4ºC. Trypton soy broth containing 0.3% yeast extract (TSY broth) was inoculated with this culture and incubated at 37ºC overnight. The cells were then transferred to 90 ml of tryptic soy broth and

incubated at 37ºC for 12hrs. Alate-exponential phase culture of *E.coli* grown in TSB was spectrophotometrically at 650 nm to absorbance of 1.0. The cells were centrifuged in refrigerated centrifuge at 7,000 xg for 12 min. and suspended in sterile distilled water to an optical density at 650 nm of 1.0.

Freezing and thawing. The cell suspension in 10-ml aliquots was frozen statically at -18ºC for1, 2, 3, 4, 5, 24 and 48 hrs .The contents were thawed in a water bath at 4ºC.

Plating medium. Nonselective and selective media were used. The nonselective medium was (TGA), and the selective medium was violet red bile agar (VRBA).The pH of the medium was aajusted 7.1- 7.3. This medium supplemented with various concentrations of compounds that either have an antioxidant function or degrade hydrogen peroxide (H_2O_2) or blocks its formation (Table (1)).Sodium pyruvate and sodium thioglycolate were added before autoclaving (TGA) or boiling (VRBA) medium.

n-Propyl gallate was sterilized by filtration and added to the medium after cooling to 45˚C. The media were poured into petri dishes and allowed to dry at 25 ˚C for 48 hrs before use.

Enumeration of cells

Serial dilutions were made in peptone saline water as needed. Cells were enumerated before and after being stressed by pour plating with nonselective (TGA) and selective violet red bile agar (VRBA) medium.

Injury repair studies

Stressed cells were serially diluted and plated on 15 ml of media. The plates were incubated at 37˚C for 24 hrs, and the colonies were enumerated. Further incubation did not increase the colony count significantly. Coli form colonies in the repair detection procedure appear to coliform colonies in VRBA [17]. All plates were incubated 24 hrs at 35 ˚C, after which all pink to-red colonies were counted.

Microbiological analysis

The Percentage was determined according to the following equation:

$$P = \frac{\text{Counts in supplemented medium - counts in unsupplemented medium}}{\text{counts in unsupplemeneted medium}} \times 100 \quad [18].$$

Statistical Analysis

Bacterial populations were converted to log10 cfu ml±1 and analysed statistically by the SAS General (19). Differences among treatments were examined for level of significance.

Results and Discussion

E.coli cells stressed by freezing at -18˚C for 1, 2, 3, 4, 5, 24, and 48 hrs were enumerated by pour plating simultaneously with unsupplemented and supplemented TGA or VRBA.VRBA is specified in standard methods procedures to enumerate coli forms in food and dairy products .Coli form organisms form red colonies [Fig.(1)]. The colonies that develop on the nonselective media represent both injured and uninjured cells ; only the uninjured cells develop on the selective media, and the difference between the number of colonies on the two media was a measure of the number of injured cells in the original culture or population(Fig.(2)).The plating of cells at zero time and up to 5 hrs of freezing on nonselective (TGA) and selective (VRBA) revealed only a slight reduction in numbers on TGA, while the numbers on VRBA were reduced considerably indicating a high degree of injury relative to a level of bile salt.

Fig. (3) showed that the same slight reduction in number on nonselective media than on selective media (VRBA).These organisms may be expected to manifest injury via increased lag phases of growth, increased sensitivity to a variety of selective media agents, damage to cell membranes and TCA-cycle enzymes, and breakdown of ribosomes [20] .If the outer cell membrane is damage, cells are unable to grow on selective media containing bile salts (such as VRBA) [21]. The results in Table (2) showed that VRBA counts were reduced approximately 50% after freezing and thawing. *E.coli* after freezing and thawing consisted of three kinds of cells: dead, injured and uninjured [17]. The cells which lost their ability to multiply in a nutritionally rich medium, such as VRBA, were considered dead. During incubation the count on the nonselective agar (TGA) reduces but the count on the selective agar (VRBA) increased after 48 hrs indicating that repair of the injured cells occurred. Similar observations were reported before [21, 23].

The percentage increase in colony counts in supplemented medium over the counts in a corresponding unsupplemented medium was determined (Table (3)). In VRBA, the largest percentage increase was obtained when the medium was supplemented with pyruvate (0.4) and thioglycolate (0.04).The same results were calculated (Table (4)) when the TGA supplementation with pyruvate and thioglycolate (0.4%+0.04%). Some compounds, such as thioglycolate (0.03%) decreased recovery in VRBA but enhanced recovery in TGA. As a result the type of injury influenced the effectiveness of the various supplement. For example, thioglycolate improved recovery of TGA but was detrimental recovery of VRBA counts. Pyruvate act to degrade peroxides, and the effectiveness of these agents was allowing for the recovery of injured cells suggests that the normal abilities of uninjured cells to destroyed peroxides were effected during injury [11]. Significantly ($P \leq 0.05$) higher counts were noted the improved repair detection procedure when either pyruvate or pyruvate with thioglycolate was used as a supplement. An increased in the number of false positives was observed in pyruvate supplemented media than in unsuplemented VRBA. Antioxidants containing phenolic group, i.e., *n*-propyle gallate was lethal to cells (data not shown).

Our results, strongly suggest an important role of H_2O_2 in the enumeration of microorganisms. The presence of sodium pyruvate and thioglycolate in the media, has been shown to overcome the effects of H_2O_2 and to increase enumeration of injured and normal microorganisms [21].

Table (1)
Type, concentration, and function of supplements.

Supplement	*Conc. (%)*	*Function*	*Time added relative to sterilization*
Sodium pyruvate	0.1and 0.5	Degrades H_2O_2 (14)	Before
Thioglycolate	0.03 and 0.06	Blocks formation of H_2O_2 [22]	Before
n-Propyl gallate	0.15 and 0.30	Antioxidizes [(23]	Before
Sodium pyruvate + Thioglycolate	0.4+ 0.04		Before

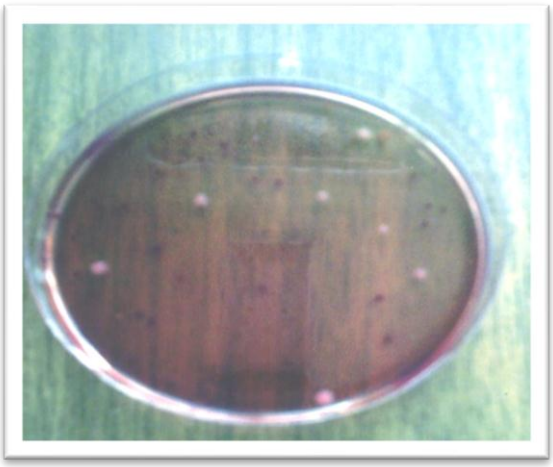

Fig.(1) E. coli grown on VRBA.

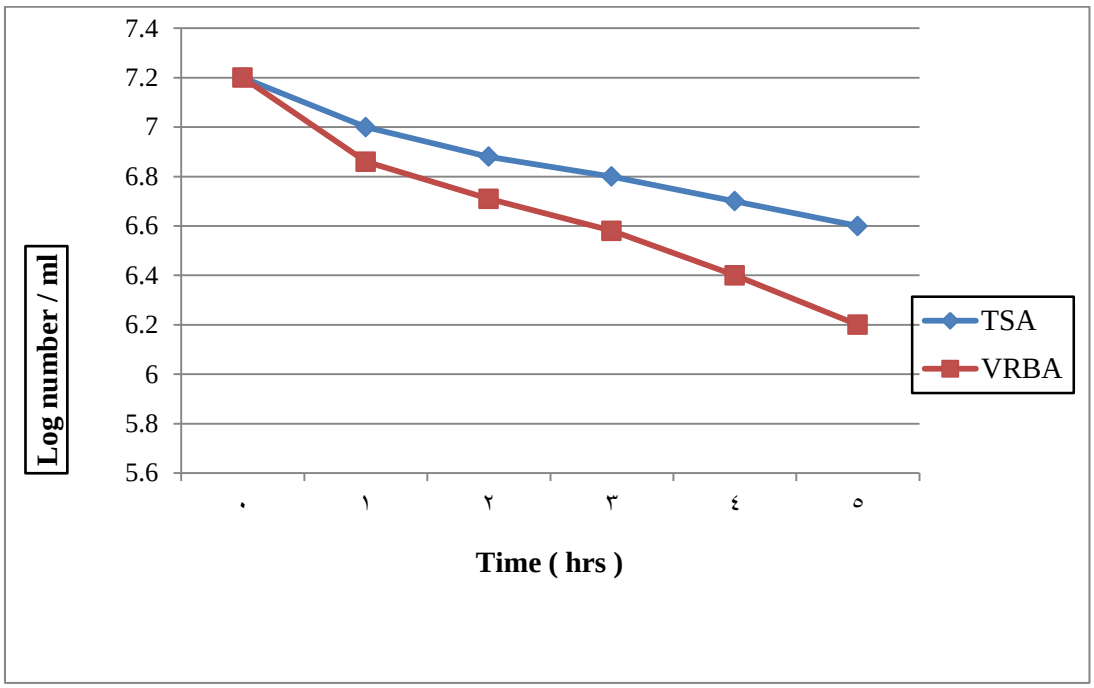

Fig.(2) Effect of freezing at -18°C on E. coli growth on TSA and VRBA.

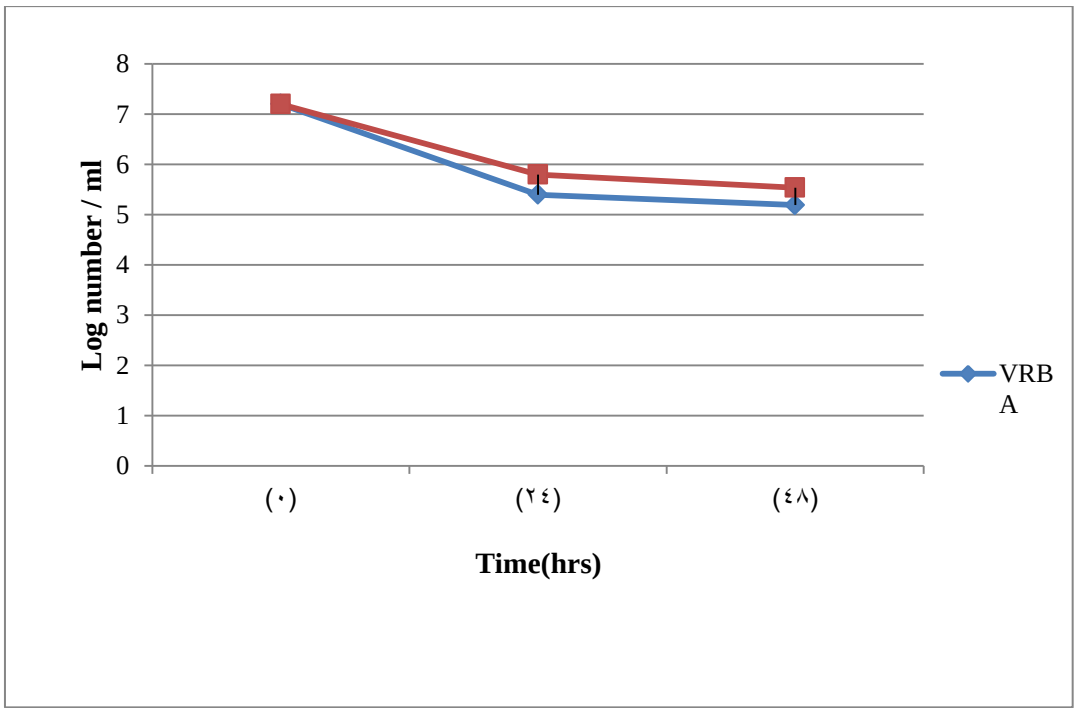

Fig.(3) Colony counts on TSA and VRBA ...for untreated and freeze-treated E. col.

Table (2)
Effect of freezing on the count of E.coli cells in VRBA and TGA media.

CFU\ml before freezing (10^4 X)	*Freezing time (hours) at -18C*	*CFU\ml in VRBA After freezing (10^7 X)*	*CFU\ml in TGA After freezing (10^7 X)*
1750	1	820	1080
1750	2	470	944
1750	4	190	620
1750	24	31	72
1750	48	142	64
	(LSD)	16.733*	21.649*

* ($P \leq 0.05$)

Table (3)
Effect of VRBA supplementation on the percentage of colony count of E.coli after freezing at -18˚C.

Supplement	*Concentration (%)*	*CFU in unsupplemented medium (10^4 X)*	*CFU in supplemented medium (10^4 X)*	*Increasment %*
Sodium pyruvate	**0.1**	**31**	**190**	**512**
	0.5	**31**	**410**	**1222**
Thioglycolate	**0.03**	**31**	**27**	**13-**
	0.06	**31**	**54**	**171**
n- propyl gallate	**0.15**	**31**	-	-
	0.30	**31**	-	-
Sodium pyruvate + thioglycolate	**0.40+ 0.04**	**31**	**610**	**1867**
(LSD)	--	--	--	**46.774***

*** ($P \leq 0.05$)**

Table (4)
Effect of TGA supplementation on the percentage of colony count of E.coli after freezing cells at -18˚C.

Supplement	*Concentration (%)*	*CFU in unsupplemented medium (10^4 X)*	*CFU in supplemented medium (10^4 X)*	*(Increase) %*
Sodium pyruvate	**0.1**	**31**	**240**	**674**
	0.5	**31**	**590**	**1803**
Thioglycolate	**0.03**	**31**	**44**	**42**
	0.06	**31**	**74**	**139**
n- propylgallate	**0.15**	**31**	-	-
	0.30	**31**	-	-
Sodium pyruvate + thioglycolate	**0.40+ 0.04**	**31**	**820**	**2545**
(LSD)	--	--	--	**71.591***

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الخلاصة

تتعرض بكتريا *E.coli* للإجهاد خلال التجميد على – ١٨°م، يتم استعادة الخلايا التي حافظت على عيوشيتها في بيئة مستخلص التربتون و الكلوكوز المتصلبة بالآكر وهي غير اختيارية. أو في بيئة أملاح الصفراء الحمراء البنفسجية المتصلبة بالآكر وهي بيئة اختيارية. واللتان تدعمان بالعديد من المواد التي تعمل إما على تحطيم بيروكسيد الهيدروجين أو تقوم بإيقاف تكوينه. اختبرت تراكيز متباينة للمواد التالية: بيروفيد الصوديوم ، صوديوم ثايوكلايكوليت ، ن–بروبيل – كاليت. سبب إضافة المادتين الأولى والثانية إلى كلتا البيئتين أعلاه إلى زيادة المعنوية في كمية الخلايا المستعادة. إن تدعيم بيئة أملاح الصفراء الحمراء البنفسجية زاد في أعداد الخلايا المستعادة ، غير أن أعدادها يمكن اعتبارها أقل من تلك التي تم استعادتها عند أستعمال بيئة مستخلص التربتون و الكلوكوز. إن استعادة خلايا *E. coli* المجهدة بالتجميد قد تحسن بشكل كبير بإضافة بايروفيت الصوديوم والثايوكلايكوليت وعلية توصي الدراسة الحالية باستعمال ٠.٠٤% من البيروفيت و ٠.٠٠٤% من الثايوكلايكوليت لتدعيم البيئة المستعملة في طريقة عد الخلايا المستعادة .