

**STAPHYLOCOCCAL GROWTH AND ENTEROTOXIN PRODUCTION
IN READY TO EAT FOOD
AT DIFFERENT STORAGE TIME AND TEMPERATURES**

**BY
AMJAD G. MOHAMED**

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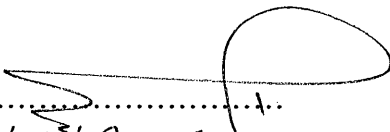
DEDICATION

I dedicate this work to my husband, Emad, who believing, encouraging, tolerating and understanding me through the whole studying period, and to my daughter Dana for being my power source of success.

This dedication is also extended with special thanks to my loving father and mother for their sincere love and ever-encouragement towards higher education and success.

DECLARATION

I certify that this work has not been accepted in substance for any degree, and is not concurrently being submitted for any degree other than of (Master of Food Safety and Quality Management) being studied at the University of Greenwich. I also declare that this work is the result of my own investigations except where otherwise identified by references and that I have not plagiarised another's work.

Signature:
Date: 28/8/2007

Supervisor Signature:
Date: 28/8/2007

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ABSTRACT

Foodborne intoxication caused by *Staphylococcus aureus* is one of the commonly reported illnesses that is caused by the ingestion of staphylococcal enterotoxin preformed in contaminated foods. Although *Staphylococcus aureus* and other coagulase positive *Staphylococcus* species are the main responsible for foodborne intoxication, investigators emphasise that staphylococcal enterotoxins are able to be produced by some coagulase negative staphylococci strains that could be a potential cause of foodborne intoxication.

In the present study, the effect of different storage time and temperatures on the growth and subsequent enterotoxin production by coagulase positive and negative *Staphylococci* representative strains (*Staphylococcus aureus* (NCTC 10657), *Staphylococcus aureus* (NCTC 8532), and *Staphylococcus epidermidis* (NCTC 12100)) was observed in a laboratory system. The growth and enterotoxins production by these commercially available cultures was examined at 3 different temperatures (5°C, 30°C and 44°C) considering the food product which is likely to encounter under the anticipated duration and storage (5°C), the storage temperature to which the product may be subjected by retailer to consumer (30°C) and the temperature (44°C - the average summer temperature in Bahrain) at which the product may be subjected by retailer or consumer during transferring and also supporting the growth and enterotoxin production (Maximum enterotoxin production temperature) by enterotoxigenic *Staphylococci*.

Rapid analytical methods are essential for routine monitoring purpose and public health protection. Different enterotoxins detection methods have been developed recently and in this project reversed passive latex agglutination (RPLA) assay has been used for staphylococcal enterotoxins A, B, C and D detection and semi – quantitative estimation in ready to served custard samples filtrates which have

been spiked with the enterotoxigenic *Staphylococcus aureus* NCTC 10657 and *Staphylococcus epidermidis* NCTC 12100.

The obtained results suggest that SET-RPLA toxin detection kit can be applied directly to food with good correspondence of culture results.

Neither significant changes in the original cell's count nor toxin production were observed among the three examined strains at 5°C during the 3 storage days. Lowering the incubation (storage) temperature of the contaminated food samples inhibit the growth and enterotoxins production by the coagulase positive and coagulase negative staphylococci (CPS & CNS) strains.

The optimum growth temperature for the examined staphylococci species was at 30°C, therefore, the long storage of foodstuff at this Staphylococcal - critical growth temperature encourages the growth and the subsequent enterotoxins production. However, the optimum enterotoxins production temperature was at 44°C where the growth rate of *Staphylococcus* species is fast due to high metabolic activity.

The growth rate of *Staphylococcus epidermidis* was observed to be slower than the other examined *Staphylococcus aureus* species therefore no enterotoxins was detected in the first and second storage days at both 30°C and 44°C though the bacterial count was more than 10⁷CFU/ml. However, SEC was detected in the third day at 30°C and 44°C with an agglutination degree of (+2) and (+3) respectively.

There was no direct effect observed of changing the pH value on the staphylococcal enterotoxin production during the 3 incubation day. The drop in pH value due to microbial growth decreases the rate of growth and the subsequent enterotoxin production.

Staphylococcal growth and enterotoxin production can not be controlled by single factor and the effectiveness of the storage temperature on the growth and the

subsequent and enterotoxin production by staphylococci influenced by the effectiveness of other growth factors such as time, pH, water activity (section 1.7).

Coagulase activity and mannitol fermentation test have been suggested by several studies as good indicators of enterotoxigenicity. However, neither mannitol fermentation nor coagulase activities appeared to be specific for enterotoxigenicity of all *Staphylococcus* species (e.g. *Staphylococcus epidermidis* NCTC 12100). Both tests can be useful to differentiate between these species and to detect the potentially enterotoxigenic CPS and CNS strains.

The most reliable test for enterotoxigenicity is the direct detection of these enterotoxins directly in food. In this study, SET – RPLA toxin detection kit represent a simple, sensitive, quick and versatile method for routine monitoring purposes, however, its regarded as presumptive and must be confirmed by other standard methods (Feng, 1996).

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CHAPTER ONE

INTRODUCTION

INTRODUCTION

1.1 Aims and objectives

Among the predominant foodborne diseases, foodborne intoxication caused by *Staphylococcus aureus* is one of the commonly reported illnesses caused by the ingestion of preformed staphylococcal enterotoxin in foods. The average weather temperature and relative humidity in Bahrain increase the potential of staphylococcal food poisoning outbreaks specially during the summer season (July – September) where the average temperature is 42°C - 45°C (optimum production temperature for staphylococcal enterotoxins). With some exception, this disease is associated with *Staphylococcus aureus*; however, other coagulase positive and negative staphylococci such as *Staph. intermedius*, *Staph. xylosus*, *Staph. haemolyticus* *Staph. epidermidis* and *Staph. hyicus* have been associated with foodborne intoxication (Victor *et al.*, 1969; Hoover *et al.*, 1983; Bautista *et al.*, 1988; Valle *et al.*, 1990; Jay, 1992; Khambaty *et al.*, 1994; Bennett, 1996; Becker *et al.*, 2001; Loir *et al.*, 2003; Cunha *et al.*, 2005).

The purpose of this study is to determine and compare the effect of different storage time and temperature on the growth rate and enterotoxins production of two different enterotoxigenic species of *Staphylococcus* (CNS & CPS) in ready to eat food. In addition, this study is undertaken to determine the relation between the growth and enterotoxin production under different storage temperature for the two groups of organisms.

1.2 Background

In spite of the introduced advances in production technologies, distribution, hygienic standards and consumer education during the last few decade, the food poisoning in most of the developed and developing countries continues to increase (Doyle, 1994; Maurice, 1994; Coyhlan, 1998; Russell and Gould, 2003).

Staphylococcal food poisoning causes an acute gastroenteritis foodborne intoxication which follows the consumption of food contaminated with pre-formed staphylococcal enterotoxins produced by certain strains of staphylococci (Gilbert and Wieneke, 1973; Hobbs and Roberts, 1993). It concerned as one of the most common types foodborne intoxication worldwide (Garthright *et al.*, 1988; Levine *et al.*, 1991; Bunning *et al.*, 1997; Archer and Young, 1998; Deshpande, 2000; Dipinto *et al.*, 2004). The relative occurrence of Staphylococcal food poisoning varies in different countries due to differences in eating habits and locations (Todd, 1978; Anon, 1985; Roberts 1988; Tranter and Brehm, 1990, Mossel and Netten, 1990).

1.3 History

Staphylococcus aureus has been incriminated in a number of foodborne diseases in humans since 1880 (Ogston, 1880). In 1884, the characteristics of two pigmented colony types (white and orange – colour cells) of the genus *Staphylococcus* were studied by Rosenbach. He named the orange – colony forming organism *Staphylococcus pyogenes aureus* and the white – colony forming organism *Staphylococcus pyogenes albus* (Baird – Parker, 1990).

In 1830, Olliver linked a food poisoning to meat pie in France and he noted that after the consumption of bacon, sausages, ham and cheese, similar symptoms were often developed (Mossel and Netten, 1990). In 1884, a large outbreak caused by eating contaminated cheese with Staphylococci in Michigan had been illustrated by Vaughan and Sternberg (Dack, 1949; Baird – Parker, 1990).

The first illustration of the etiological role of *Staphylococcus aureus* in food poisoning was in USA by Sternberg in 1885. Later on Sternberg's observations were verified by (Denys, 1894) in Lovain, (Owen, 1907) in USA and (Barber, 1914) in Philippines (Mossel and Netten, 1990).

Staphylococcal food poisoning caused by filterable toxin (enterotoxin) was primarily demonstrated by Dack and his fellow researchers (1930) who identified a toxin in an organism isolated from Christmas cake cream filling that caused an outbreak in Chicago (Baird – Parker, 1990; Mossel and Netten, 1990).

Various studies used human volunteers and young cats to establish a link between the organisms which had been isolated from foods and the enterotoxicity of them. Using monkeys in such experiment by (Surgalla *et al.*, 1953) developed a challenge test on intragastric feeding of extracts/culture fluids and gave more cohesive results than those obtained with humans and cats (Mossel and Netten, 1990). Since that time, many research papers on Staphylococci growth, survival and enterotoxin production in food have been published.

In 1959, (Omori and Kato) were reported a food poisoning outbreak due to enterotoxin – producing coagulase negative staphylococci among Japanese high school students. Another outbreak caused by (CNS) has been illustrated by (Breckinridge and Bergdoll, 1971).

Over 2000 incidents of Staphylococcal food poisoning were reported in England and Wales in the period 1949 – 1971 (Gilbert and Wieneke, 1972). However, this number of outbreaks has been declined to 35 cases in the period 1992 – 2006 (HPA, 2007). In the Kingdom of Bahrain, around 29 Staphylococcal food poisoning outbreaks were reported by public health laboratory in the period 2004 – 2006.

1.4 Staphylococci (general characteristics)

Staphylococci (Greek: staphyli = a bunch of grapes) are Gram – positive, non – motile, catalase – positive, oxidase negative and non – spore forming cocci (0.5 – 15 µm in diameter) that are capable of dividing in more than one plane to form irregular, grape- like, three – dimensional clusters of cells (Figure 1-1) (Cohen, 1972). The cell wall of this genus consists of two main component, peptidoglycan and teichoic acids. This peptidoglycan characterize by the pentaglycine cross – bridge between the peptide subunit (Cohen, 1972).

Staphylococci are facultative anaerobic bacteria which grow better in the presence of oxygen. In contrast to their morphologically similar members of the genus *Micrococci* (obligate aerobes), *Staphylococci* have the ability to create fermentative metabolism (Cohen, 1972; Baird – Parker, 1990). The main characteristics separating the genera *Staphylococcus* from *Micrococcus* are illustrated in Table 1-1.

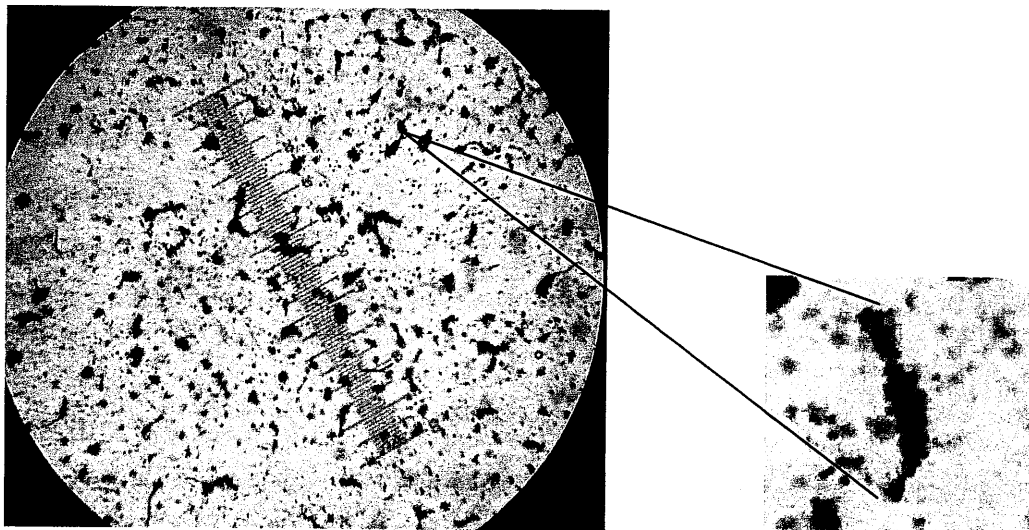


Figure 1-1 Irregular (grape- like) cells arrangement of the Gram + ve *Staphylococcus aureus* (NCTC 10657).

Table 1-1 The main characteristics separating *Staphylococcus* spp from *Micrococcus* spp.

Characteristic	<i>Staphylococcus</i> spp	<i>Micrococcus</i> spp
Nucleic acid		
Mol % g + c in DNA	30-37	66-75
Cell wall		
Teichoic acid	+	-
Glycine in interpeptide	+	-
Bridge of peptidoglycan		
Respiratory chain		
Cytochromes <i>c</i> and <i>d</i>	-	+
Major menaquinones	MK- 6 to 8 (unsaturated)_	Mk-7 (H ₂) to 9 (H ₂) (partially hydrogenated)

1.5 Staphylococcal Enterotoxins

Staphylococcal enterotoxins (SEs) are simple proteins with relatively low molecular weights polypeptide chain range between 27500 – 30000 (Tranter and Breham, 1990). They were classified in to seven serologically recognisable types (SEA, SEB, SEC1, SEC2, SEC3, SED, and SEE) in which lysine, tyrosine, aspartic acid and glutamic acid are relatively their predominant building units (Baird – Parker, 1990; Tranter and Breham, 1990). Recently, further different types of Staphylococcal enterotoxins (SEF, SEG, SHE, SEI, SEK, SEL, SEM, SEN, SEO, SEU) that share sequence and structure similarities (Table 1-2) have been identified (Ren *et al.*, 1994; Su and Wong, 1995; Zhang *et al.*, 1998; Munson *et al.*, 1998; Jarraud *et al.*, 2001; Orwin *et al.*, 2001; Omoe *et al.*, 2003; Loir *et al.*, 2003; Sergreev *et al.*, 2004; Lina *et al.*, 2004; Nakayama *et al.*, 2006). These Staphylococcal enterotoxins are commonly found in foodborne isolates of *Staphylococcus aureus* and several studies reported their roles as staphylococcal food poisoning – pathogenic factors (McLauchlin *et al.*, 2000; Jarraud *et al.*, 2001;

Omoe *et al.*, 2002; Rosec and Giraud, 2002; Becker *et al.*, 2003; Nakayama *et al.*, 2006). Staphylococcal enterotoxins are soluble in water and saline solution (Loir *et al.*, 2003), therefore saline solution is used in some toxin detection kit such as Set- REPLA (Oxoid). They are highly stable molecules that resist most of proteolytic enzymes such as pepsin and trypsin, therefore their activity remains after ingestion in the digestive tract. In addition, SEs resists several enzymes such as chymotrypsine, rennin and papain (Loir *et al.*, 2003). In some cases SEs could be destroyed by these enzymes. For example, Bergdoll (1983) reported that enterotoxin B (SEB) could be destroyed by pepsin enzyme at a pH level of 2, however, under normal stomach conditions (especially after food ingestion) the pH level mostly above 2 and the pepsin enzyme will not affect the activity of the SEB. Staphylococcal enterotoxins are heat stable molecules (Tranter and Brehm, 1990; Lori *et al.*, 2003), and its heat stability in food stuffs more than those produced in laboratory media (Bergdoll, 1983).

Table 1-2 Percentage of amino acid identity in different staphylococcal enterotoxins (SEs) (Lori *et al.*, 2003)

Toxin	SEA	SEB	SEC	SED	SEE	SEG	SEH	SEI	SEJ	SEN	SEM	SEO
SEA	100	33	30	50	83	27	37	39	64	35	39	37
SEB		100	68	35	32	43	33	31	33	29	32	36
SEC			100	31	29	41	27	26	30	26	29	33
SED				100	52	27	35	33	51	41	38	39
SEE					100	27	35	35	63	37	39	37
SEG						100	34	28	29	28	31	30
SEH							100	33	35	38	34	31
SEI								100	34	31	31	57
SEJ									100	38	42	33
SEM										100	28	31
SEN											100	42
SEO												100

1.5.1 Production of Staphylococcal Enterotoxins

The most frequent staphylococcal enterotoxins are produced by *Staphylococcus aureus* *Staphylococcus aureus* - human isolates followed by bovine and ovine mastitis isolates (Dabrowski and Medralla, 2005). Both SEB and SEC are similar in their amino acids composition and their production occurs at the end of the stationary phase where the levels of *Staphylococcus* species are 10^5 CFU/ml. They are mainly produced under plasmid control in optimal environmental condition (Bergdoll, 1989; Baird – Parker, 1990). On the other hand, SEA, SED and SEE have similar amino acids composition and their production occurs through the logarithmic growth phase under chromosomal controlled (Bergdoll, 1989; Baird – Parker, 1990). Unlike SEB and SEC, SEA and SED produced under wide range of environmental conditions, therefore these enterotoxins (SEA and SED) involved in most food poisoning outbreaks (Bergdoll, 1989; Baird – Parker, 1990).

The production of Staphylococcal enterotoxins can occur in both foodstuff and laboratory culture but the rate and level of production is affected by the media type and it also depends upon the toxin producing types as well as between the strain of the same toxin type (Spero *et al.*, 1988).

A study shows that production of SEA by mixed *Staphylococcus aureus* strains producing SEA, SEB, and SEC when they were grown in Brain Heart Infusion (BHI) broth is much higher than the other enterotoxins under wide range of growth conditions (Baird – Parker, 1990).

The media used for outturns the production of staphylococcal enterotoxins production mainly contain the essential energy source (amino acids, glucose or glutamate), vitamins, and salts, or those which achieved the minimal requirements for Staphylococcal enterotoxins production (Tranter and Brehm, 1990). For example, Brain Heart Infusion broth (BHI) (Casman and Bennett, 1963), casein hydrolysate containing media (Bergdoll *et al.*, 1959, 1961) and NZ anime NAK reported in other studies to produce SEC and SED (Ewald, 1987).

Examples of enterotoxigenic-coagulase negative *Staphylococcus* species and their enterotoxins are shown in Table 1-4.

Similarly, (Valle *et al.*, 1990) found 9 CNS – enterotoxin producer strains isolated from healthy goats, two of them produced only one enterotoxin (*Staph. chromogens* produced SEC and *Staph. lentus* produced SEE) and the remaining 7 isolates produced more than one enterotoxins (*Staph. caprae*, *Staph. epidermis*, *Staph. haemolyticus*, *Staph. sciuri*, *Staph. saprophyticus* *Staph. warneri* and *Staph. xylosus*).

Enterotoxigenic CNS strains were isolated from workers hands in 50 restaurants in Kuwait City (Udo *et al.*, 1999). Six percent (6%) of these isolates produced one or more Staphylococcal enterotoxins (SEA, SEB and SEC). Such enterotoxigenic strains could be a source of cross contamination to the prepared food which might progress to outbreaks of food poisoning if this prepared food is held at a storage temperature that support the growth of *Staphylococcus* species and permits the subsequent enterotoxins production (Hobbs, 1973).

A recent study created by (Cunha *et al.*, 2005) on a total of 88 food samples emphasised genetically the capability of CNS to produce staphylococcal enterotoxins. The percentage of the isolated CNS among other bacteria in the tested food samples was 22.7% which include the following strains; *Staphylococcus epidermidis* (40%), *Staphylococcus xylosus* (20%), *Staphylococcus warneri* (20%), *Staphylococcus saccharolyticus* (15%), *Staphylococcus hominis* (5%). In this study, the enterotoxin genes were detected for four isolates (Table 1-5) and these include *Staph. epidermidis* (SEA), *Staph. xylosus* (SEA), *Staph. xylosus* (SEC1) and *Staph. hominis* (SEA). The predominant gene detected in this study was SEA that most food poisoning outbreaks involved (Baird – Parker, 1990).

Table 1-3 Characteristics of the enterotoxigenic *Staphylococcus* species known to produce enterotoxins (Jay, 1992; Baird-Parker, 2000).

Species	Coagulase production	TNase production	Mannitol production	Enterotoxin production	Hemolysis production
<i>Staph. aureus</i>	+/-	+	+	+	+
<i>Staph. intermedius</i>	+	+	+/-	+	+/-
<i>Staph. hyicus</i>	+	+		+	-
<i>Staph. cohnii</i>	-	-	+/-	+	-
<i>Staph. chromogens</i>	-	-	+/-	+	-
<i>Staph. epidermis</i>	-	-	+	+	+/-
<i>Staph. haemolyticus</i>	-	-	+	+	+/-
<i>Staph. hominis</i>	-	-	-	+	-
<i>Staph. intermedius</i>	-	-	+	+	-
<i>Staph. lentus</i>	-	-	+	+	-
<i>Staph. xylosus</i>	-	-	+/-	+	+

1.4.1.1 Coagulase positive *Staphylococcus* species (CPS)

Among coagulase positive staphylococcal species, it was thought previously that only *Staphylococcus aureus* had been implicated in human foodborne disease and other species were not (Bennett, 1996). However, other food predominant coagulase positive staphylococci such as *Staphylococcus intermedius* have been reported to produce staphylococcal enterotoxins (Baird – Parker, 1990; Jay, 1992; Bennett, 1996; Becker *et al.*, 2001) and clearly involved in food poisoning outbreaks (Khambaty *et al.*, 1994; Lori *et al.*, 2003). Unlike *Staphylococcus aureus*, this species does not ferment mannitol under anaerobic conditions (Evans, 1948; Mossel, 1962; Waart *et al.*, 1968; Mossel & Netten, 1990). In a study reported by (Hirooka *et al.*, 1988) on 73 of staphylococci species which were recovered from pyodermitis in dogs in Brazil, 13 of 52 isolated *Staph.*

intermedius were TNase and enterotoxins producer. Five of the enterotoxigenic strain produced staphylococcal enterotoxin (SE) E (SEE), four produced SED, one produced SEB, one produced SEC, one produced SED/E and one produced SEA/C (Jay, 1992).

Another CPS is *Staph. hyicus* which has the ability to produced enterotoxins demonstrated in several studies. One of these studies created by (Valle *et al.*, 1990) showed that two of six coagulase positive *Staph. hyicus* which isolated from healthy goats were capable to produce SEC. Similarly, two other studies demonstrated the ability of this species to produce enterotoxin and create enterotoxin response in cynomologus monkeys (Hoover *et al.*, 1983; Adesiyun *et al.*, 1984; Jay, 1992).

1.6.2 Coagulase negative *Staphylococcus* species (CNS)

CPS have been considered as the strain of importance in many food laboratory and routine tests ignoring the existence of Coagulase negative and its important as enterotoxin producing strains though it have been incriminated in many staphylococcal food poisoning outbreaks (Jay, 1992; Bennett, 1996; Cunha *et al.*, 2005).

In 1969, 18 out of 31 CNS strains had been reported to produce enterotoxins (Victor *et al.*, 1969). Other study done by (Hoover *et al.*, 1983) demonstrated the existence of 5 enterotoxigenic, CNS and TNase producer strains (Jay, 1992). However, none of the two studies identified the *Staphylococcus* species.

A few years' later, other study illustrated the CNS species which have been isolated from sheep milk along with *Staph. aureus*. These species were: one strain of *Staphylococcus cohnii* produced SEC, 3 strains of *Staphylococcus epidermis* produced SEC and SEB/C/D, 5 strains of *Staphylococcus haemolyticus* produced SEA, SED and SEB/C/D and SEC/D and 4 strains of *Staphylococcus xylosus* produced SED (Baustista *et al.*, 1988). *Staphylococcus haemolyticus* is also

reported in other studies to produce SEC and SED (Ewald, 1987). Examples of enterotoxigenic–coagulase negative *Staphylococcus* species and their enterotoxins are shown in Table 1-4.

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A recent study created by (Cunha *et al.*, 2005) on a total of 88 food samples emphasised genetically the capability of CNS to produce staphylococcal enterotoxins. The percentage of the isolated CNS among other bacteria in the tested food samples was 22.7% which include the following strains; *Staphylococcus epidermidis* (40%), *Staphylococcus xylosus* (20%), *Staphylococcus warneri* (20%), *Staphylococcus saccharolyticus* (15%), *Staphylococcus hominis* (5%). In this study, the enterotoxin genes were detected for four isolates (Table 1-5) and these include *Staph. epidermidis* (SEA), *Staph. xylosus* (SEA), *Staph. xylosus* (SEC1) and *Staph. hominis* (SEA). The predominant gene detected in this study was SEA that most food poisoning outbreaks involved (Baird – Parker, 1990).

These studies emphasise that CNS are able to produce Staphylococcal enterotoxins that could be a potential cause of food poisoning outbreaks, therefore CNS should not be ignored especially in the suspected cases of staphylococcal food poisoning. In addition, more attention, investigation and studies are required towards its toxigenic capacity.

1.7 The effect of environmental factors on the growth and enterotoxin production of by *Staph. aureus* and related staphylococci

1.7.1 Effect of temperature

Staphylococci are typical mesophilic organisms that grow optimally in a temperature range between 35° - 40°C (Cohen, 1972; Baird – Parker, 1990). Generally, they are grow at temperature range of 6.5° - 46°C (Cohen, 1972) and their enterotoxins are produced within the temperature range of 10 - 48°C ((Baird – Parker, 1990) and optimum between 40 - 45°C (Baird-Parker, 1990; Jay, 1992).

Although they are mesophilic organisms, some food poisoning strain are capable to grow at low storage temperature of 6.7°C (Angelotti *et al.*, 1961). Palumba (1986) discussed the effect of low temperature (slightly above 5°C up to 12°C) on the growth of certain foodborne pathogens including *Staphylococcus aureus*. He reported that refrigeration (5°C) can not keep the foods absolutely safe due to the ability of some pathogenic organisms to survive and grow at this low temperature. Angelotli and his follow workers (1961) have been reported that *Staphylococcus aureus* grew in sterile custard at 7.7°C. Similarly, *Staph. aureus* -S6 found to produce enterotoxin at 10°C in laboratory cured ham (Genigeorgis *et al.*; Palumba, 1986). Rather than other enterotoxigenic staphylococcal strains, there are a vast studies and information on various intrinsic and extrinsic factors (Table 1-6) affecting the growth and subsequent enterotoxin production of *Staphylococcus aureus* in foods (Baird – Parker, 1990; Mossel & Netten, 1990).

Table 1-6 Factors affecting growth and enterotoxin production by *Staphylococcus aureus* (Baird – Parker, 1990).

Factor	Optimum Growth	Growth Range	Optimum Toxin Production	Toxin production range
Temperature (°C)	37	7 - 48	40 – 45	10 - 48
pH	6 – 7	4-10	7 – 8	4 – 9.6
Water activity (a_w)	0.98	0.83 - >0.99	0.98	0.85 - >0.99
E_H	+ 200 MV	<-200 MV to >+ 200 MV	?	?
Atmosphere	aerobic	anaerobic-aerobic	aerobic (5 – 20% dissolved O ₂)	anaerobic-aerobic

1.7.2 Effect of salts and other chemicals

Most *Staphylococcus* species capable of withstanding evaluated salt concentration conditions, for example, all of *Staphylococcus* species have the ability to grow in the presence of 10% of NaCl (Jay, 1992) and some can tolerate higher percentage up to 15% of NaCl (Cohen, 1972).

Staphylococcus aureus grow perfectly in NaCl – free media as well as in a concentration of 7 – 10 % NaCl and some strain can adapt as high as 20% (Jay, 1992). The maximum concentration that support the growth and the enterotoxin production is highly influenced by other parameters such as temperature, pH, Eh and a_w (Jay, 1992). Compound such as tellurite, acriflavine, mercuric chloride, neomycin, polymyxin, and sodium azide is highly resisted by *Staphylococcus aureus*, therefore this compounds are widely used as selective agents in culture media (Jay, 1992).

Staphylococci are resistant to lysis by lysozyme, however, they are very sensitive to lysostaphin endopeptidase lysis which breaks down their cell wall by breaking the peptide bridges (glycyl - glycine links) of the peptidoglycan (Cohen, 1972). Both *Staphylococcus aureus* and *Staphylococcus epidermidis* are sensitive to novobiocin, however, *Staphylococcus saprophyticus* is not (Jay, 1992). *Staphylococcus aureus* is sensitive to borate, whereas *Staphylococcus epidermidis* is not (Jay, 1970; Jay, 1992).

1.7.3 Effect of pH

In general, *Staphylococcus* species are capable of growth over a wide range of pH values range from 4.5 – 9.3 with an optimum of between 7.0 – 7.5 (Cohen, 1972). In particular, *Staphylococcus aureus* is able to grow over pH range of 4 – 10 (Baird – Parker, 1990) and its optimum temperature is in the range of 6 – 7 (Baird – Parker, 1990; Jay, 1992). Staphylococcal enterotoxins production occurs within a pH range of 4 – 9.6 and optimum production of 7 – 8 (Baird – Parker, 1990). The production of SEA is less sensitive to pH level than SEB (Jay, 1992).

An example of the effect of pH and NaCl on the SEC production by *Staph. aureus* 137 at 37°C is illustrated in (Table 1-7).

Table 1-7 The effect of pH and NaCl on the SEC production by an inoculum of 10^8 C.F.U/ml of *Staph. aureus* 137 in a protein hydrolysate medium incubated at 37°C for 8 days (Genigeorgis *et al.*, 1971; Jay, 1992).

pH range	4.00 – 9.83	4.4 – 9.43	4.50 – 8.55	5.45 – 7.30	4.50 – 8.55
NaCl %	0	4	8	10	12
Enterotoxins production	+	+	+	+	-

1.7.4 Effect of water activity (a_w)

Staphylococci are unique in their growth ability at in lower level of water activity compared to the other non halophilic bacteria and foodborne pathogens (Jay, 1992; Baird-parker, 2000). Under optimal condition of other staphylococcal growth parameters, they can adapt a_w as low as 0.83 (Jay, 1992; Bremer *et al.*, 2004). The optimum growth and staphylococcal enterotoxins production occur at a_w level of 0.99 (Bremer *et al.*, 2004). Regarding staphylococcal enterotoxins, Baird-Parker (2000) demonstrated that under all conditions of growth, the production of staphylococcal enterotoxin (SE) A (SEA) occur at much lower water activity than SEB and SEC. The minimal level of a_w supporting *Staphylococcus aureus* growth and subsequent enterotoxins production depends on several factors such as humectants, temperature, Redox potential and pH (Bergdoll, 1989; Watson, 1998; Baird-Parker, 2000). The effect of a_w on the growth and enterotoxin production is shown in Table 1-6.

1.7.5 Effect of other bacterial growth

The growth of enterotoxigenic *Staphylococcus* strains are also affected by the competitive growth of some microorganisms in food (Troller & Frazier, 1963). As illustrated by Peterson and his follow workers (1962) naturally occurring bacteria in foods repress the growth of staphylococci and this repression varies under different environmental condition, for example:

- The inhibition of staphylococci increases as the pH level increases above 8 or decreases below 6;
- The inhibition of staphylococci increase when stored/held at room temperature;
- The inhibition of staphylococci increase as the proportional number of Staphylococci cells decreased in food;
- The inhibition of staphylococci decrease in food with salt concentration above 3.5 % w/w.

In addition, it had been reported that staphylococci are suppressed by the growth of naturally occurring - saprophytes in milk (Newman, 1943), in creamed chicken (Straka & Combs, 1952) and in ground pork (Miller, 1955). Similar result has been achieved by Troller and Frazier (1963) when they confirmed the sensitivity of enterotoxigenic Staphylococci in the presence of competitive organisms under different environmental conditions. In this experiment, 5 types of inhibitory bacteria (*Bacillus cereus*, *Proteus vulgaris*, *Serratia marcescens*, *Escherichia coli* H-52 and *Aerobacter aerogenes*) were tested against *Staph. aureus* 196. The inhibition of the enterotoxigenic *Staph. aureus* 196 was greatly increased in the presence of *Escherichia coli* H-52 in nutrient broth at 20°C (Troller & Frazier, 1963). Nevertheless, several studies later verified that *Staph. aureus* is sensitive and a weak competitor with genera of food spoilage microorganisms (Table 1-8) such as *Pseudomonas* sp and Lactobacilli (Landol *et al.*, 1965; Digiacinto & Frazier, 1966; Kao & Frazier, 1966; Seminiano & Frazier, 1966; Gilliland & Speck, 1972; Niiskanen & Nurmi, 1976; Metaxopoulos *et al.*, 1981). However, if the water activity of food is less than 0.95, the growth of these spoilage microorganisms inhibited allowing staphylococci to develop in the food without any competition unless the storage temperature limits their growth (Mossel & Netten, 1990).

Table 1-8 Genera and species of spoilage organisms demonstrated to act as antagonist against *Staphylococcus aureus* (Mossel & Netten, 1990).

No.	<i>Staphylococcus aureus</i> antagonist organisms
1	<i>Acinetobacter</i>
2	<i>Aeromonas</i>
3	<i>Bacillus</i>
4	Enterobacteriaceae
5	<i>Enterococcus</i>
6	Lactobacillaceae
7	<i>Pseudomonas</i>
8	<i>Staphylococcus epidermidis</i>
9	<i>Streptococcus</i>
10	Saprophytic flora

The control mechanism of staphylococcal enterotoxigenesis should be taken initially by preventing the enterotoxigenic organism's growth in food (Mossel & Netten, 1990). Due to the wide spread of *Staphylococcus aureus* in human and animals, it is difficult to prevent contamination of food, and the greatest way to limit contamination of food is by strict adherence to good hygienic practices (Mossel & Netten, 1990). The formation of Staphylococcal enterotoxin can be managed completely by controlling the temperature (storage food below 7°C); however, this low temperature rarely remains constantly in catering and retailing where most of food poisoning outbreaks occur (Beckers, 1988; Bryan, 1988; Roberts, 1988; Todd, 1989; Mossel & Netten, 1990).

1.8 Habitat of enterotoxigenic types and sources of contamination

Staphylococci are wide spread in the environment which can be found in water, air, sewage, dust, environmental surfaces, varieties of food (see section 1.4.9) and food equipments, humans and animals (FDA, 2006).

In animal, staphylococci mainly exist on skins and mucosal membrane surfaces. They are highly adapted to living on the warm-blooded animals surfaces; for instance, *Staphylococcus gallinarum* is generally associated with poultry, *Staphylococcus Caprae* associated with goats and *Staph. hyicus* associated with pig's skin (Baird – Parker, 1990). *Staphylococcus aureus* is widely spread and found associated on most terrestrial and marine mammals (Baird – Parker, 1990).

In human, staphylococci found in the nasal passages and throats as well as on more than 50% of healthy individuals hair and skin (FDA, 2006). *Staph. aureus* in particular, are commonly found in the nasal cavity (main reservoir), arms, hands, face, eyes, throat with boies and spds and intestinal tract from which *Staph. aureus* find its way to air, dust, food, cloths and the environment (Jay, 1992). The proportion of enterotoxigenic *Staph. aureus* varies according to its origin source (Table 1-9), and the majority of food *Staph. aureus* strains are enterotoxin

negative (Mossel, 1983; Bergdoll, 1989; Loir *et al.*, 2003) and its presence in food indicates poor hygiene (Mossel & Netten, 1990).

Both *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* are found on human skin, whereas *Staphylococcus xylosus* habitat is the skin of lower primates and other mammals (Jay, 1992). *Staphylococcus cohnii* generally found on human's skin, urinary tract and wound infection whereas *Staphylococcus intermedius* is rarely associated with human and it is mostly found on the skin and nasals of the carnivores and horses (Jay, 1992).

The main source of enterotoxin producing staphylococci in food is the food handler hands, nose or septic lesions which contaminate the food with one or more enterotoxigenic Staphylococci species, followed by suitable storage conditions (Table 1-10) that support the growth of staphylococcus species and the subsequent enterotoxins production (Gilbert and Wieneke, 1972; Hobbs, 1973; Mossel & Netten, 1990).

Another source of food infection by Staphylococci is cow, raw milk and raw milk products such as cheeses and cream which gave a high rate of outbreaks in various countries (Gilbert and Wieneke, 1972). Gilmour and Harvey (1990) reported that milk and milk product become contaminated with *Staphylococcus* species if:

- The good hygienic practices are absent in the farms;
- Milk pasteurized inadequately;
- Precautions during the manufacturing process have not been taken to prevent the staphylococci contamination or growth in the final products.

Table 1-9 The incidence of enterotoxinogenicity in *Staphylococcus aureus* isolated from various sources (Mossel & Netten, 1990).

Origin	Enterotoxin types included in assay	Positive percentage	Reference
Human	A and B	76 – 100%	(Reali, 1982)
	A and B	51%	(Melconian <i>et al.</i> , 1983)
Poultry	A – D	25%	(Harvey <i>et al.</i> , 1982)
	—	57%	(Gibbs <i>et al.</i> , 1978)
Various foods of animal origin	A – E	25 – 26%	(Wieneke, 1974; Melconian <i>et al.</i> , 1983; Ewald, 1987)
Sausage	A and B	29%	(Reali, 1982)
Aviation meal	A – E	51%	(Ewald & Christensen, 1987)
Ovine milk	—	49%	(Gutierrez <i>et al.</i> , 1982)
Bovine milk	A and B	52%	(Reali, 1982)
Abscesses of slaughtered animals	A – E	74%	(Menes <i>et al.</i> , 1984)
Food poisoning outbreaks	A - D	94%	(Wieneke, 1974)

Table 1-10 Principle mechanisms of outbreaks transmitted through foods and meals in United State (Mossel, 1988).

Defaults	Percentage
Contamination	
• Naturally contaminated food inadequately heated.	47%
• Recontamination from human source.	32%
• Recontamination from food environment.	15%
Colonization	
• Slow cooling after heat treatment.	48%
• Too long storage before use.	34%
• Inadequate refrigeration.	19%

1.9 Mechanism of pathogenicity

In general, 10^5 cfu of enterotoxigenic strain growth is required for sufficient production of Staphylococcal enterotoxin to induce intoxication (Brett, 1998). Staphylococcal enterotoxin A and D are responsible for the majority of Staphylococcal food poisoning outbreaks (Bergdoll, 1989; Brett, 1998) and the infected dose that capable of causing illnesses in human after ingestion is less than 100ng (Evenson *et al.*, 1988; Brett, 1998). Several studies using human volunteers have been shown that 0.4µg/Kg body weight of the SEA, SEB and SEC will cause the illness (Watson, 1998). The mode of enterotoxin action is unknown (Baird – Parker, 1990), however, tow hypothesises have been reported on the mechanism of the SEs (Dabrowski and Medralla, 2005). One of these hypotheses suggested that the degranulation that occurs through mast cells receptor result in inflammation. The other hypothesises (neurogenic hypothesises) suggested that a neuropeptide that released by sensory nerves stimulate the mast cells (Scheuber *et al.*, 1987; Dinges *et al.*, 2000; Dabrowski and Medralla, 2005). The vomiting and diarrhoeal responses are due to the stimulation of the intestinal tract local neuroreceptors that result in stimulation of the vomiting centre of the brain through vagus and sympathetic nervous system (Baird – Parker, 1990).

1.10 The Staphylococcal Food Poisoning Symptoms

As a result of ingestion of food contaminating with staphylococcal enterotoxins, the following symptoms will be developed within 1 -6 hours (Tranter and Breham, 1990):

- Acute gastroenteritis
- Nausea
- Vomiting
- Abdominal crumbs
- Diarrhoea

The intensity of symptoms may vary among individuals (FDA, 2006) and the recovery of this disease will mostly take 1 – 3 days; however, it could be fatal to young children and elderly people (Tranter and Breham, 1990).

1.11 Food associated with food poisoning outbreaks

Wide ranges of foodstuff that consist of the essential nutritious elements support the growth of staphylococci and the subsequent enterotoxins production under optimal growth conditions. Bremer (2004) reported that rich protein content is highly associated with staphylococcal food poisoning intoxication. Staphylococci are chemo-organotrophs that requires amino acids, one or more group B vitamins, and other growth factors containing media for their aerobic growth; however, it requires fermentable carbon source and uracil during anaerobic growth (Cohen, 1972; Baird – Parker, 1990). Staphylococcal enterotoxins production requires the essential energy source (amino acids, glucose or glutamate), vitamins, and salts, or those requirements that achieved the minimal requirements for its production (Tranter and Breham, 1990). For enterotoxin B production, the amino acid (arginine) is also required (Wu and Bergdoll, 1971; Jay, 1992).

According to the Food and Drug Administration (FDA) the potentially hazardous foods are those natural or synthetic foods that support the rapid growth of infective or toxigenic microorganisms and therefore require temperature control. The most frequently types of food contaminated with *Staphylococcus aureus* are meat and meat product; poultry and eggs; prepares foods such as custard, trifles and cream product; bakery products; salads and foods which require considerable handling during preparation (Hobbs and Roberts, 1993; FDA, 2006).

In general, food and food products that associated with foodborne intoxication caused by *Staphylococcus aureus* are high protein content, cooked, contaminated during handling, and held in improper temperature (more than 7.2°C or less than 60°C) (Watson, 1998; FDA, 2006).

In central Europe, the most important vehicles of staphylococci are home – made creams, cakes and vegetable salad (Dabrowski and Medralla, 2005). The food of

concern in this project is ready to served custard which intended to be used for filling and designing cakes bakeries and other desserts (Figure1-2).

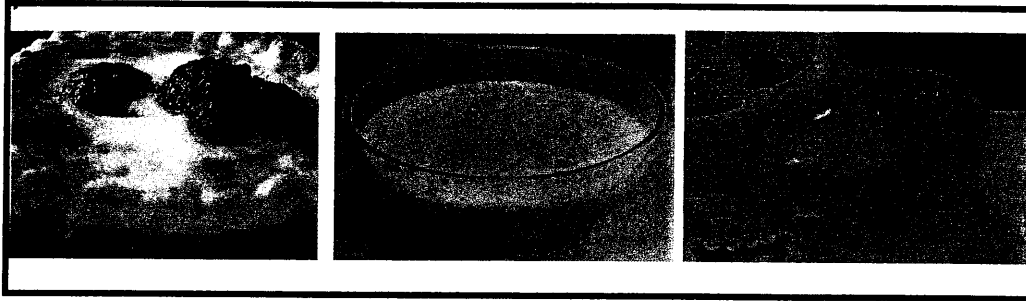


Figure 1-2 Different custard desserts (Wikipedia, 2007).

Custard distributed over wide areas and it's prepared in most homes. It is a combination of milk or cream, egg yolks, sugar, and flavorings such as vanilla that support the staphylococci growth. It is highly possible that custard or other cold sweets can be contaminated by been touched with hands. At the same time they will act as suitable media for staphylococci and streptococci as well as other organisms (Hobbs and Roberts, 1993). Major outbreaks associated with custard have been mostly associated to staphylococcal enterotoxins which have initiated from boils, ulcers, witlows, or abrasions on hands as well as from healthy looking hands (Hobbs and Roberts, 1993).

Vanilla cakes, in which *Staph. aureus* – were grown from a mixing bowl to high number (18 – 200 million per gram). This cackes were eaten by 5 families who reported after 3-6 hours later abdominal pain and in some diarrhoea. The cakes found to contain enterotoxin while one of the bakery workers who prepared the cakes had eczematous lesions on his right hand (Hobbs and Roberts, 1993).

Milk and milk product (high protein content foods) are also associated with foodborne intoxication caused by *Staphylococcus aureus*. Row milk was implicated in 20 staphylococcal food poisoning outbreaks (590 cases) in UK between 1951 and 1960. Many outbreaks associated with ice-cream have been reported before the ice-cream regulations were enforced in UK. One of these

outbreaks affected more than 7000 individuals due to formation of staphylococcal enterotoxin (Hobbs and Roberts, 1993).

The most frequently implicated foods in staphylococcal food poisoning outbreaks in UK and USA are meat, poultry and their products (Wieneke *et al.*, 1993; Watson, 1998). In UK (July 1975), 71 out of 147 pupils and staff became sick due to consumption of cooked chicken in school lunch. The isolated *Staphylococcus aureus* (from the chicken, the affected individuals and one food handler) found to produce staphylococcal enterotoxin A. SEA was also detected in the left over chicken which had been thawed overnight, roasted in the following day and held at the ambient temperature in order to be served (Wieneke *et al.*, 1993; Watson, 1998).

1.12 Frequency of Staphylococcal Food Poisoning Disease

The actual incidence of the staphylococcal food poisoning disease is unknown due to the following reasons (FAD, 2006):

- Low responses from victims during the health official's interviews.
- Inadequate sample collection for laboratory analyses.
- High similarity in the disease symptoms with other food poisoning types (e.g. food poisoning cause by *Bacillus cereus* toxin) which result in misdiagnosis of this Disease.
- Improper laboratory examination.

As a result of bacterial foodborne disease in the United States, reports that about 76 million people get sick, greater than 300,000 are hospitalized, and 5,000 people die annually (CDC, 2007). Regarding Staphylococcal food poisoning, the outbreaks, cases and death for the years 1973 – 2002 are presented in (Table 1-11).

In England and Wales, the estimated annual impact of foodborne disease from 1996 – 2000 are 1,724,315 cases that resulted in 21,997 hospitalizations and 687 deaths (Adak *et al.*, 2005). Among these foodborne diseases, Staphylococcal food poisoning caused 9,196 cases (3,678 general practitioner cases), 232 were hospitalized within 278 days and no deaths were recorded (Adak *et al.*, 2005). However, this number of outbreaks has been declined to 35 cases (Table 1-12) in the period 1992 – 2006 (HPA, 2007).

In the Kingdom of Bahrain, around 29 Staphylococcal food poisoning outbreaks were reported by public health laboratory in the period 2004 – 2006. The percentage of *Staphylococcus aureus* in variety of food and water samples during the last three years was in the range of (2.2 – 2.9%) of the total food samples that have been examined in Public Health Laboratory – Bahrain (PHL-BAH, 2007). About 92 – 96% of these positive results are belonging to ready to eat foods such as sandwiches, salads, desserts and other prepared hazardous foods which also reported to be the most common reported vehicle of transmission in staphylococcal food poisoning (Table 1-13). The results tested for *Staphylococcus aureus* in variety of food and water samples including the during the last three years (Bahrain) are shown in (Table 1-14)

The optimum samples receiving from food poisoning outbreaks to Public Health Laboratory in Bahrain are in the summer season (July – September) where the relative humidity rang between 70 – 85% and the average temperature is 42°C - 45°C (optimum SEs production temperature) . This emphasizes the importance of good hygienic practices and adequate refrigeration in preventing staphylococcal food poisoning.

Table 1-11 Number of reported Staphylococcal food poisoning outbreaks, cases and death in the United States, 1973 – 2002.

Years	Outbreaks	Cases	Death	References
1973 - 1987	367	17,248	?	(Bean and Griffin, 1990)
1983	14	1,257	?	(Bean <i>et al.</i> , 1990)
1984	11	1,153	?	(Bean <i>et al.</i> , 1990)
1985	14	421	?	(Bean <i>et al.</i> , 1990)
1986	7	250	?	(Bean <i>et al.</i> , 1990)
1987	1	100	?	(Bean <i>et al.</i> , 1990)
1988	8	245	0	(Bean and Goulding, 1996)
1989	14	524	0	(Bean and Goulding, 1996)
1990	13	372	0	(Bean and Goulding, 1996)
1991	9	331	0	(Bean and Goulding, 1996)
1993	6	206	0	(Bean and Goulding, 1996)
1993 – 1997	42	1,413	1	(Olsen <i>et al.</i> , 2000)
1998	15	615	0	(Lynch <i>et al.</i> , 2006)
1999	19	353	0	(Lynch <i>et al.</i> , 2006)
2000	23	657	2	(Lynch <i>et al.</i> , 2006)
2001	23	646	0	(Lynch <i>et al.</i> , 2006)
2002	21	405	0	(Lynch <i>et al.</i> , 2006)

Table 1-12 *Staphylococcus aureus* food poisoning reported to the Health Protection Agency Centre for Infections - England & Wales, 1992-2006 (HPA, 2007).

Year	Number of Outbreaks	Number Affected
1992	7	107
1993	1	15
1994	2	17
1995	3	46
1996	5	146
1997	2	18
1998	0	0
1999	4	81
2000	0	0
2001	6	25
2002	2	14
2003	0	0
2004	2	31
2005	0	0
2006	1	5

Table 1-13 Number and type of samples incriminated in Staphylococcal food poisoning outbreaks in Kingdom of Bahrain during 2002 – 2006 (PHL-BAH., 2007)

Year	No. of Samples Recorded	Type of samples	No. of Samples per Type
2002	1	Green Salad	1
2003	No records found	-	-
2004	5	Beef Burger Sandwich	1
		Egg Sandwich	1
		Frozen Chicken Spring Rolls	1
		Green Salad	1
		Qeema sandwich	1
2005	13	Boiled Rice	1
		Chicken Tika	1
		Chicken Teriyaki with Rice	1
		Cooked Chicken Cut	1
		Humus	1
		Meat burger	1
		Green Salad	6
		Vegetable Sandwich	1
2006	11	Beef Shawarma	2
		Chicken Shawarma	2
		Falafel Sandwich	1
		Green Salad	4
		Liver Sandwich	1
		Mutable	1

Table 1-14 The result of various sample tested in Public Health Laboratory – Food Microbiology (Bahrain) for *Staphylococcus aureus* from 2004 – 2006 (PHL-BAH., 2007).

Type of sample	2004			2005			2006		
	No. samples received	SAT (-ve)	UNSAT (+ve)	No. samples received	SAT (-ve)	UNSAT (+ve)	No. samples received	SAT (-ve)	UNSAT (+ve)
DAIRY PRODUCTS (CAT # 100)	71	71	0	47	47	0	70	70	0
INFANTS,CHILDREN & CERTAIN CATEGORIES OF DIETETIC FOODS (CAT # 200)	70	70	0	76	76	0	54	54	0
BEVERAGES,FRUIT JUICE,TEA AND COFFEE (CAT # 300)	0	0	0	5	5	0	12	12	0
CANNED FOODS AND INGREDIENTS FOR CANNING (CAT # 400)	2	2	0	0	0	0	2	2	0
FOODS CONTAINERS (CAT # 500)	0	0	0	0	0	0	0	0	0
MEAT,POULTRY AND ITS PRODUCTS (CAT # 600)	177	176	1	202	199	3	193	192	1
FISH AND SHELLFISH (CAT # 700)	6	6	0	19	19	0	21	21	0
EGG PRODUCTS, MARGARINE AND NUT BUTTERS (CAT # 800)	1	1	0	4	4	0	2	2	0
CEREALS,PULSES,OIL SEEDS,NUTS,& ITS PRODUCTS (CAT # 900)	7	7	0	12	12	0	23	23	0
JELLY ANDJAM PODUCTS (CAT # 1000)	0	0	0	1	1	0	0	0	0
TOMATO PRODUCTS ,SALAD,VINEGAR, AND SPICES,SAFFRON (CAT # 1100)	21	21	0	47	47	0	27	27	0
VEGETABLES AND FRUITS (CAT # 1200)	2	2	0	6	5	1	0	0	0
INGREDIENTS FOR FOODS INDUSTRIES (CAT # 1300)	0	0	0	0	0	0	0	0	0
CHOCOLATE AND CANDY PRODUCTS (CAT # 1400)	1	1	0	0	0	0	1	1	0
OILS AND FATS (CAT # 1500)	2	2	0	0	0	0	0	0	0
DRINKING WATER AND OTHERS WATER (CAT # 1600)	3	2	1	3	3	0	1	1	0
READY TO EAT FOODS (CAT # 1700)	1307	1261	46	1666	1611	55	1508	1469	39
ANIMAL FEEDS (CAT # 1800)	0	0	0	0	0	0	0	0	0
BIOLOGICAL FLUIDS (CAT # 1900)	0	0	0	1	0	1	3	3	0
MISCELLANEOUS PRODUCTS (CAT # 2000)	30	30	0	14	14	0	26	24	2
GRAND TOTAL	1700	1652	48	2103	2043	60	1943	1901	42

All the reported positive results that incriminated *Staphylococci* in any routine outbreaks in Bahrain do not give the actual implication of this pathogen to the food poisoning disease due to the following reasons:

- All these records were positive for *Staphylococcus aureus* only; however other coagulase positive and negative were reported to produce Staphylococcal enterotoxins and clearly involve in staphylococcal food poisoning outbreaks (see sections 1.6.1 and 1.6.2). In addition, enterotoxins could be produced by coagulase negative *Staphylococcus aureus* strain (Wilson *et al.*, 1991; Kroll *et al.*, 1993).
- Inadequate sample collection for laboratory analyses, that most of foods which were sent to Public Health Laboratory as food poisoning samples were not necessarily incriminated for Staphylococcal food poisoning as there was no records showing if they are left over samples or not.
- *Staph. aureus* positive results (Unsatisfactory) reported for these samples because the bacterial count which obtained from the conventional method (viable *Staph. aureus* count) was more than 100 cfu /g, however, the extreme food processing conditions (e.g. cooking) kills or injures the bacterial cells but not the heat stable – enterotoxins that may produced by the enterotoxigenic strains and survive this conditions (Wilson *et al.*, 1993; Kroll *et al.*, 1993). In addition the most of the diagnostic media such as (Baird – Parker Agar and Rabbit Plasma Fibrinogen Agar) which designed to detect the coagulase positive strains.
- All samples were not subject to any enterotoxins test (disease causative agent).
- Misdiagnosis of this disease due to the symptoms similarity with other food poisoning diseases results in sending these samples to the laboratory for other pathogens test.

1.13 Staphylococcal food poisoning control and preventative measure.

The contamination of food with *Staphylococcus* species must be prevented before the production of the staphylococcal enterotoxins (CDC, 2006) and this prevention could be achieved by good hygiene practices (GHP), good manufacture practices (GMP) and through the application of Hazard Analysis Critical Control Point (HACCP) system. The application of the HACCP to a particular food – handling operation or any food business can assess, minimize and control the *Staphylococcus aureus* contamination, growth and the subsequent enterotoxins production in food (Watson, 1998). HACCP is a cost effective approach that ensure the safety of food product through controlling *Staphylococcus aureus* at all manufacture production line, storage, transport, retail stages (Hobbs and Roberts, 1993). Examples of control and preventative measures are illustrated in the following points:

- Good hygienic practices such as washing hands vigorously and cleaning under fingernails with bactericidal soap/cream and water before handling or preparing foods is an important practice to reduce the food contamination incidence by handlers (Bremer, 2004; CDC, 2006).
- The food should not prepare or serve by handlers that have a nose infection, eye infection or wounds or skin infections in hands ((CDMP, 2001; CDC, 2006).
- The kitchens and food-serving areas must be kept clean and sanitized (CDC, 2006).
- Implementation of control procedure for cooking and chilling to prevent the growth and subsequent enterotoxin production, for instance, the good temperatures control that prevent the growth of Staphylococci are 60°C or above and 7-7.2°C or below (Bremer, 2004; CDC, 2006).
- The cooked/prepared foods should be rapidly cooled and stored in refrigerator (<7.2°C) in wide and shallow containers (Bremer, 2004; CDC, 2006).
- Improving the food handler education in strict food hygiene (CDMP, 2001).
- Reduce the time of food handling (the time from the initial preparation to service) to less than 4 hours in the ambient temperature (CDMP, 2001).

1.13.1 Inactivation of Staphylococcal Enterotoxins

Although staphylococcal enterotoxins are highly heat resistant, they could be inactivated (when they are in low concentration) by heat treatments that are the protection of used in canned food (Bergdoll, 1983). However, in other study created by (Schwabe *et al.*, 1990) on staphylococcal enterotoxins inactivation by heat, the inactivation of the enterotoxins were varies depending on the food matrix and pH. In general, heat inactivation method of SEs is quite difficult because it varies among the type and concentration of SE, food matrix, and pH level (Loir *et al.*, 2003). Nevertheless, this method could be reversible by an alkaline pH (Schwabe, *et al.*, 1990; Loir *et al.*, 2003). Schwabe and his follow workers demonstrated that at high pH treatment (pH 11), heating of different preparations containing SE (gelatin (pH 7.0), cauliflower extract (pH 4.0 or pH 7.0), milk (pH 4.0), casein (pH 6.0), rice extract (pH 7.0), noodles extract (pH 4.0) and oat-flakes extract (pH 7.0)) at 80°C or 100° C for 10 min had a lower effect on the staphylococcal enterotoxin's immunological activity (activity greater than or equal to 25%).

Chemically, electrolyzed anodic NaCl solution is one of the greatest inactivation methods of Staphylococcal enterotoxins. The ability of this solution to inactivate Staphylococcal enterotoxin A (SEA) has been demonstrated to be effective and cause fragmentation of SEA (Suzuki, *et al.*, 2001). This result estimated by amino acid test showed loss in amino acids content of SEA, the process which prevents its reorganisation by reversed-phase passive latex agglutination (RPLA) test due to loss of immuno-reactivity between SEA and a specific anti-SEA antibody (Suzuki, *et al.*, 2001).

Biological inactivation of SEs varies among the types of the enterotoxins due to the slight differences in their structure. For example, SEI is more susceptible to stomach proteases inactivation than SEA (Erickson, 2003). This result is clarified when both enterotoxins were exposed to monkey's stomach fluid for grater than 40 min at 37°C. Complete degradation of SEI was achieved within one hour,

however, SEA remained stable for more than one hour. In general, Staphylococcal enterotoxins are very stable molecules towards proteolytic enzyme and biological inactivation (Erickson, 2003; Loir *et al.*, 2003).

Using (Irradiation) as a method for SEs inactivation is more effective in buffer rather than complex media such as food (Erickson, 2003). For instance, the dose required to inactivate 98% SEB (31µg/ml) in buffer (0.04 M, pH 7.2) was 50 KGy, however, 200 KGy was required in order to inactivate the same percentage of SEB in milk. Similarly, the complete inactivation of SEA in a gelatine phosphate buffer (100 – 110 ng/ml) required 8 KGy, whereas 27 – 34% of SEA activity remains in 15% minced beef sample after subjection to similar dose. In most cases, the required irradiation doses for enterotoxins inactivation are usually high, therefore this method is not an effective solution for enterotoxin elimination from the contaminated foods (Erickson, 2003).

Altogether, this data shows that Staphylococcal enterotoxins are highly resistant molecules; therefore it is easily to destroy the enterotoxigenic bacteria rather than destroy or inactivate them.

1.14 Diagnosis of staphylococcal foodborne intoxication

As the number of food poisoning outbreaks increase over the years, the requirement of rapid, sensitive, and specific detection methods are important as well as the improvement of food safety, hygiene and quality within the local factories, restaurants and any food supplier.

1.14.1 Quantitative Isolation and Identification Methods

Staphylococci can be grown in any nutrient medium containing peptone (Cohen, 1972). Variety of various media have been developed for the isolation and enumeration of Staphylococci especially *Staph. aureus*. Schleifer and Kramer (1980) developed a selective media for staphylococci isolation depends on furoxine and furazolidone addition that inhibited all micrococci and allow all staphylococci to grow well (except *Staph. intermedius*) (Baird-Parker, 1990).

Staph. aureus – media should be selective that could be achieved by different ways such as addition of 1.0% glucose, 8.0% sodium chloride, or incubation under anaerobic conditions (Cohen, 1972; Bouwer-Hertzbery *et al.*, 1982).

The oldest selective media designed for *Staphylococcus aureus* were depending on high NaCl salt concentration (5 – 10%) in order to reduce the water activity that prevent non halophilic or halotolerant organisms to grow (Chapman, 1945; Bouwer-Hertzbery *et al.*, 1982). Mannitol Salt Agar (MSA), a selective medium with high NaCl (selective agent) concentration (7.5%) that was used for isolation of presumptive pathogenic staphylococci (Oxoid, 2001). In addition, MSA is also a differential medium which contains mannitol and phenol red as pH indicator (Johnson, 2006). Organisms that can ferment mannitol and produce acid (e.g. *Staphylococcus aureus*) that converts the media colour to bright yellow (Fig.1-3), whereas the non- pathogenic staphylococci (e.g. non pathogenic-*Staphylococcus epidermidis*) appears red (Oxoid, 2001; Johnson, 2006). This media was recommended by (Chapman, 1945) and (Bleuir *et al.*, 1967), however, this media has been demonstrated to support the growth of other competitive organisms

growth such as *Bacillus* species (Bouwer-Hertzbery *et al.*, 1982) and few halophilic species (Oxoid, 2001).

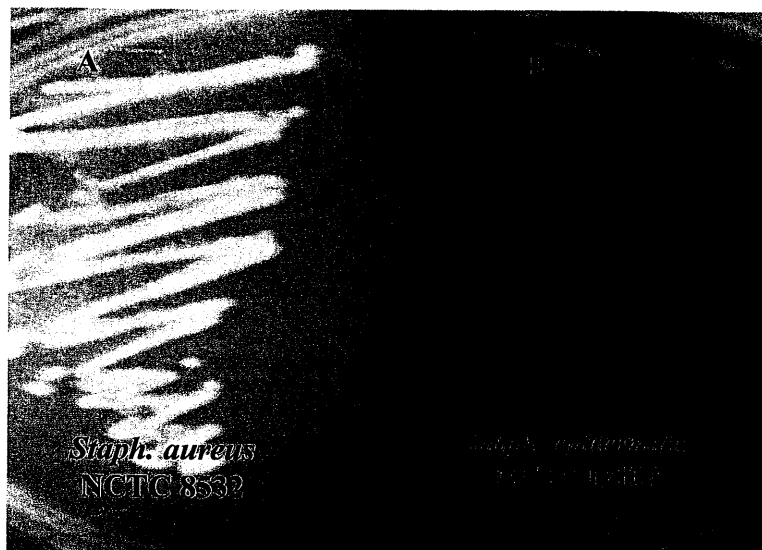


Figure 1-3 Different *Staphylococcus* species growing on Mannitol Salt Agar (MSA). On the left (A) bright yellow colonies of *Staphylococcus aureus* is positive for mannitol fermentation. On the right (B) pink-red colonies of *Staphylococcus epidermidis* shows negative mannitol fermentation.

Mannitol as illustrated in (Table 1-1) can be utilized by most enterotoxigenic coagulase positive strains as well as coagulase negative *Staphylococcus* species (Jay, 1992), and it was reported by Bautista and his follow workers (1987) that mannitol utilization was the best test for differentiation between enterotoxigenic and nonenterotoxigenic staphylococci.

Phenolphthalein Diphosphate Agar Polymyxin (PPAP), a medium developed by (Barber and Kuper, 1951) that relies on the addition of polymyxin (selective agent) to inhibit many organisms other than *Staphylococcus aureus*. This media used also to determine the phosphates enzyme production by the isolated organisms; however, this is not a distinguishable characteristic or unique feature of *Staphylococcus aureus* (Bouwer-Hertzbery *et al.*, 1982). This medium also lacks selectivity in which other interfering organisms such as *Bacillus* species are

able to grow, in addition, (Hobbs, 1967) reported that polymyxin had an inhibitory effect towards some *Staphylococcus aureus* strains, particularly those found particularly in cheese (Bouwer-Hertzbery *et al.*, 1982).

Baird – Parker Agar is the medium of choice for *Staphylococcus aureus* detection in food samples. It has been used since the early 1960s and succeeded several selective media that were used for *Staphylococcus aureus* isolation (Bouwer-Hertzbery *et al.*, 1982). This medium depends upon two main characteristics of *Staphylococcus aureus*: first, the egg yolk lipoprotein utilization which results in a clearing zone surrounding the bacterial colonies after 24 hours incubation and often with white precipitate (Figure 1-4) due to calcium and magnesium salts formation of the fatty acids (Bouwer-Hertzbery *et al.*, 1982). On further incubation (48 hours) opaque zones are formed around the *Staphylococcus aureus* colonies as a result of lipase enzyme action (lipolysis activity) (Oxoid, 2001).

The second characteristic is the ability of this organism to reduce tellurite (an ingredient in the medium) to produce black colour colonies. This tellurite also inhibits many interfering organisms. In addition, the agar contains selective growth stimulants (sodium pyruvate and glycine) which also make the media non-toxic for stressed populations (Baird – Parker and Davenport, 1965; Mossel *et al.*, 1970; Gray *et al.*, 1974; Idziak and Mossel, 1980; Bouwer-Hertzbery *et al.*, 1982).

In the other hand, the egg yolk lipoprotein hydrolysis is not a conclusive result for *Staphylococcus aureus* identification as both egg yolk lipoprotein hydrolysis positive and negative colonies have been shown to be *Staph. aureus* (Crisley *et al.*, 1965; De waart *et al.*, 1968; Untermann *et al.*, 1973; Bouwer-Hertzbery *et al.*, 1982). In 1976, Stadhouders *et al.* suggested to use the coagulase activity as a diagnostic character instead of clearing activity by using pig plasma instead of egg yolk in Baird – Parker agar to overcome the egg yolk hydrolysis disadvantage; However, the recognition of the fibrin halo around the colonies due to coagulase activity was difficult in this media due to low fibrinogen in some of the prepared pig plasma. Therefore, the researchers (Hauschild *et al.*, 1979) recommended a

modification of this media by using Baird – Parker agar containing bovine fibrinogen in addition to pig plasma (Bouwer-Hertzbery *et al.*, 1982).



Figure 1-4 Characteristic zones (clear and opaque zones) formed around *Staphylococcus aureus* (NCTC 10657) colonies as a result of proteolysis (24 hr) and lipolysis (48 hr) activities on Baird – Parker agar.

Tellurite – polymyxin egg yolk agar (TPEY) media is similar to Baird – Parker agar system which use egg yolk as a diagnostic agent, however, the presence of polymyxin limits the usage of this agar for *Staph. aureus* isolation since this agent inhibits some of the target strains (Criseley *et al.*, 1964; Bouwer-Hertzbery *et al.*, 1982).

Potassium thiocyanate – actidione - sodium azaide - egg yolk pyruvate (KRANEP) agar, a popular diagnostic medium especially in Germany that uses egg yolk as the diagnostic agent. In this medium, sodium azaide and actidione are used as selective agents as well as inhibitory agents for aerobic contaminants and most yeast and moulds respectively (Sinell and Baumgart, 1967; Bouwer-Hertzbery *et al.*, 1982).

Rabbit Plasma Fibrinogen Agar (RPF Agar) is a modification media of Baird-Parker agar which used for highly selective isolation and enumeration as well as

the confirmation of *Staphylococcus aureus* and coagulase – positive staphylococci from food and other specimens (Beckers *et al.*, 1984; Oxoid, 2001). This Agar depends on the Baird-Parker agar base; however, it differs in that the egg yolk emulsion has been replaced by fibrinogen to enhance the coagulase reaction (Hauschild *et al.*, 1979), rabbit plasma and trypsin inhibitor. (Beckers *et al.*, 1984) compared several plasma sources and found that the rabbit plasma was the more specific for the coagulase activity than the other sources. Trypsin inhibitor was added to RPF agar to avoid fibrinolysis (Oxoid, 2001). The potassium tellurite concentration was reduced in RPF agar by four fold (from 0.01% to 0.0025% w/v) due to some *Staphylococcus aureus* strains sensitivity when Potassium tellurite used at 0.01 % in RPF agar. The reduction of potassium tellurite results in white or grey or black *Staphylococcus aureus* colonies surrounded by an opaque halo of precipitation (Oxoid, 2001).

1.14.2 Identification Methods

After isolation and conformation of purity, the colonies on Baird-Parker agar should be first checked by Gram staining and catalase productions test (Cohen, 1972). Gram's stain is one of the most useful and widespread staining method that used for identifying of unknown bacterial species in the first stage of any diagnostic procedure. It was developed by the Danish bacteriologist Hans Christian Gram in 1884 and it distinguishes between the bacterial species according to there chemical and physical properties of their cell wall (Brock, 1926; Gillies and Dodds, 1973). Staphylococci are Gram – positive organisms (Fig. 1-5) that have a thick layer of peptidoglycan and thin lipid content cell wall (Cohen, 1972). This characteristic result in retaining the crystal violet stain during the Gram's staining method and appears purple in colour (Brock, 1926).

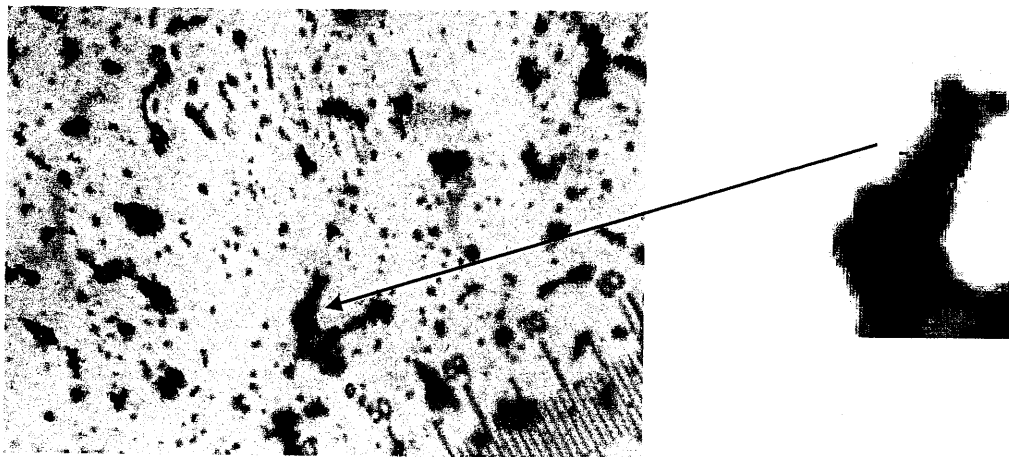


Figure 1-5 Gram positive *Staphylococcus aureus* NCTC 10657 (purple cocci).

Most of *Staphylococcus* species produce range of enzymes such as glycosidases, proteases, esterases and lipases on different substrates by which the laboratory Staphylococci identification methods depends on (Baird – Parker, 1990).

Catalase production test is a biochemical test that used also to differentiate between bacterial species in the diagnostic laboratories (e.g. differentiation between Staphylococci (+ve) and Micrococci (-ve). In this test the Catalase enzyme that produced by the organism decompose the hydrogen peroxide to water and oxygen. The organism is said to be catalase +ve if bubbles forms after smear the examined cells in the microscopic slide containing a drop of 3% w/v hydrogen peroxide ($2 \text{ H}_2\text{O}_2 \rightarrow 2 \text{ H}_2\text{O} + \text{O}_2$ (bubbles)) and if not, the organism is catalase-ve (Cohen, 1972; Wikipedia, 2006).

The two typical conformational and differential tests for Staphylococci are the coagulase activity test and thermostable nuclease test (Bouwer-Hertzbery *et al.*, 1982; Baird-Parker, 1990). However, neither of the two tests is specific for *Staphylococcus aureus* (Baird-Parker, 1990). *Staphylococcus aureus* produce the coagulase which binds to the host pro-thrombin to form staphylothrombin complex (Foster, 2001). This complex activates the thrombin protease activity that results in converting the fibrinogen to fibrin (Foster, 2001; Wikipedia, 2007). This extracellular protein (coagulase) used by CPS as a defence mechanism by clotting the areas of plasma around them thereby protecting themselves from the host immune system cells and resist phagocytosis (Wikipedia, 2007) . Staphylase test

(e.g. Oxoid-DR0595) is one of these assays that depend on the fact that all *Staphylococcus aureus* have the ability to produce free and bound coagulase (clumping factor) that result immediately in agglutination (Fig.1-6) after mixing the test reagent (fibrinogen sensitized sheep blood cells) with the suspected *Staphylococcus aureus* cells that contains the clumping factor (coagulase enzyme) which convert the fibrinogen to fibrin (Oxoid, 2001).

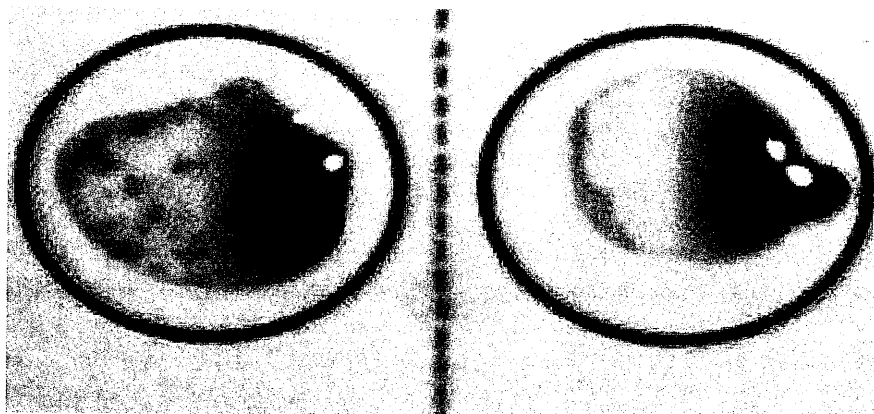
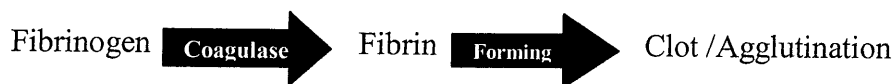


Figure 1-6 Detection of coagulase activity of *Staphylococcus aureus* (NCTC 8532). On the left agglutination of rabbit plasma (positive result) and the smooth emulsion on the right shows negative result for Coagulase.

Beside coagulase activity, Deoxyribonuclease (DNase) activity has been reported also to identify the pathogenic *Staphylococci* (Weckman and Catlin, 1957). Deoxyribonuclease (DNase) is an enzyme produced by some organisms and catalyzed the Phosphodiester bonds – hydrolytic cleavage in the DNA backbone (Wikipedia, 2007). A method of detection of DNase activity provided by the addition of DNA in to the agar medium (Jeffries *et al.*, 1957; Oxoid, 2001). The colonies that produce DNase enzyme will hydrolyse the DNA which found in the media and then if the plate flooded with 1N hydrochloric acid (HCl), clear zone will appear around the DNase – positive colonies (Fig. 1-7) due to hydrolyzation of the DNA molecules, while the DNase – negative surrounding area and the other

remains will appear opaque after addition of 1N HCl due to the precipitation of the polymerised DNA (Bouwer-Hertzberg *et al.*, 1982; Oxoid, 2001). Further modification of this test reached by incorporation of dyes such as Toluidine blue (Schreier *et al.*, 1969) and Methyl-green (Smith *et al.*, 1969) into the medium in order to distinguish DNA hydrolysis and avoiding the use of HCl acid (Oxoid, 2007).

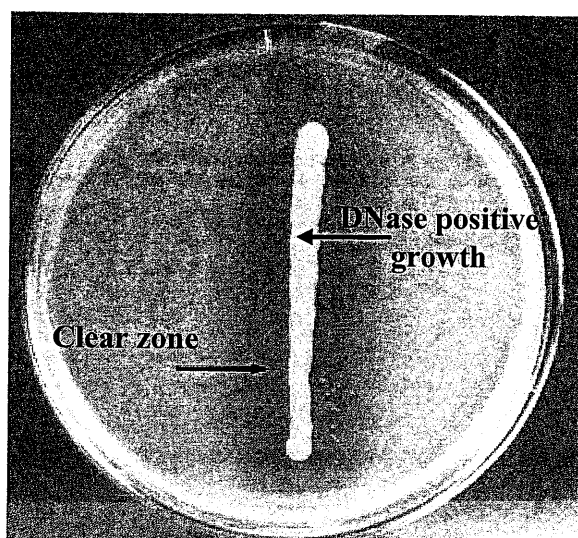


Figure 1-7 Positive DNase Test: a clear zone surrounds the bacterial growth after 1N HCl was added (Kaiser, 2002).

The recent rapid detection and identification methods which are based on different principles of direct or indirect detecting of microorganisms are faster, more sensitive, accurate and specific than conventional bacteriological methods (Kroll, *et al.*, 1998).

BD - Phoenix Automated Microbiology System (Fig. 1-8), is a new automated, logical, flexible microbiology identification system that provides a complete reportable result within short period of time (<24 hours) and high degree of accuracy. It uses MPD (Multi – parameter Determination) technology that utilizes multiple data inputs for rapid and proper identification (BD, 2007). Phoenix system is useful for both food and medical microbiology identification purposes in which it can identify the organism's genus and species as well as its sensitivity

to different antibiotics. The identification in phoenix depends on the organism's reaction with the fluorogenic and chromogenic substrates that found in the system's kits.

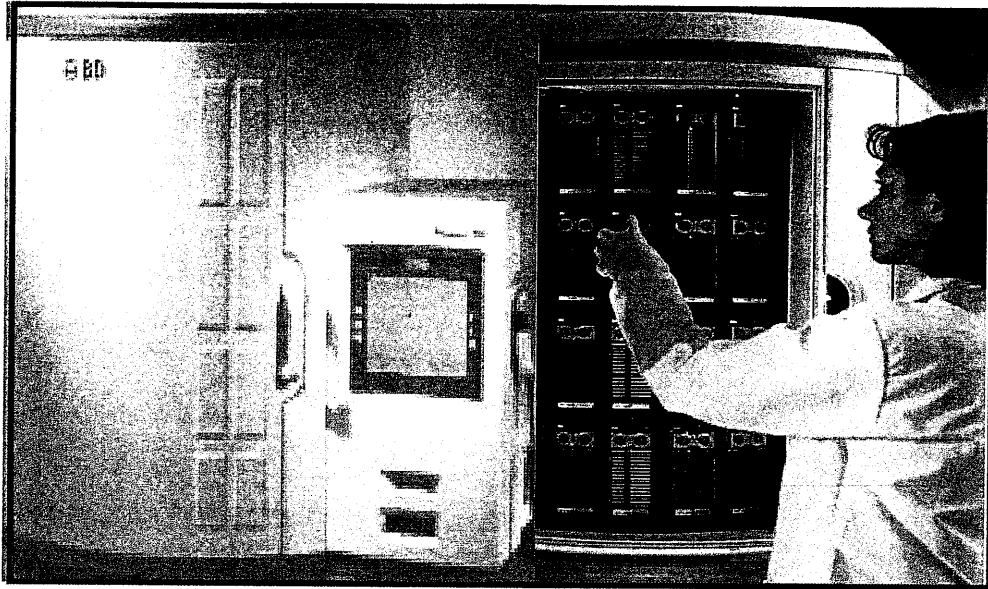


Figure 1-8 BD Phoenix Automated Microbiology System (BD, 2007).

In contrast to other identification system that concentrate on coagulase positive *Staphylococci*, this system can identify various variety of coagulase negative *Staphylococci* (Table 1-15). In the evaluation of this system among 295 *Staphylococcus* species (100 *Staphylococcus aureus* & 195 non - *Staphylococcus aureus*), phoenix system gave a 100% correct identification of *Staphylococcus aureus* and 93.6% of the total 295 examined species (Williams, 2004).

Table 1-15 Coagulase negative Staphylococci (CNS) species identified by Phoenix Automated Microbiology System (BD, 2007).

Bacteria	Phoenix ID
Coagulase negative Staphylococci (CNS)	<i>Staph. cohnii</i>
	<i>Staph. chromogens</i>
	<i>Staph. epidermidis</i>
	<i>Staph. hyicus</i>
	<i>Staph. haemolyticus</i>
	<i>Staph. hominis</i>
	<i>Staph. caprae</i>
	<i>Staph. warneri</i>
	<i>Staph. xylosus</i>
	<i>Staph. capitis</i>
	<i>Staph. aureus</i>
	<i>Staph. saprophyticus</i>
	<i>Staph. lugdenensis</i>

Other Rapid detection method of *Staphylococcus aureus* is Bioluminescent Enzyme Immunoassay that developed for direct rapid detection or identification of *Staph. aureus* – protein A. Staphylococcal protein A is one of the most characteristic proteins that produced by *Staphylococcus aureus* (Fukuda *et al.*, 2000). It was demonstrated by (Fukuda, 1970) that 98% of coagulase positive Staphylococcal strains which had been isolated from human are able to produce protein A. Bioluminescent Enzyme Immunoassay is highly rapid and sensitive in which it can detect as low as one pg of protein A and 10^3 c.f.u./ml within 7 hours and by using appropriate immunological reagents, this method also could be adapted to detect the toxins as well as other foodborne pathogens (Fukuda *et al.*, 2000).

A powerful rapid detection method of variety of pathogens is Polymerase Chain Reaction (PCR), is an invitro methods used for replicating a defined target of DNA sequence (Gerhardt *et al.*, 2004). This technique was proposed by many authors to replace conventional methods for the detection of food-born pathogens in the last 10 years as it rapid, sensitive, specific and easy to handle (Pinto *et al.*,

amplification and hybridization of Staphylococcal enterotoxins genes using Multiplex PCR – enzyme immunoassay (Pinto *et al.*, 2004).

More recently, real time PCR (RT-PCR) achieved more rapid, specific and reliable detection for *Staph. aureus* (Letertre *et al.*, 2003) as well as staphylococcal enterotoxins (Klotz *et al.*, 2003) than the other immunological assays (Pinto *et al.*, 2004).

1.14.3 Detection of staphylococcal enterotoxins

Over the recent years, varieties of different conventional and modern rapid methods of detecting food-born pathogens and toxins have been improved (Kroll *et al.*, 1993). Directly testing for the enterotoxigenicity of the isolated *Staphylococcus* species using one of the rapid new methods is the more useful approach to determine their pathogenicity (Bennett, 1996).

Staphylococcal enterotoxins were detected by biological test (animal test) either by intragastric feeding of young monkeys or by food extracts intraperitoneal injection in to kittens (Bergdoll, 1978). Other classic Traditional method is a combination of extraction, concentration and final detection of the toxin by use of microslide ouchterlony technique. It is time consuming (3-6 days) and labor – intensive method with a sensitivity of 0.1µg/100g of food (ICMSF, 1996; Kroll *et al.*, 1993).

The analytical methods for Staphylococcal enterotoxins detection in food requires very high sensitivity, at least of (0.4ng/g food) as this concentration reported to cause disease (Evenson *et al.*, 1988). The new methods based on different principles of direct or indirect detecting and have been improved in sensitivity, speed, precision and accuracy (Kroll *et al.*, 1993).

Immunological assays replaced the biological test by using other rapid and sensitive technique (Antibody – based assays). One of the most common rapid

Antibody – based detection method is ELISA (Enzyme Linked Immuno-Sorbent Assay) which is a very sensitive immunological test, detect the presence of an antibody or an antigen (enterotoxins) in a sample as low as 0.1ng (Lapeyre *et al.*, 1988; Windemann *et al.*, 1989; Baird – Parker, 1990). A solid phase double antibody sandwich ELISA techniques has been used for Staphylococcal enterotoxins detection in a variety of food implicated in food poisoning outbreaks, where (Freed *et al.*, 1982) found less than 1ng of enterotoxin per gram of food (>1ng/g) could be detected within 8hours working day.

Latex agglutination method is very practical and simple immunological test but less sensitive than ELISA (Kroll *et al.*, 1993). Several disadvantages associated with immunological assays that limits it used for SEs detection in food, for example, loss of immunological activity and the subsequent biological activity of SEs after heat treatment of foods (e.g. cooking) specially in an acidic medium (Loir *et al.*, 2003). It has been reported by (Bennet, 1992) that after a heat treatment of SEA and SED, the serological recognition of the two enterotoxin have been loosen and their detection by serological method have been prevented though both enterotoxins were perfectly active after an in vivo test on kittens. In addition, false positive reaction could be obtained due to non – specific interaction of the food component in the immunological tests (Kroll *et al.*, 1993). The quality of the antibody and the purity of the detected antigen determine the quality of the immunoassay for SEs detection, however, the potential of non – specific reaction is always there even if a pure antigen is used (Brett, 1998).

Chemical methods were also used in isolation, quantification and identification of varieties of Staphylococcal enterotoxins which have been considered to important human virulence. Isoelectric focusing, repeated chromatographic separation mass spectrometry, electrospray ionization (ESI), and matrix – assisted laser desorption ionization (MALDI) are an example of useful chemical methods for analysis of proteins molecules such as Staphylococcal enterotoxins, however, both repeated chromatographic separation and Isoelectric focusing methods are time consuming and usually requires purification steps (Duran-Reynals, 1942; Forsgren and

Sjoquist, 1966; Navarre and Schneewind, 1999; Takeuchi *et al.*, 1999; Kawano *et al.*, 2000).

On the other hand, mass spectrometry methods, particularly electrospray ionization (ESI) mass spectrometry focus in protein chemistry and determine rapidly accurate molecular masses of the interest protein (Fenn *et al.*, 1990; Mann and Willm, 1995; Kawano *et al.*, 2000). A rapid isolation, quantification and identification of Staphylococcal exoproteins obtained by (Kawano *et al.*, 2000) using reverse capillary performance liquid chromatography electrospray ionization mass spectrometry (LC-ESI/MS). In this assay Staphylococcal enterotoxin C3 (SEC3) was detected by (LC-ESI/MS) and followed by amino acid (N-terminal sequences) detection from the high resolution separated peaks.

The sensitivity and specificity of Staphylococcal enterotoxins detection methods has been significantly increased by using of polymerase chain reaction (PCR) and other amplification techniques. Although the result of PCR technique can be obtained in one day, it is only show the existence of the enterotoxin genes (virulence factors) in the producer organism do not indicate the presence or production of staphylococcal enterotoxins in food (Kroll *et al.*, 1993). This problem has been overcome recently by using the advanced PCR technology that improved the sensitivity and specificity for *Staphylococcus aureus* and staphylococcal enterotoxins detection. This powerful detection method based on the coupling of the Enzyme – Linked Immunosorbent Assay (ELISA) and Polymerase Chain Reaction (PCR) at which the enterotoxin - specific antibody detect the enterotoxin and then amplified by PCR, thus the detection sensitivity increased by 50 – 1000 times than the ELISA test (Fischer *et al.*, 2006).

1.14.4 Rapid detection kit of staphylococcal enterotoxins

Toxin detection kits are convenience for rapid and routine laboratory work as the entire test reagents are supplied as a package. All of the available commercial detection kit of Staphylococcal enterotoxins are immunoassay and the currently kits available in UK (Table 1-16) are six namely; BOMMELI, RIDASCREEN, TECRA, TRANSIA, SET – RPLA and VIDAS (Brett, 1998). The reason of doing the test will determine the most suitable kit to choice. At the end, the result must be interpreted and confirmed by other test or corroborated by other findings what ever the test kit is used (Brett, 1998). Comparison between these kits is outlined in (Table 1-17).

Table 1-16 Kit for Staphylococcal enterotoxins detection (Brett, 1998).

Kit	Manufacture	Supplier in the UK
BOMMELI	Dr Bommeli AG, Berne, Switzerland	Dr Bommeli AG, Berne, Switzerland
RIDASCREEN	R-Biopharm GnbH, Darmstadt, Germany	Digen Ltd, Oxford
TECRA	Bioenterprises Pty Ltd, Roseville, Australia	TECRA Diagnostics, Reading
TRANSIA	Transinia Diffchamb – SA, Lyons, France	Microgen Bioproducts Camberley
SET-RPLA	Denka, Japan	Oxoid, Basingstoke
VIDAS	bioMerieux, Marcy- PEtoile, France	bioMerieux UK Ltd, Basingstoke

Table 1-17 Comparison between the immunoassay kits for Staphylococcal enterotoxins detection (Brett, 1998).

	BOMMELI	RIDASCREEN	TECRA	TRANSIA	SET-RPLA	VIDAS
Toxins detected	A-D (H*)	A-E	A-E	A-E	A-D (E+)	A-E
Toxins differentiated	Yes	Yes	No	No	Yes	No
Sensitivity	High	High	Medium	Medium	Low	Medium
Specificity	High	High	Low	Low	High	Medium
Controls	-, IgG	+, -, IgG	+, -	+, -	+, -, IgG	+, -
Sample size	≤ 20 ml	0.7 ml	0.2 ml	0.5 ml	0.2 ml	0.5 ml
Duration of test	24 h	3 h	4.5 h	1.5-2 h	16 h	1.5 h
Duration of extraction	4-8 h	2 h	0.5-24 h	2 h	0.5 h	0.5-2 h
Complexity of test	High	Medium	Medium	Medium	Low	Automated
Complexity of extraction	High	Medium	Medium/high	Medium	Low	Low
Results subjective	No	No	Yes or no	Yes or no	Yes	No
Specialized equipment	Yes	Yes	Yes	Yes or no	No	Yes
Cost of kit	Medium	High	Small no.: high Large no.: low	High	Medium	High
Number of tests per kit	10	12	12-44	10-40	20	30
Format	ELISA polystyrene balls	ELISA microtitre strip	ELISA microtitre plate	ELISA tubes	Reverse passive latex agglutination	ELISA microtitre strip

1.14.4.1 BOMMELI

A highly sensitive solid- phase (ELISA) method that used antibodies – coating polystyrene beds to detect SEA – SED and SHE, but not SEES (Brett, 1998; Pimbley and Patel, 1998). It confirmed all of the positive results obtained from food poisoning outbreaks – food samples (140 samples) during 1984 – 1990 when it has been used by Food Hygiene Laboratory. However, some week false results have been reported by (Ewald, 1988) by using BOMMELI method.

BOMMELI is labour – intensive and impractical method for screening high number of food samples, however it considered as a method of choice for food poisoning outbreaks (Wieneke, 1991; Brett, 1998).

1.14.4.2 TRANSIA

TRANSIA is a tube-based sandwich immunoassay that used a mixture of monoclonal and polyclonal antibodies for the detection of Staphylococcal enterotoxins A – E (Pimbley and Patel, 1998), however it dose not preformed a specific identification of the enterotoxins types (Brett, 1998). The results of this test are determined either by photometer at 450 nm or by visual comparison with a positive control. It has been reported that TRANSIA method has less sensitivity than BOMMELI and RIDASCREEN (Brett, 1998).

1.14.4.3 TECRA

TECRA Staphylococcal enterotoxin detection kit (Fig. 1-9) is a Convenient, rapid microtitre plate – based immunoassay used for specific identification of Staphylococcal enterotoxins A – E by using polyvalent antisera (Pimbley and Patel, 1998). The sensitivity of this test is equal to 1 ng/ml of food/culture filtrates and the analysis required 3 – 4 hours, however, some foods which are thermally processed required 21 hours due to urea treatment step that responsible for enterotoxins renaturation (Pimbley and Patel, 1998).

The results obtained from this test can be read by absorbance measurement of 414nm or visually with colour card. This method achieved AOAC adoption for large number of samples preliminary screening (Bennett and McClure, 1994; Brett, 1998).

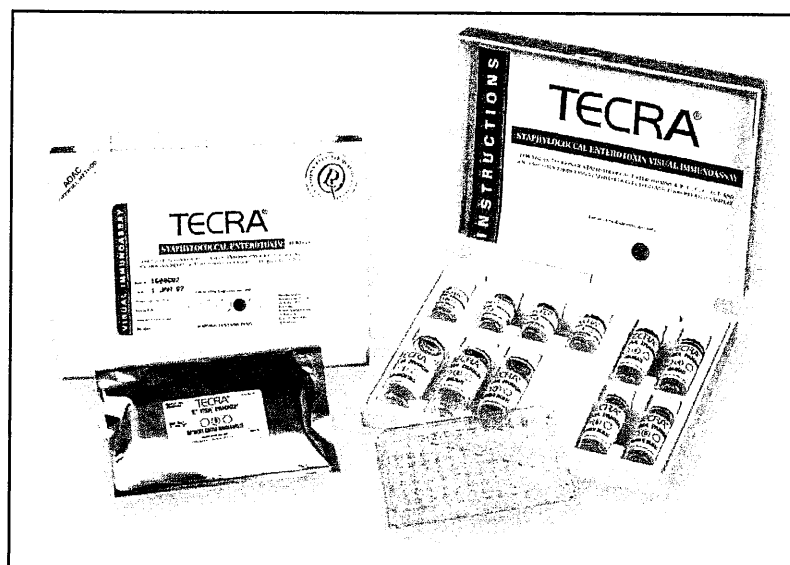


Figure 1-9 TECRA Staphylococcal enterotoxin detection kit (Biotrace, 2004)

In evaluation of TECRA method, the result showed that its sensitivity similar to SET – RPLA immunoassay, however, with samples contaminated with bacteria other than *Staphylococcus aureus*, this kit showed non specific reaction (Pimbley and Patel, 1998). Endogenous peroxidaes enzyme in some food such as pickles and some brine is similar to the peroxidaes of the conjugate in TECRA kit which caused false positive reactions (Deibel *et al.*, 1993; Brett, 1998). When the food treated with sodium azide, the endogenous peroxidaes inactivated, however, the amount of Staphylococcal enterotoxins reduced by 20 – 30% (Park *et al.*, 1994) moreover, (Pimbley and Patel, 1998) reported false positive result when samples treated with heat for 2 min at 100°C were examined.

1.14.4.4 RIDASCREEN

A very sensitive and specific immunoassay (Fig. 1-10) used for rapid and accurate staphylococcal enterotoxin detection in fluid and solid foods as well as in crude culture extracts (Arrow Scientific, 2007). The assay used for quantitative analysis and it was reported to detect as low as (0.5 – 0.75 ng/g food) of staphylococcal enterotoxin in contaminated food (Park *et al.*, 1994; Brett, 1998) and (0.2 – 0.25 ng/g food) in artificially contaminated food (Pimbley and Patel, 1998).

This method has been used by 12 laboratories for staphylococcal enterotoxin testing in food in a collaborated study. As a result, no false positive results were obtained and only 0.7% gave weak positive results recorded by one analyst (Park *et al.*, 1994; Brett, 1998). Several foods inoculated with enterotoxigenic *Staphylococcus aureus*, non – toxigenic staphylococci and *Serratia marcescens*, and tested with RIDASCREEN-SET assay and TECRA kit, as a result, only the enterotoxigenic *Staphylococcus aureus* – containing samples gave positive result, however TECRA kit result gave false positive result with samples contaminated with *Serratia marcescens* (Pimbley and Patel, 1998). Similar to TECRA kit, RIDASCREEN-SET assay is also based on peroxidase – coupled detection system which gave positive false results with naturally occurring peroxidase in food samples.

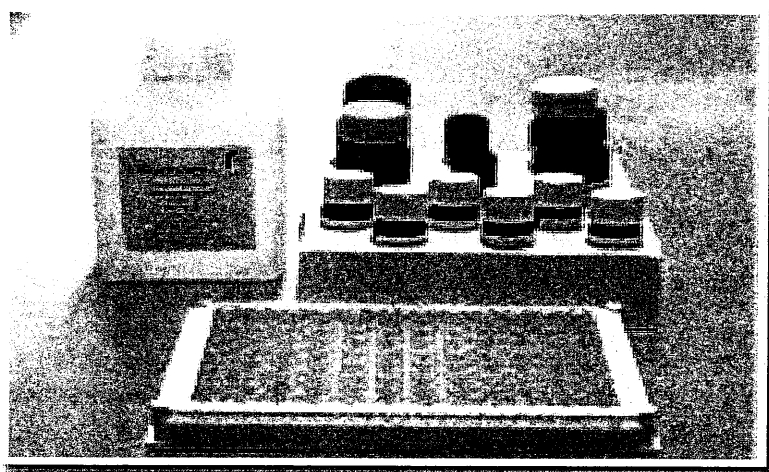


Figure1-10 RIDASCREEN - Staphylococcal enterotoxins (SET) A, B, C, D, E
(Arrow Scientific, 2007).

1.14.4.5 SET – RPLA

Reversed passive latex-based agglutination immunoassay used for Staphylococcal enterotoxins A, B, C and D detection in wide variety food samples or culture filtrates in which specific antibody which attached to particles (latex) reacts with soluble antigen (enterotoxin) result in visible latex agglutination reaction (Oxoid, 2001). The result in this test read by eye (Fig.1-11) and classified according to the agglutination degree from (-) to strongly positive (+ + +) (Brett, 1998).

None specific reaction reported by (Haines and Stunnard, 1987) during SEs detection in some food such as cheeses and prawns. Other study created by (Wienke, 1991) for SEs detection in food using 4 immunological test kits shows that no single method was superior, however SEF-RPLA was the lowest price and the easiest test to be performed (Pimbley and Patel, 1998).

Although SET – RPLA has the lowest sensitivity among the other immunoassays (Wieneke and Gilbert, 1987; Brett, 1998), relatively expensive and gave only semi – quantitative results (Pimbley and Patel, 1998), it remains the method of choice for culture filtrate (Wieneke, 1988; Brett, 1998).

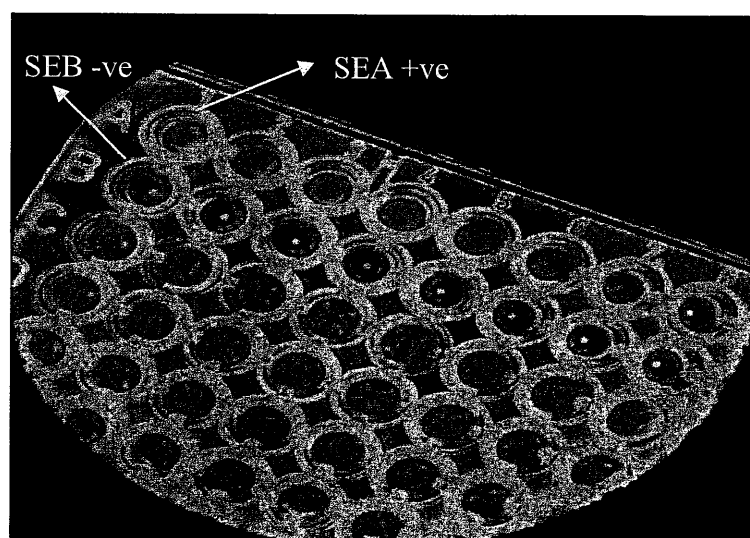


Figure 1-11 Positive result for SEA (Row A) and negative result for SEB (Row B) detected in contaminated ready to eat humus by SET-RPLA (PHL-Bahrain, 2005).

1.14.4.6 VIDAS

VICAS Staphylococcal enterotoxin –SET assay (BioMérieux) is an automated enzyme – linked fluorescent immunoassay (ELFA) for Staphylococcal enterotoxin A, B, C1, C2, C3, D, and E detection in foods (Pimbley and Patel, 1998; BioMérieux, 2005). Its sensitivity range is between $< 1 - 1.5$ ng / ml in artificially contaminated foods and this sensitivity is good sensitivity to most SEs types except SEE (Pimbley and Patel, 1998).

In VIDAS - SET2 (BioMérieux) assay, a new combination of complementary polyclonal and monoclonal antibodies directed to Staphylococcal enterotoxin antigenic sites (Fig. 1-12). Removal of Fc (crystallizable region) fragment from the antibodies used in this technology increases the specificity of this kit as well as reduces the potential of false positive results due to the interference with the food matrices and non – specific binding of other bacteria. In addition, removal of the two Fab (fragment antigen binding) units increases the recognition of the antigen which results in better sensitivity (BioMérieux, 2005).

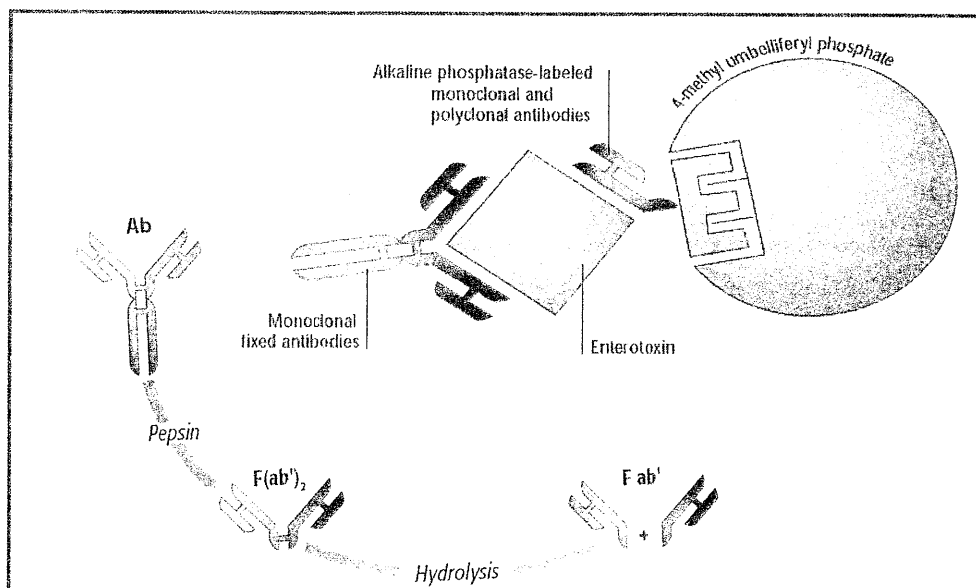


Figure 1-12 The principle of VIDAS Staphylococcal enterotoxin - SET2 assay (BioMérieux, 2005)

The detection limit of VIDAS Staphylococcal enterotoxin - SET2 assay increase to reach 0.1 – 0.25 ng/g of food and 0.05 – 0.1 ng/g in purified toxin (Fig. 1-13). Beside the higher specificity and sensitivity, this assay also reduces the handling time in sample preparation by using trichloro acetic acid (TCA) precipitation (BioMérieux, 2005).

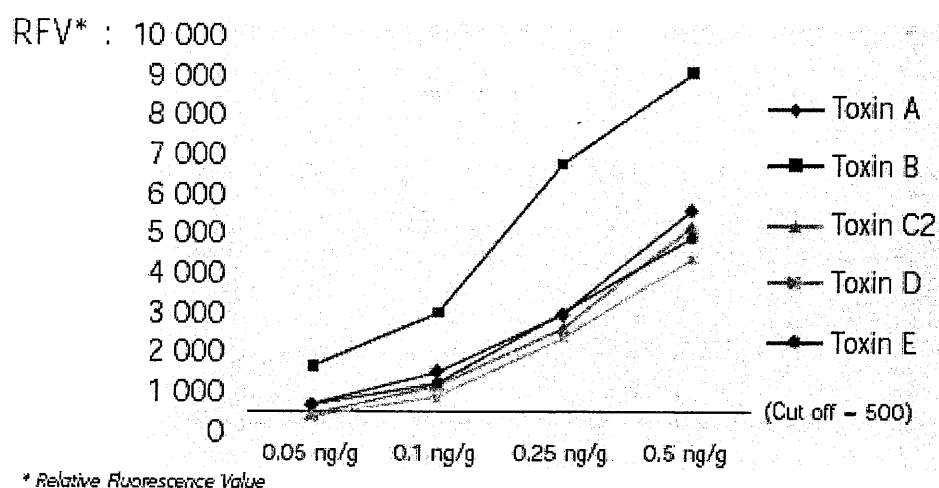


Figure 1-13 Detection limits of purified enterotoxins in VIDAS Staphylococcal enterotoxin – SET 2 assay (BioMérieux, 2005)

1.15 Conclusion

Staphylococcal human intoxication is caused by ingesting the enterotoxins which were produced in food by some strains of *Staphylococcus* due to improper holding temperature of food that must be kept either $\leq 7.2^{\circ}\text{C}$ or $\geq 60^{\circ}\text{C}$ (FDA, 2006)

Beside *Staphylococcus aureus*, other Staphylococci species (CPS and CNS) are capable of producing Staphylococcal enterotoxins in foods. Although CPS are the main enterotoxin producer, several CNS have been involved in plenty of food poisoning outbreaks besides several studies that emphasise its toxigenic capacity (Breckinridge & Bergdoll, 1971; Bergdoll, 1982; Olsvik *et al.*, 1982; Evans *et al.*, 1983; Crass & Bergdoll, 1986; Ewald, 1987; Aliu & Bergdoll, 1988; Baustista *et al.*, 1988; Gomez-Lucia *et al.*, 1989; Valle *et al.*, 1990; Bennett, 1996; Cunha *et al.*, 2005). This capacity could be a significant potential cause of food poisoning outbreaks; therefore detection of these strains should not be ignored in reference or routine food microbiology laboratories especially in the suspected cases of staphylococcal food poisoning. In addition, more attention, investigation and studies are required towards the toxigenic capacity of CNS.

According to (Ingram, 1960) recommendation “all Staphylococci should be considered in assessing the possibility of an enterotoxin hazard”

The growth and the enterotoxin production is highly influenced by several parameters such as temperature, pH, Eh and a_w (Jay, 1992). Staphylococcal enterotoxins are highly resistant molecules, therefore it is easily to destroy the enterotoxigenic bacteria rather than destroy or inactivate them.

The actual incidence of the staphylococcal food poisoning disease is unknown due to the following reasons (FAD, 2006):

- Low responses from victims during the health official's interviews.
- Inadequate sample collection for laboratory analyses.

- High similarity in the disease symptoms with other food poisoning types (e.g. food poisoning cause by *Bacillus cereus* toxin) which result in misdiagnosis of this Disease.
- Improper laboratory examination.

The traditional detection methods of *Staphylococcus aureus* based on the use of selective media such as (Baird – Parker agar, Mannitol agar, RPF agar....etc) that used for enumeration of the Staphylococcal - food isolates. The obtained positive colonies then subjected to other conformational test such as DNase and coagulase production and other biochemical tests. The recent rapid detection and identification methods which are based on different principles of direct or indirect detecting of microorganisms are faster, more sensitive, accurate and specific than conventional bacteriological methods (Kroll, *et al.*, 1998).

Directly testing for the enterotoxigenicity of the isolated *Staphylococcus* species using one of the rapid new methods is the more useful approach to determine their pathogenicity (Bennett, 1996).

CHAPTER TWO

METHODOLOGY

2.0 Methodology (Experiments)

2.1 Bacterial Culture

Three strains of *Staphylococcus* species (*Staphylococcus aureus* NCTC 8532 and *Staphylococcus aureus* NCTC 10657, which are coagulase positive and *Staphylococcus epidermidis* NCTC 12100 which is coagulase negative) were obtained from National collection of Type Cultures (NCTC), Central Public Health Laboratory, Colindale Avenue, London, UK. The bacterial cells were resuspended in 10 ml Trypticase soy broth (TSB) medium (Appendix I) and then incubated for 18 hours at 37°C. Cells were precultured overnight on Nutrient Agar (NA) medium to check for purity. A loopful of each culture broth was streaked on a Baird-Parker agar (BP) plate (Oxoid-CM275) - (Appendix I) surface to confirm the presence of the required strain and to obtain pure colonies. The plates were then incubated at 37°C for 24 hours. Purity was further confirmed (section 2.8) by Gram's stain and Staphylase diagnostic kit (Oxoid-DR0595). The cultures were maintained on Nutrient Agar (NA) slopes at 5°C.

2.3 Sterility testing of the food sample used as the laboratory test system (ready to served custard)

Ready to serve custard was used as the substrate for this laboratory study to evaluate the growth and staphylococcal enterotoxins production by the three *Staphylococcus* species at 3 different incubation temperatures.

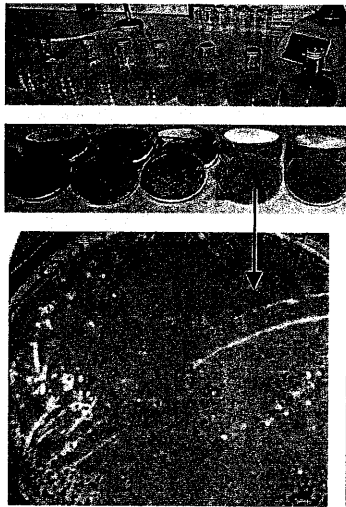
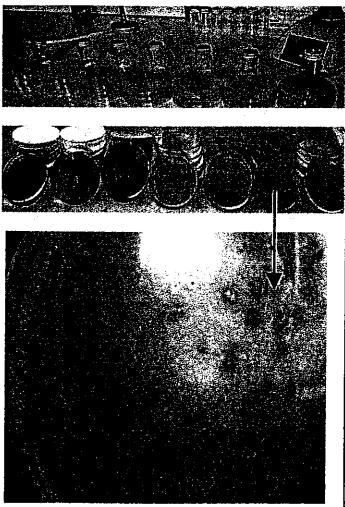
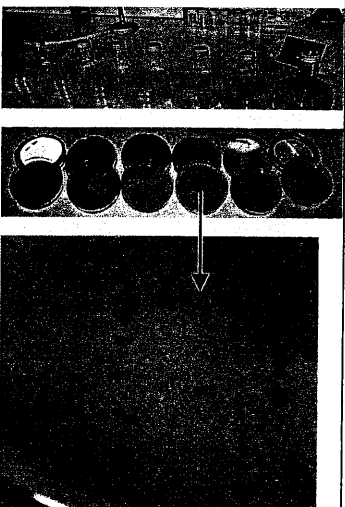
A sample of 10 g of custard was suspended in 90 ml of Maximum Recovery Diluent (MRD) (Appendix I), and homogenized in a stomacher for one minute. From this, appropriate serial dilutions were prepared. Aliquots of 0.1 ml from each dilution were distributed evenly over the surface of Baird – Parker agar (Oxoid). The plates were incubated at 37°C for 24 hours.

2.4 Experiment 1: Standardisation of evaluation bacterial inocula for sample contamination (challenge test)

2.3.1 Materials and methods

To identify the required level of bacterial inoculum (10^4 cfu/ml) to spike the custard sample for this work, overnight cultures were grown in Trypticase Soy Broth (TSB) medium (one pure colony from each strain was aseptically transferred into 10 ml TSB). The cultures were then incubated at 37°C for 18 hours. One millilitre of each culture was then diluted with 9 millilitres of Maximum Recovery Diluent (MRD) to give 10^{-1} dilution. From this, serial of 10 – fold dilutions (10^{-1} - 10^{-7}) were made. The approximate level of each strain was quantified by using the spread plate technique (APHA, 1976). The number of bacteria per ml of the original sample was calculated (Appendix II). The evaluation of the bacterial inoculums for sample contamination is illustrated in (Table 2-1).

Table 2-1 The evaluation of the bacterial inocula for sample contamination

<i>Staphylococcus aureus</i> NCTC 8532	<i>Staphylococcus aureus</i> NCTC 10657	<i>Staphylococcus epidermidis</i> NCTC 8532
		

2.3.3 Results

The counts obtained of the 3 strains (*Staphylococcus aureus* NCTC 8532, *Staphylococcus aureus* NCTC 10657, and *Staphylococcus epidermidis* NCTC 12100) were recorded after 24 hours incubation at 37°C (Appendix II) and the expected level of each strain was calculated (Table 2-1).

Table 2-2 The evaluated bacterial inocula for sample contamination

Species	Bacterial inocula required /100ml custard to give 10^4 cfu/ml
<i>Staphylococcus aureus</i> NCTC 8532	1.3 ml of the dilution 10^{-3}
<i>Staphylococcus aureus</i> NCTC 10657	1.4 ml of the dilution 10^{-3}
<i>Staphylococcus epidermidis</i> NCTC 12100	1.8 ml of the dilution 10^{-3}

2.4 Experiment 2: Evaluation of the effect of different storage times and temperatures on the growth of *Staphylococcus aureus* - NCTC 10657, *Staphylococcus aureus* - NCTC 8532, and *Staphylococcus epidermidis* - NCTC 12100.

2.4.1 Materials and methods

The laboratory system was prepared by adding 1.4 ml, 1.3 ml and 1.8 ml of the dilution 10^{-3} of the, and *Staphylococcus epidermidis* NCTC 12100 culture's broth respectively to each 100ml of custard and homogenised for one min by the stomacher to give an initial bacterial concentration of approximately 10^4 cfu/ml. The spiked custard was then transferred aseptically into 9 sterile pots and each 3 pots were incubated at 3 different temperatures (5°C, 30°C and 44°C), thus triplicate spiked custard pots were prepared for each strain at each examined temperature. 100 millilitres of Non-spiked custard were also transferred in to 3 sterile pots (control). The growth and enterotoxins productions were examined after 1, 2, and 3 days. The preparation of the contaminated custard sample is demonstrated in (Table 2-3).

After each incubation day, samples (25 g) were taken aseptically and diluted in 225 ml Maximum Recovery Diluents (MRD) - (Oxoid). The food suspensions were homogenised for one min in the stomacher to give a dilution of 10^{-1} . Further ten – folds dilutions (10^{-2} and 10^{-3}) were made (Table 2-4). The enumeration of staphylococcal strains (viable bacterial counts) was recovered on the surface of predried Baird – Parker agar plates. Aliquots of 0.1 ml of each dilution was agitated and aseptically transferred in to the surface of the agar plate. The aliquots were spread with a sterile disposable spreader. Plating was carried out within 15 min of dilution preparation of each sample. The whole plates were incubated in inverted position at 35°C and examined after 24 and 48 hours. *Staphylococcus aureus* and *Staphylococcus epidermidis* colonies were quantified after 24 hours incubation at 37°C and examined for coagulase reaction for further conformation. *Staphylococcus* species results were reported per millilitre of food.

Table 2-3 The preparation of the contaminated custard for each of the examined strain in this study.

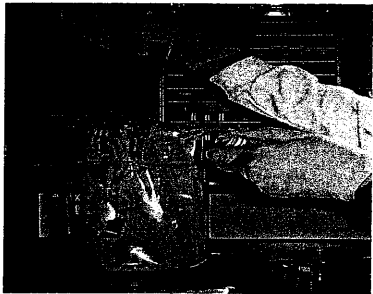
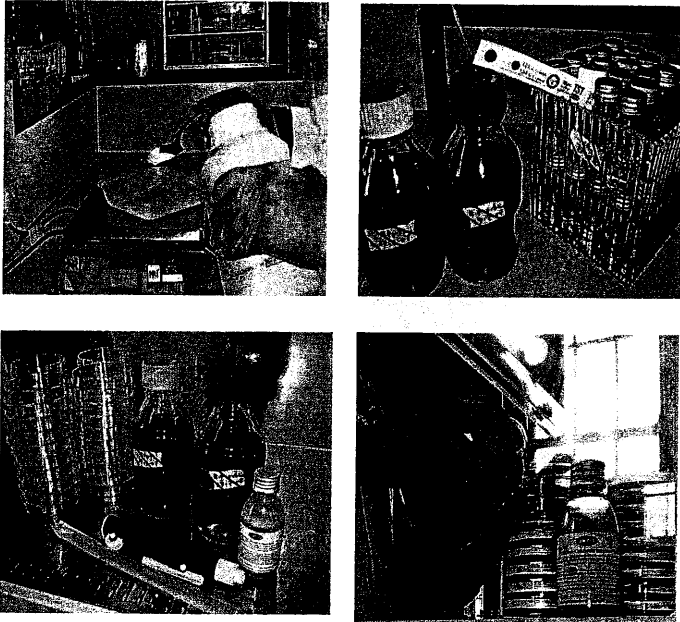
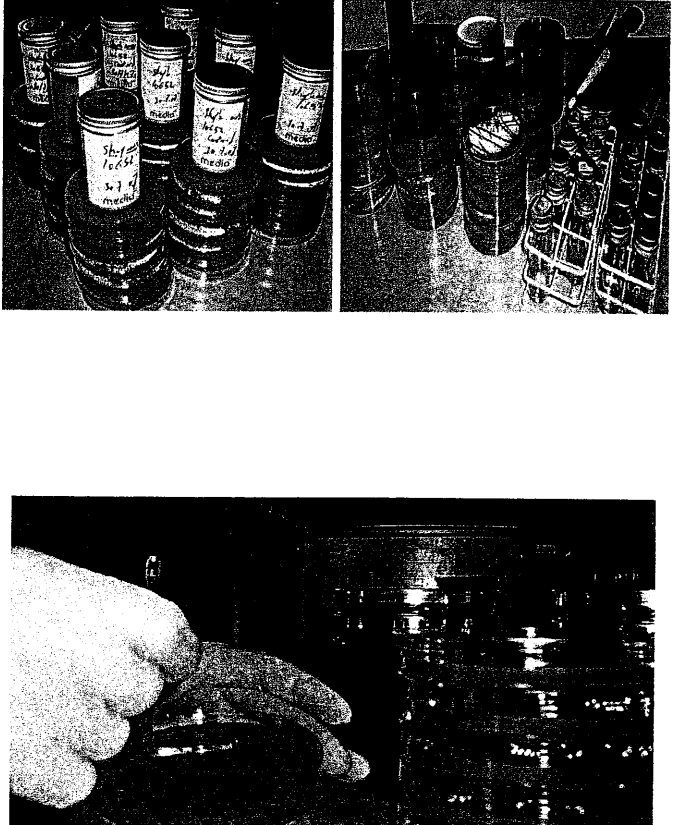
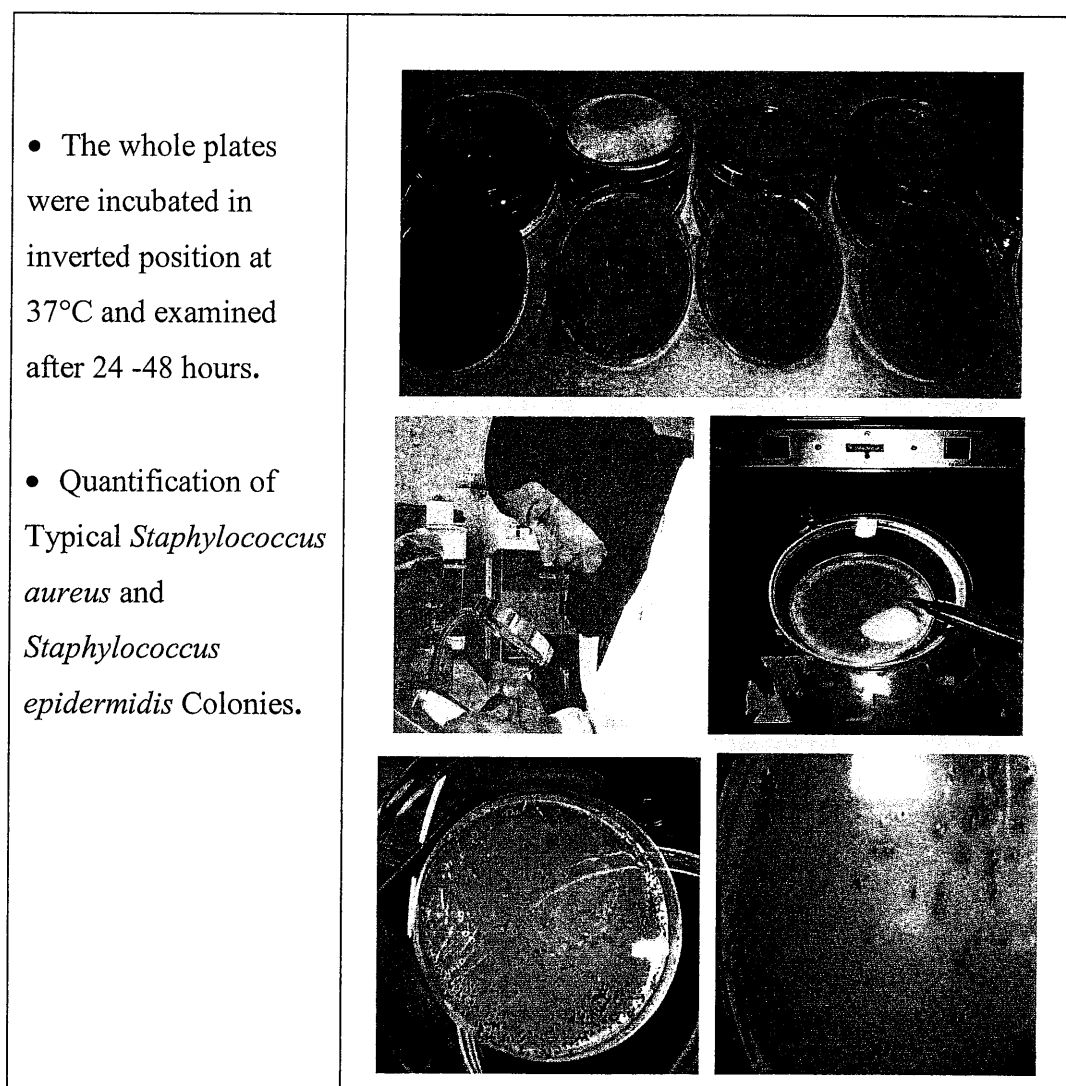
Procedure Steps	Picture
Addition of appropriate volume of the bacterial culture's dilution (10^{-3}) to the whole custard sample and homogenise thoroughly for one min in the stomacher to give an initial bacterial concentration of approximately 10^4 cfu/ml.	
Transfer 100 millilitres of the spiked custard aseptically in to 9 sterile pots. 100 millilitres of Non-spiked custard were also transferred in to 3 sterile pots (Control).	
Incubation of the spiked custard pots (triplicate) with non-spiked (control) custard pot in to 3 different temperature (5°C, 30°C and 44°C) incubators.	

Table 2-4 Staphylococcal Growth Detection.

Procedure steps	Related Picture
• Preparation of Baird – Parker agar plates for staphylococcal - enumerations. • <i>Staphylococcus</i> cells viable counts were carried out by using Spread plate method.	 The top row of images shows Baird-Parker agar plates on the left and bottles of media on the right. The bottom row shows a rack of test tubes on the left and a rack of test tubes on the right.
• 25 g of each samples pot was taken aseptically and diluted in 225 ml (MRD) and homogenised for one min in the stomacher to give a dilution of 10^{-1}. • Ten – folds dilutions (10^{-2} and 10^{-3}) were prepared. • Plating was carried out within 15 min of dilution preparation of each sample	 The top row of images shows bottles of media on the left and a rack of test tubes on the right. The bottom image shows a hand holding a petri dish.



2.4.2 Result

The effect of different storage times and temperatures on the growth of *Staphylococcus aureus* (NCTC 10657 and 8532) and *Staphylococcus epidermidis* (NCTC 12100) in the spiked custard was evaluated over the 3 storage days using the spread plate method on Baird – Parker agar (BP).

The colonies with typical morphology of each strain were counted every 24 hours and confirmed by Gram's stain and staphylase test (Oxoid – DR0595). The typical *Staphylococcus aureus* colonies on Baird – Parker agar were black, shiny, smooth and convex with narrow white entire margins which gave visible clearing zone (2-5mm) after 48 hours incubation (Figure 2-1). In contrast to *Staphylococcus aureus*

colonies, *Staphylococcus epidermidis* colonies appeared small, non – shiny black colour on Baird – Parker agar after overnight incubation. The typical colony forming unit of *Staphylococcus epidermidis* on Baird – Parker and Nutrient agar plates is shown in (Figure 2-2).

The counts of a total of nine samples per each strain (Triplicate in each storage temperature) during the 3 storage days are illustrated in (Appendix II). The amount of variability present in the growth records at each temperature for each species was measured using *t*-test and SPSS – descriptive statistical analysis are demonstrated in (Appendix II).

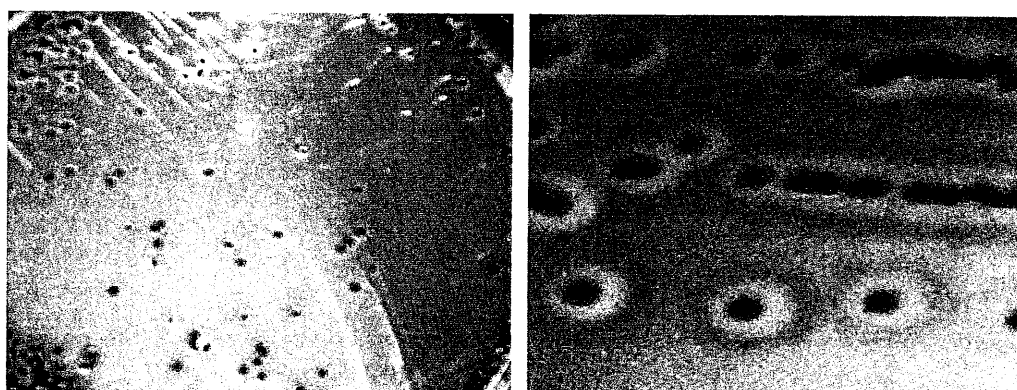


Figure 2-1 Typical *Staphylococcus aureus* (NCTC 10657) colonies on Baird-Parker agar. Colonies on the right shows clear zones as a result of proteolysis activity (24 hr) and opaque zone due to lipolysis activity (48 hr).



Figure 2-2 Typical *Staphylococcus epidermidis* (NCTC 12100) colonies on Baird-Parker agar. The small white colonies on the left are *Staph. epidermidis* on Nutrient agar plate.

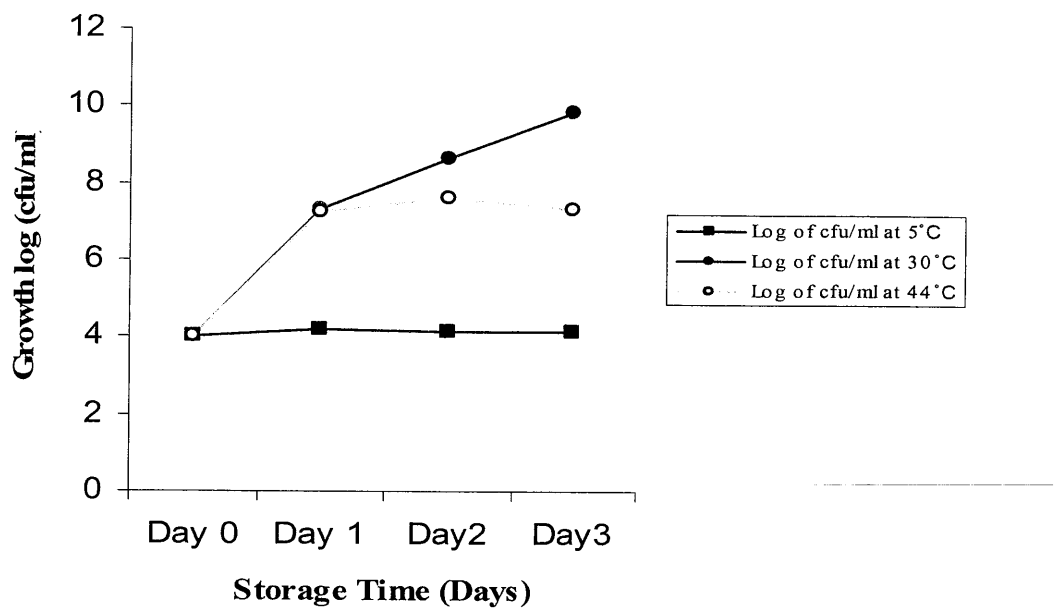
The growth results of *Staphylococcus aureus* (NCTC 10657) at 5°C within the 3 storage days shows no significant increase in the total cell's counts at storage temperature of 5°C within the 3 days of storage ($p=0.076$ at 95 % confidence interval). There was a slight increase in the total cell counts at the end of the third storage day by only 0.12 log units, however, the counts remains constant during the second and third days (Graph 2-1). At 30°C, a gradual increase in bacterial growth was observed over the 3 storage days. This difference was significant ($p=0.02$ at the % 95 confidence interval) in which the level of this strain was increased by 3.43-log units after one incubation day. The growth continued to increase until reach its maximum after the third incubation day at which the original cell counts increased by 5.87 log units.

A sharp increase in the growth rate of this strain was observed at 44°C (by 3.23 log units) within 24 hours incubation. This rate increased slightly after the second incubation day reach its maximum by 3.62 log units from the origin cell counts, however, this rate was reduced after the third incubation day. It was found that there was also a significant deference in the total number of bacterial counts at the % 95 confidence interval ($p=0.001$) at the % 95 confidence interval.

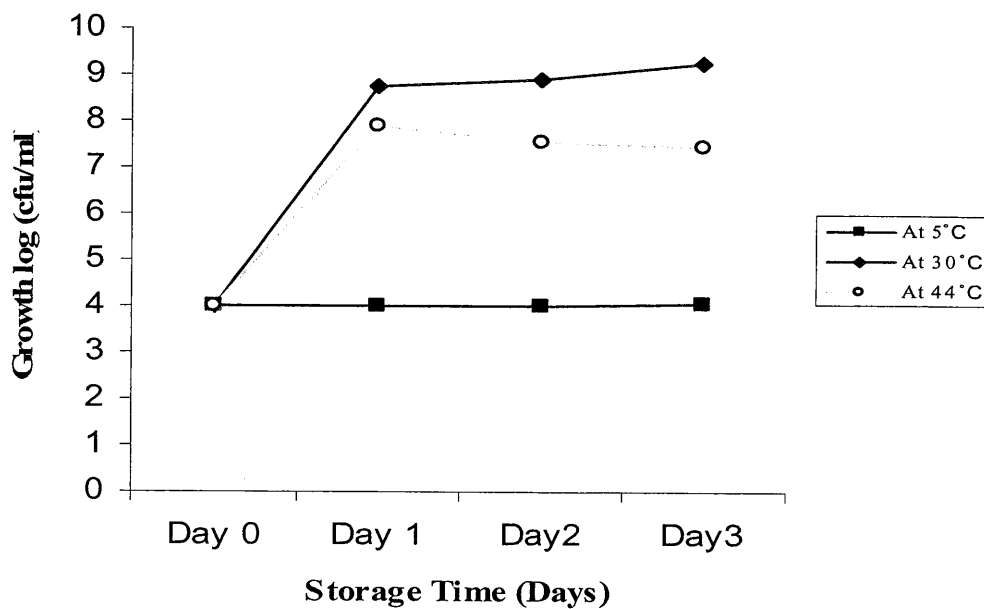
After the third day of incubation, the growth of *Staphylococcus aureus* (NCTC 10657) decreased at 44°C, increased at 30°C, and remains constant at 5°C.

As with *Staph. aureus* (NCTC 10657), the level of *Staph. aureus* (NCTC 8532) remained constant at 5°C and there was no significant increases in the original cell counts ($p= 0.39$ at the % 95 confidence interval). At 30°C, there was a sharp increase in the total cell counts after one day incubation (Graph 2-2) in which the original counts increase by 4.73 log units. This increase was continued to reach it maximum in the third day by 5.26 log units. This difference was statistically significant ($p= 0.001$ at the % 95 confidence interval). At 44°C, the cell counts was sharply increased (by 3.89 log units) within 24 hours incubation. However, this rate decreased gradually within the next two days. The difference in the original cell counts at this temperature was also significant ($p= 0.001$ at the % 95 confidence interval).

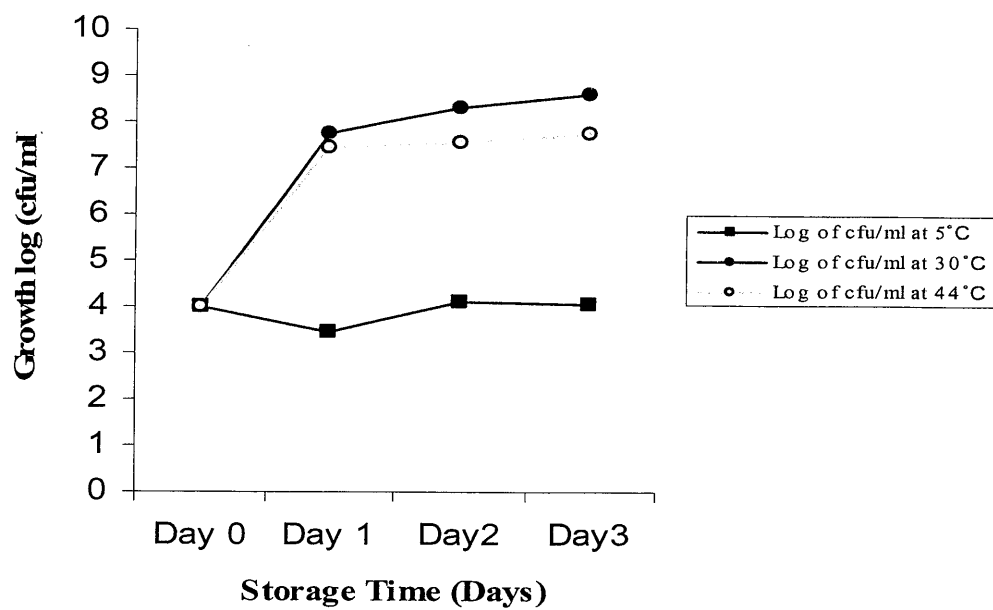
As with both *Staphylococcus aureus* species, *Staph. epidermidis* (NCTC 12100) shows no significant difference ($P= 0.184$ at 95% confidence interval) in levels from the original cell counts at 5°C. However, there was a significant difference observed at 30°C ($P= 0.004$ at 95% confidence interval) where the total cell counts at the end of the third day increased by 4.62 log units. At 44°C, the cell counts were sharply increased by 3.43 log units within 24 hours incubation. In contrast to both examined species of *Staphylococcus aureus*, this cell counts at 44°C was continued to increase within the next and third incubation days (Graph 2-3). This difference was significant around the sample count mean at 95% confidence interval ($P= 0.001$).



Graph 2-1 Effect of different storage temperatures on the growth rate of *Staphylococcus aureus* (NCTC 10657) over 3 storage days



Graph 2-2 Effect of different storage temperatures on the growth rate of *Staphylococcus aureus* (NCTC 8532) over 3 storage days



Graph 2-3 Effect of different storage temperatures on the growth rate of *Staphylococcus epidermidis* (NCTC 12100) over 3 storage days

2.5 Experiment 3: Evaluation of the effect of different storage times and temperatures on the staphylococcal enterotoxins production in the spiked custard samples by *Staphylococcus aureus* - NCTC 10657 and *Staphylococcus epidermidis* - NCTC 12100.

The detection of staphylococcal enterotoxins in the spiked food matrices and the culture fluid of the isolated *Staphylococcus* species were carried out with SET - Reversed passive latex-based agglutination immunoassay kit toxin detection (Oxoid TD0900) following the manufactures instructions.

The latex- control reagents (staphylococcal enterotoxin A, B, C and D control) were used from time to time to confirm the correct working of latex test.

The reagents were reconstituted by adding 0.5ml of the latex diluent (TD910) to each reagent's vial. The vials were shaken gently until the contents were dissolved. The reagents were examined for agglutination with its respective sensitised latex in the assay of toxin content (Table 2-5).

2.7.1 Methods and materials

The presence and level of staphylococcal enterotoxins in spiked custard, inoculated with *Staphylococcus aureus* - NCTC 10657 and *Staphylococcus epidermidis* - NCTC 12100 were examined to evaluate the effect of different storage times and temperatures on the staphylococcal enterotoxins production. 10 ml portions of custard samples along with 10 ml of a 0.85% NaCl solution was homogenised in a stomacher. The homogenate was centrifuged at 900g at 4° C for 30 minutes, and the supernatant was filtered with a low-protein-binding membrane filter (0.2mm-0.45mm).

The staphylococcal enterotoxins production in culture fluid by pure isolated colonies of *Staphylococcus aureus* - NCTC 10657 and *Staphylococcus epidermidis* - NCTC 12100 were also examined. The isolated organisms were inoculated in to Tryptone Soy Broth (TSB) and incubated at 37°C for 18 – 24 hours. After growth, each broth culture filtrated through 0.45µm low protein binding filter (Table 2-5). The filtrate was used for assay of toxin content.

Non-spiked custard sample which incubated in the 3 examined storage temperature (as a control) were examined for enterotoxin during the three incubation days for staphylococcal enterotoxins.

All extracted samples (filtrates) were examined for staphylococcal enterotoxins following the manufacture method:

The microtitre plates (V-well) were arranged, so that each sample had 5 rows and each row consists of 8 wells. 25µl of diluent was dispensed in each well of the 5 rows by using a pipette. 25µl of the filtrated supernatant (tested sample) was dispensed to first well of each of the 5 rows. Starting with the first well of each row, 25µl portion was picked up and doubling dilutions were preformed along each of the 5 rows until the 7th well reached. The last well was left only with diluent. 25µl of latex sensitised with anti-enterotoxin **A** was added to each well in the first row. 25µl of latex sensitised with anti-enterotoxin **B** was added to each

well in the second row. 25µl of latex sensitised with anti-enterotoxin **C** was added to each well in the third row. 25µl of latex sensitised with anti-enterotoxin **D** was added to each well in the fourth row. 25µl of latex control was added to each well in the fifth row. Each sample plate was agitated gently by hand to mix the contents of each well. The microtiter plate of each sample was covered with a microtitre plate lid to avoid evaporation and left undisturbed on vibration – free surface for 20 – 24 hours at room temperature (Oxoid, 2001).

Against a black background, each well in each row of the microtiter plate was examined for agglutination. The agglutination patterns (if any) were compared with the following illustration for result interpretation (Fig. 2-4):

- The positive results were classified as (+), (++), and (+++).
- The last well in all rows should be negative as well as the last row of wells which contain the latex control.

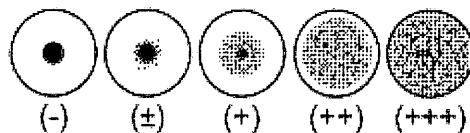
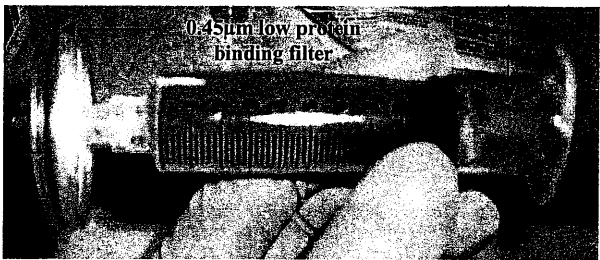
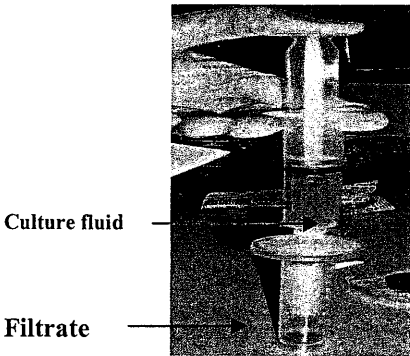
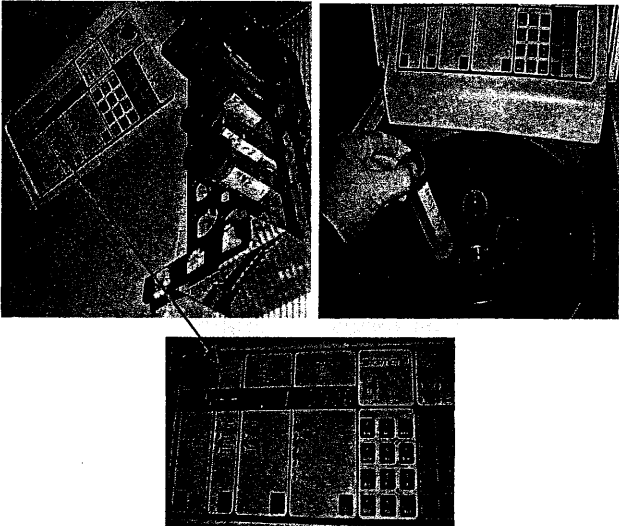
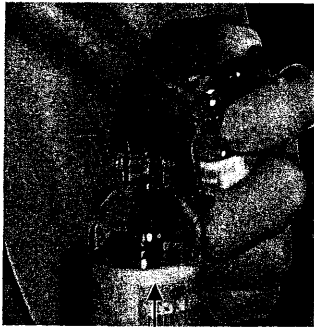
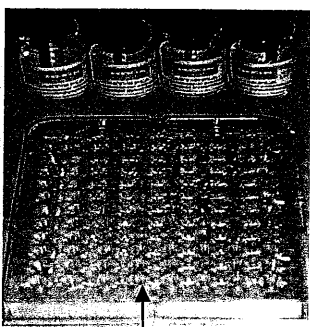
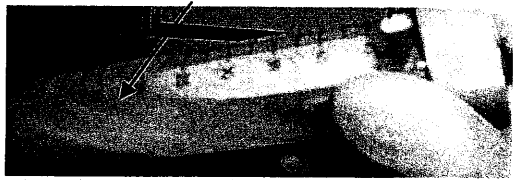
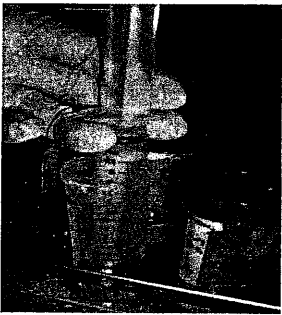
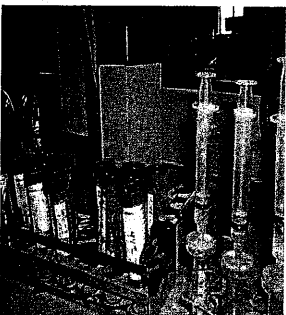
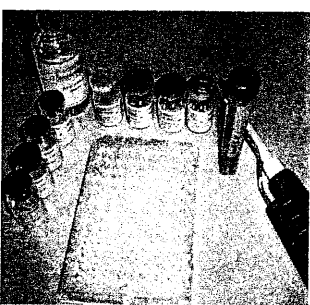


Figure 2-4 The agglutination patterns for enterotoxins result interpretation.

Table 2-5 The procedure steps of the evaluation of staphylococcal enterotoxins concentration in the spiked custard samples (Enterotoxin Assay)

Procedure	Picture
Overnight cultures of the 3 tested strains were grown on Trypticase soy broth (TSB) medium (one pure colony from each strain in to 10 ml TSB). The cultures were then incubated at 37°C for 18 hours.	
Each strain's broth culture was filtrated through 0.45µm low protein binding filter and the filtrate was used then for assay of toxin content.	
10ml portions of custard samples homogenised with 10 ml of a 0.85% NaCl solution. The homogenate was centrifuged at 900g (3000rpm) at 4° C for 30 minutes.	

The latex control reagents were used after reconstituted with 0.5 ml latex diluent to confirm the correct working of latex test.	Latex control reagents   Latex diluent Microtiter plate (V shape)
The supernatant was filtered after centrifugation with a low-protein-binding membrane filter (0.2mm-0.45mm).	Supernatant   
The diluent, filtrated supernatant (tested sample) and anti-enterotoxin A, B, C, and D were dispensed in the appropriate wells of the microtiter plate following the manufactures instructions.	 

2.7.2 Result

The effect of different storage times and temperatures on the Staphylococcal enterotoxin production by *Staphylococcus aureus* (NCTC 10657) and *Staphylococcus epidermidis* (NCTC 12100) in the spiked custard was evaluated over the 3 storage days using reversed passive latex-based agglutination immunoassay (SET - RPLA) diagnostic test (Oxoid). *Staphylococcus aureus* (NCTC 8532), the coagulase positive non – enterotoxin producer *Staphylococcus* species strain was used as a negative control in this study. The results were read visually and classified according to the agglutination degree (Fig. 2-4) from (-) to strongly positive (+++).

To confirm the correct working of latex test reagents, the SET-RPLA detection kit - control reagents (staphylococcal enterotoxin A, B, C and D control) were examined for agglutination with its respective sensitised latex in the assay of toxin content. Different agglutination patterns of SEA, SEB, SEC, and SED were detected with a level of sensitivity of 0.5 ng/ml. The agglutination degrees were read visually using the standard agglutination patterns card (Fig 2-7).

Non-spiked custard sample which incubated in the 3 examined storage temperature (as a control) were examined during the three incubation days for staphylococcal enterotoxins. Negative results were obtained for all control samples in each storage temperature (Fig. 2-8).

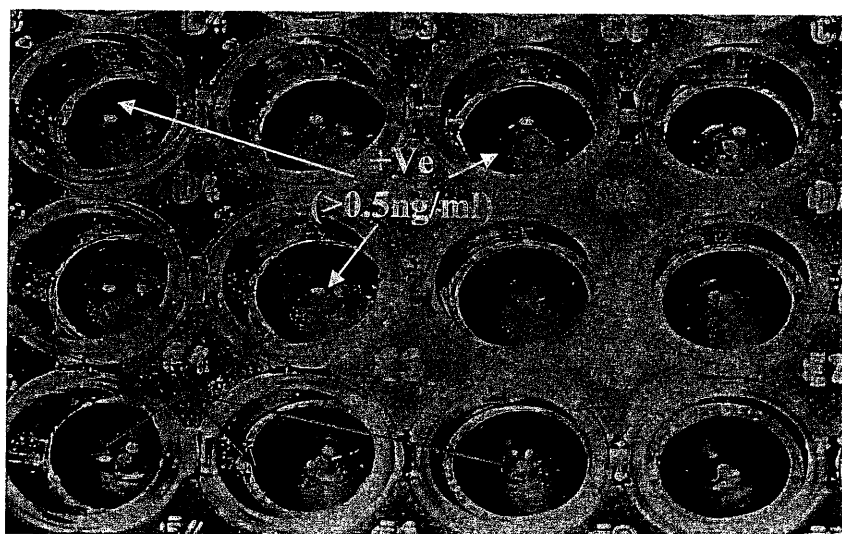


Figure 2-7 Latex test reagents effectiveness test result.

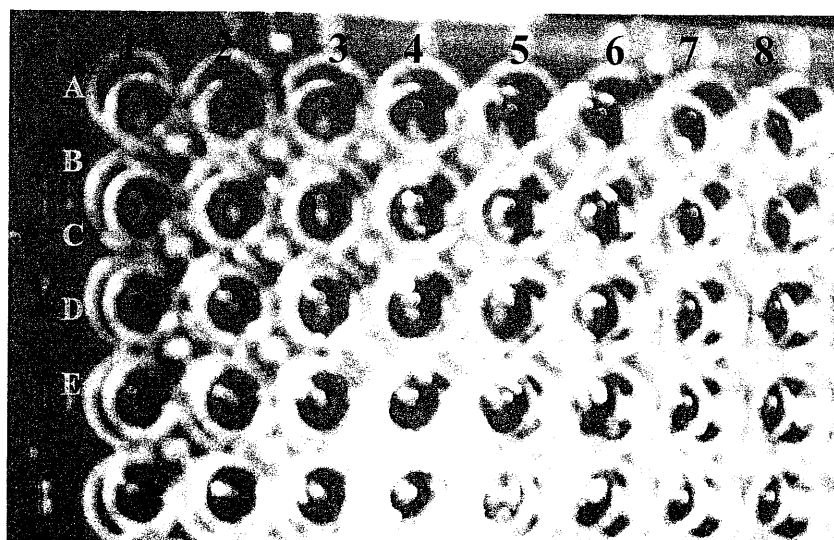


Figure 2-8 Control sample – enterotoxins examination (negative result).

The filtrates of the both 18 hours incubated enterotoxigenic organisms at 37° were examined for enterotoxins content using Set- RPLA kit. The result of pure *Staphylococcus aureus* - NCTC 10657 (7.4×10^8 cfu/ml) was found to be (+3) agglutination degree for both SEA and SEB with less than 18 hours incubation (Fig. 2-9). Similarly, the Pure culture of *Staphylococcus epidermidis* - NCTC 12100 result was found to be (+3) agglutination degree for SEC where the total cell counts was 1.0×10^9 CFU/ml. The result of pure *Staphylococcus aureus* - NCTC 10657 and *Staphylococcus epidermidis* - NCTC 12100 is shown in Table 2-6 and 2-7. *Staphylococcus aureus* -NCTC (negative control) pure culture filtrate was also examined for enterotoxin production and the obtained negative result is shown in Table 2-8.

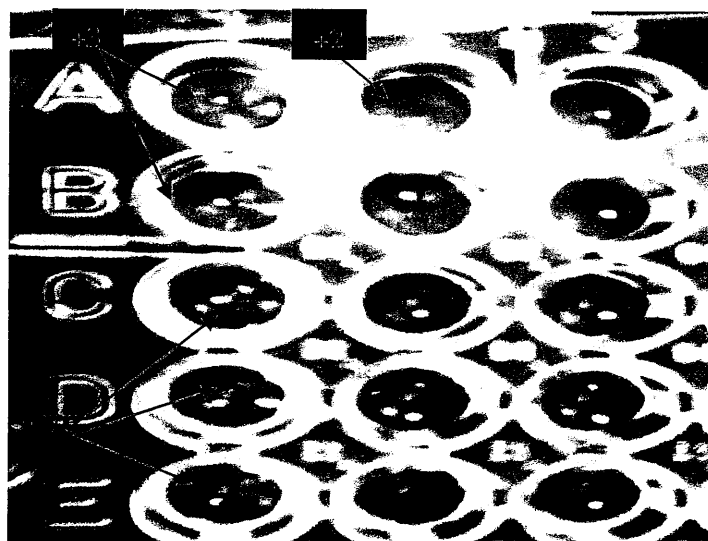


Figure 2-9 The result of pure *Staphylococcus aureus* - NCTC 10657 for both SEA and SEB with less than 18 hours incubation.

A total of nine spiked custard samples (3 in each storage temperature) per each enterotoxigenic strain (*Staphylococcus aureus* - NCTC 10657 and *Staphylococcus epidermidis* - NCTC 12100) per day were examined for staphylococcal enterotoxins using SET - RPLA diagnostic kit during day 0, 1, 2 and 3. The initial inoculum in each tested sample was approximately 10^4 cfu/ml.

At 5°C, no enterotoxin was produced by both enterotoxigenic strains. At 30°C and 44°C, *Staphylococcus aureus* - NCTC 10657 produced strong agglutination patterns (+3) for both SEA and SEB at the end of the third incubation day. The dilution through the microtiter plates indicates that SEB produced in high concentration than SEA at 30°C and 44°C. However, at 30°C the SEA production was increased during the 3 storage days and at 44°C remains constant through the diluted microtiter plate's wells and the agglutination did not exceed the forth dilution of the microtiter plate's well. The level of bacteria and enterotoxins concentration (degree of agglutination) of *Staphylococcus aureus* - NCTC 10657 during the three storage days at 5°C, 30°C and 44°C are illustrated in Table 2-9, 2-10, and 2-11 respectively.

No enterotoxin was detected for *Staphylococcus epidermidis* - NCTC 12100 in the first and second storage days at both 30°C and 44°C though the bacterial count was more than 10^7 CFU/ml. However, SEC was detected in the third day at 30°C and 44°C with an agglutination degree of (+2) and (+3) respectively. *Staphylococcus epidermidis* - NCTC 12100 results during the three storage days and temperature are shown in Table 2-12, 2-13, and 2-14. The control negative - *Staphylococcus aureus* (NCTC 8532) results is shown in Table 2-15.

Table 2-9 Results of staphylococcal enterotoxins produced by *Staphylococcus aureus* (NCTC 10657) in ready to served custard at 5°C during 3 incubations days using SET – REPLA diagnostic kit.

Storage Day	Average enterotoxin concentration	Interpretation	Bacterial colony counts (cfu/ml)
First Day	SEA: – SEB: – SEC: – SED: –	Negative	1.6 E ⁴
Second Day	SEA: – SEB: – SEC: – SED: –	Negative	1.4 E ⁴
Third Day	SEA: – SEB: – SEC: – SED: –	Negative	1.4 E ⁴

Table 2-10 Results of staphylococcal enterotoxins production by *Staphylococcus aureus* (NCTC 10657) in ready to served custard at 30°C during 3 incubations days using SET – REPLA diagnostic Kit.

Storage Day	Average enterotoxin concentration	Interpretation	Bacterial colony counts (cfu/ml)
First Day	SEA: +2 SEB: +3 SEC: – SED: –	Positive (> 0.5ng/ml) Positive (> 0.5ng/ml) Negative Negative	2.2 E ⁷
Second Day	SEA: +3 SEB: +3 SEC: – SED: –	Positive (> 0.5ng/ml) Positive (> 0.5ng/ml) Negative Negative	4.2 E ⁸
Third Day	SEA: +3 SEB: +3 SEC: – SED: –	Positive (> 0.5ng/ml) Positive (> 0.5ng/ml) Negative Negative	7.4 E ⁹

Table 2-11 Results of staphylococcal enterotoxins Produced by *Staphylococcus aureus* (NCTC 10657) in ready to served custard at 44°C during 3 incubations days using SET – REPLA diagnostic kit.

Storage Day	Average enterotoxin concentration	Interpretation	Bacterial colony counts (cfu/ml)
First Day	SEA: +3 SEB: +3 SEC: – SED: –	Positive (> 0.5ng/ml) Positive (> 0.5ng/ml) Negative Negative	1.7 E ⁷
Second Day	SEA: +3 SEB: +3 SEC: – SED: –	Positive (> 0.5ng/ml) Positive (> 0.5ng/ml) Negative Negative	4.3 E ⁷
Third Day	SEA: +3 SEB: +3 SEC: – SED: –	Positive (> 0.5ng/ml) Positive (> 0.5ng/ml) Negative Negative	1.9 E ⁷

Table 2-12 Results of staphylococcal enterotoxins produced by *Staphylococcus epidermidis* (NCTC 12100) in ready to served custard at 5°C during 3 incubations days using SET – REPLA diagnostic kit.

Storage Day	Average enterotoxin concentration	Interpretation	Bacterial colony counts (cfu/ml)
First Day	SEA: – SEB: – SEC: – SED: –	Negative	1.6 E ⁴
Second Day	SEA: – SEB: – SEC: – SED: –	Negative	1.4 E ⁴
Third Day	SEA: – SEB: – SEC: – SED: –	Negative	1.4 E ⁴

Table 2-13 Results of staphylococcal enterotoxins production by *Staphylococcus epidermidis* (NCTC 12100) in ready to served custard at 30°C during 3 incubations days using SET – REPLA diagnostic Kit.

Storage Day	Average enterotoxin concentration	Interpretation	Bacterial colony counts (cfu/ml)
First Day	SEA: –	Negative	5.4 E ⁷
	SEB: –	Negative	
	SEC: –	Negative	
	SED: –	Negative	
Second Day	SEA: –	Negative	2.1 E ⁸
	SEB: –	Negative	
	SEC: –	Negative	
	SED: –	Negative	
Third Day	SEA: –	Negative	4.2 E ⁸
	SEB: –	Negative	
	SEC: + 2	Positive (> 0.5ng/ml)	
	SED: –	Negative	

Table 2-14 Results of staphylococcal enterotoxins Produced by *Staphylococcus epidermidis* (NCTC 10657) in ready to served custard at 44°C during 3 incubations days using SET – REPLA diagnostic kit.

Storage Day	Average enterotoxin concentration	Interpretation	Bacterial colony counts (cfu/ml)
First Day	SEA: –	Positive (> 0.5ng/ml)	2.7 E ⁷
	SEB: –	Positive (> 0.5ng/ml)	
	SEC: –	Negative	
	SED: –	Negative	
Second Day	SEA: –	Positive (> 0.5ng/ml)	3.7 E ⁷
	SEB: –	Positive (> 0.5ng/ml)	
	SEC: –	Negative	
	SED: –	Negative	
Third Day	SEA: –	Negative	5.6 E ⁷
	SEB: –	Negative	
	SEC: + 3	Positive (> 0.5ng/ml)	
	SED: –	Negative	

Table 2-15 Examination for staphylococcal enterotoxins ready to served custard after inoculation of the negative control strain (*Staphylococcus aureus*-NCTC 8532) at the 3 storage temperatures during 3 incubations days using SET – REPLA diagnostic kit.

Storage Day	Enterotoxins Result at 5°C	Enterotoxins Result at 30°C	Enterotoxins Result at 44°C	Interpretation
First Day	SEA: – SEB: – SEC: – SED: –	SEA: – SEB: – SEC: – SED: –	SEA: – SEB: – SEC: – SED: –	Negative
Second Day	SEA: – SEB: – SEC: – SED: –	SEA: – SEB: – SEC: – SED: –	SEA: – SEB: – SEC: – SED: –	Negative
Third Day	SEA: – SEB: – SEC: – SED: –	SEA: – SEB: – SEC: – SED: –	SEA: – SEB: – SEC: – SED: –	Negative

2.8 Experiment 4: Evaluation of the effect of changing of the sample pH level on the growth and staphylococcal enterotoxins production of *Staphylococcus aureus* - NCTC 10657 and *Staphylococcus epidermidis* - NCTC 12100.

2.8.1 Materials and methods

The pH value of the spiked custard and controls samples in each storage temperature were measured with HI 207 (*HANNA*)-pH meter electrode (Fig. 2-10) before and after inoculation (during the 3 storage) to determine the effect of changing in pH value (if any) on the growth and enterotoxin production of the examined staphylococci.

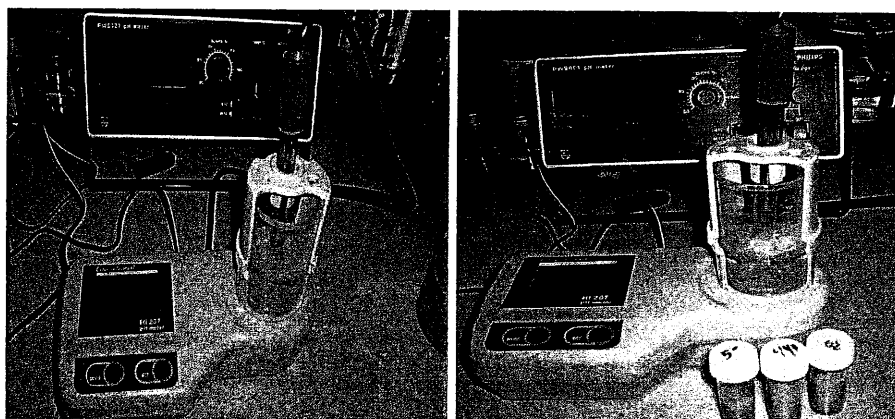


Figure 2-10 HI 207 (*HANNA*)-pH meter.

2.8.2 Result

The pH values of the spiked custard and controls samples in each storage temperature were measured before and after inoculation (during the 3 storage). The change in pH values of the spiked custard samples and controls at each temperature and storage day are shown in Table 2-16, 2-17 and 2-18.

At 5°C, there was no significant difference in the pH value (pH = 6.86) during the 3 incubation days for all of the examined strains. At 44°C, the pH of the custard sample that spiked with *Staphylococcus aureus* (NCTC 10657) was decreased

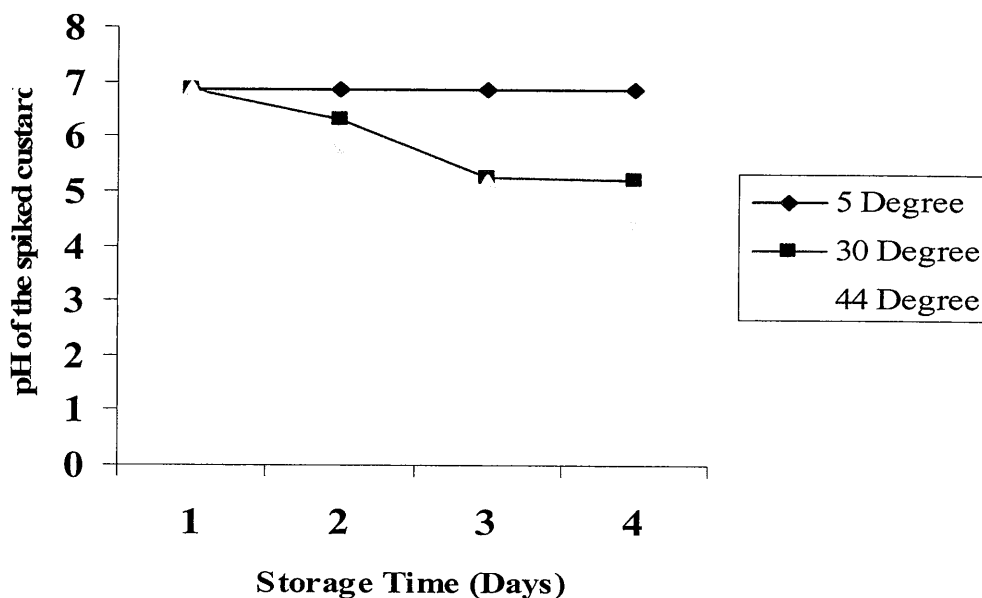
significantly by 2.4 pH units at the end of the third incubation day (Graph 2-4). This drop in the pH demonstrate the slow growth at the second day (pH = 5.16) and the decline in growth at the third incubation day where the pH value was close to the minimum growth ability of this strain (pH= 4.40). At 30°C, there was slightly decrease of the pH value by 1.6 pH units at the end of the third incubation day (pH = 5.22). This pH value within the strain growth range therefore no decrease in the total cell counts was observed.

Similarly, *Staphylococcus aureus* (NCTC 8532) showed a significant decrease in the pH value by 2.1 pH units at the end of the third incubation day at 44°C and slight decrease in the pH value by 1.1 pH units at the end of the third incubation day at 30°C (Graph 2-5).

Direct correlation was observed between growths and changing in pH value of the custard sample spiked with *Staphylococcus epidermidis* (NCTC 12100). Unlike the *Staphylococcus aureus* strains the drop in pH of the sample spiked with *Staphylococcus epidermidis* at the incubation temperature 30°C (by 1.1 units) was slightly higher than at 44°C in which the pH value decrease only by 0.62 units at the end of the third incubation days (Graph 2-6). Looking at the growth of this strain at the third incubation day demonstrate the drop of pH at 30°C, where the average cfu/ml was $4.2E^8$ whereas the average cfu/ml at 44°C was $5.6E^7$.

Table 2-16 Changing in pH value of the custard sample spiked with *Staphylococcus aureus* (NCTC 10657) during the 3 examined days and temperatures.

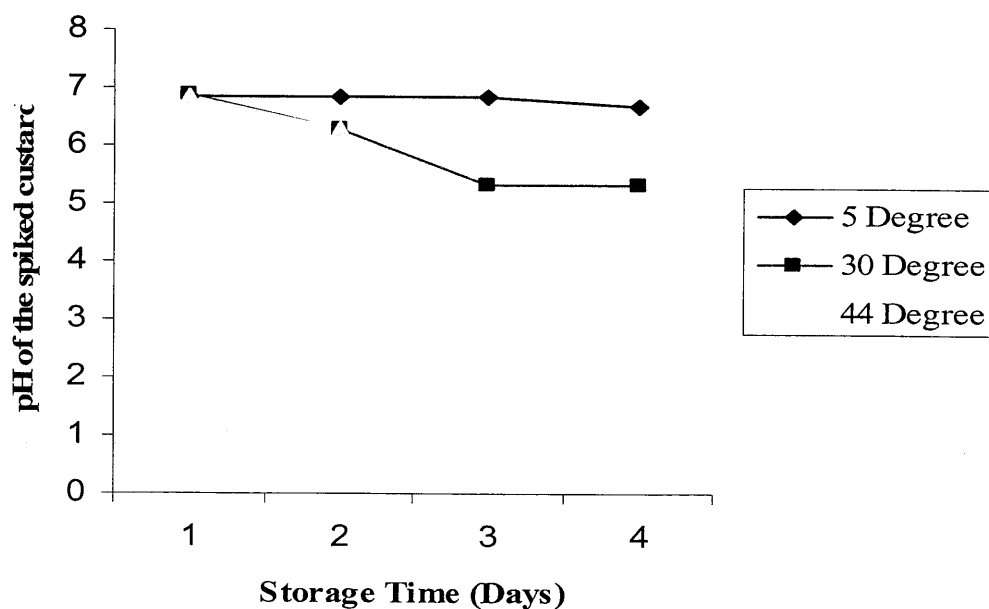
Storage Day	Storage Temperatures	Average pH reading	Control
First Day	5°C	6.86	6.86
	30°C	6.32	6.84
	44°C	5.85	6.80
Second Day	5°C	6.86	6.86
	30°C	5.26	6.84
	44°C	5.16	6.80
Third Day	5°C	6.86	6.86
	30°C	5.22	6.84
	44°C	4.40	6.80



Graph 2-4 The change in pH value of the custard sample spiked with *Staphylococcus aureus* - NCTC 10657 at different storage time and temperatures.

Table 2-17 Changing in pH value of the custard sample spiked with *Staphylococcus aureus* (NCTC 8532) during the 3 examined days and temperatures.

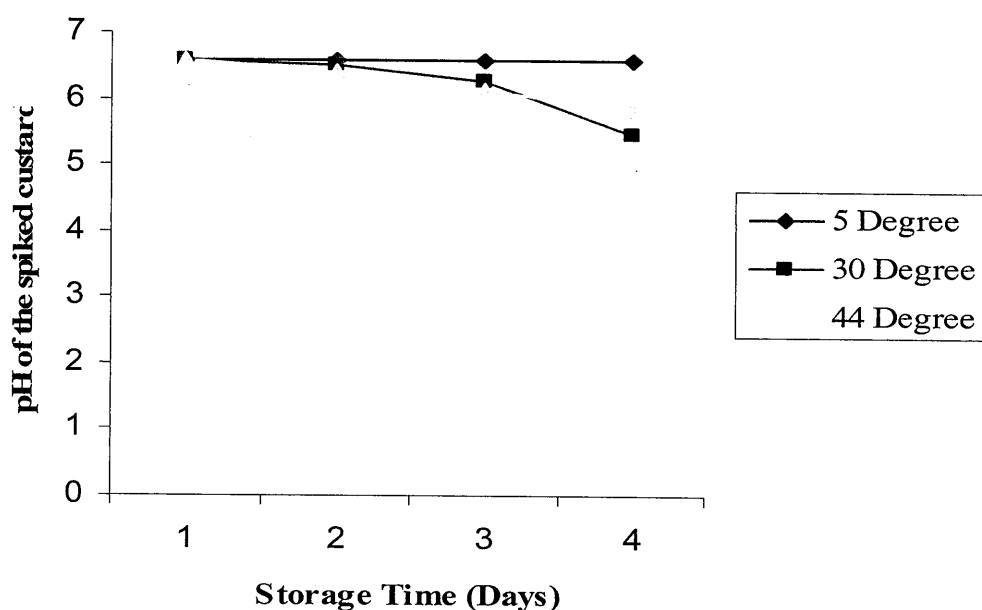
Storage Day	Storage Temperatures	Average pH	Control
First Day	5°C	6.86	6.87
	30°C	6.28	6.81
	44°C	6.30	6.80
Second Day	5°C	6.86	6.86
	30°C	5.32	6.81
	44°C	4.25	6.68
Third Day	5°C	6.70	6.80
	30°C	5.32	6.37
	44°C	4.25	6.34



Graph 2-5 The change in pH value of the custard sample spiked with *Staphylococcus aureus* - NCTC 8532 at different storage time and temperatures.

Table 2-18 Changing in pH value of the custard sample spiked with *Staphylococcus epidermidis* (NCTC 12100) during the 3 examined days and temperatures.

Storage Day	Storage Temperatures	Average pH	Control
First Day	5°C	6.57	6.57
	30°C	6.51	6.57
	44°C	6.39	6.54
Second Day	5°C	6.57	6.57
	30°C	6.25	6.51
	44°C	6.13	6.52
Third Day	5°C	6.57	6.57
	30°C	5.47	6.54
	44°C	5.84	6.46



Graph 2-6 The change in pH value of the custard sample spiked with *Staphylococcus epidermidis* -NCTC 12100 at different storage time and temperatures.

2.9 Experiment 5: Detection of mannitol fermentation

2.7.1 Materials and methods

This test was done for CNS and CPS used in this study (*Staphylococcus aureus* NCTC 8532, *Staphylococcus aureus* NCTC 10657 and *Staphylococcus epidermidis* NCTC 12100) as recommended by Bautista and his follow workers (1987) that mannitol utilization is the best test for the differentiation between enterotoxigenic and non- enterotoxigenic Staphylococci.

Representative colonies of *Staphylococcus aureus* NCTC 8532, *Staphylococcus aureus* NCTC 10657 and *Staphylococcus epidermidis* NCTC 12100 cultures were streaked in to Mannitol Salt Agar plates (Appendix I) and incubated for 36 hours at 37°C. In order to examine the lipase activity and mannitol fermentation of staphylococci simultaneously, 5% v/v egg yolk tellurite emulsion was added to mannitol salt agar media after cooled in water bath to 50°C.

2.7.2 Results

The result of the streaked *Staphylococcus aureus* NCTC 8532, *Staphylococcus aureus* NCTC 10657 and *Staphylococcus epidermidis* NCTC 12100 colonies streaked in to Mannitol Salt Agar (MSA) plates is shown in Table 2-19. Bright yellow colonies (Fig. 2-14) were obtained for both *Staphylococcus aureus* examined species (positive mannitol fermentation) with yellow halo surrounded their colonies, whereas *Staphylococcus epidermidis* gave pink-red colonies without alteration of MSA media colour (Fig. 2-15).

Table 2-19 Mannitol fermentation results of the examined *Staphylococcus* species on mannitol salt agar (MSA).

<i>Staphylococcus</i> species	Colonies appearance on MSA	Interpretation of the result
<i>Staphylococcus aureus</i> NCTC 8532	Bright yellow colonies	Positive mannitol fermentation
<i>Staphylococcus aureus</i> NCTC 10657	Bright yellow colonies	Positive mannitol fermentation
<i>Staphylococcus epidermidis</i> NCTC 12100	pink-red colonies	Negative mannitol fermentation

The lipase activity and mannitol fermentation of staphylococci were examined simultaneously by addition of 5% v/v egg yolk emulsion to mannitol salt agar media. All examined *Staphylococcus* species reduced the tellurite found in egg yolk emulsion and form black colonies. However, Both *Staphylococcus* species altered the media from pink-red to yellow (Fig. 2-16) and produced clear zones around their colonies, and the *Staphylococcus epidermidis* is not.



Figure 2-14 *Staphylococcus* species growing on Mannitol Salt Agar. On the right, Bright yellow colonies of *Staphylococcus aureus* NCTC 10657 indicate positive mannitol fermentation. Very bright yellow *Staphylococcus aureus* NCTC 8532 colonies on the left with wide halo around the growth is also positive for mannitol fermentation.

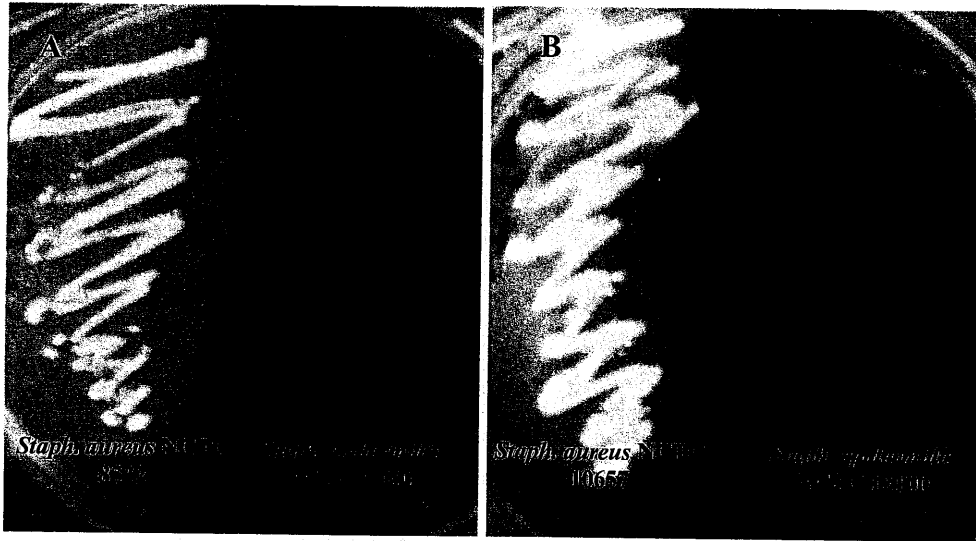


Figure 2-15 *Staphylococcus aureus* species and *Staphylococcus epidermidis* on Mannitol Salt Agar. On the right of Fig. A & B, pink-red colonies of *Staphylococcus epidermidis* without alteration of MSA media colour is negative positive for mannitol fermentation.

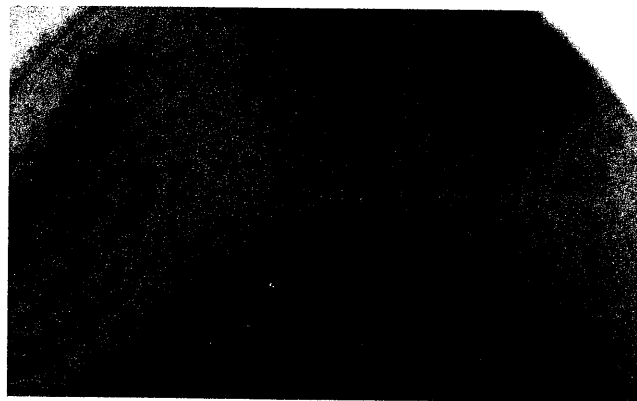


Figure 2-16 *Staphylococcus aureus* (NCTC 10657) on Mannitol Salt Agar containing 5% v/v egg yolk tellurite emulsion.

2.8 Confirmatory test

Five Representative colonies of suspected *Staphylococcus aureus* and *Staphylococcus epidermidis* colonies were examined for free and bound coagulase by Staphylase diagnostic kit (Oxoid-DR0595), and Gram's staining.

For staphylase test, overnight subcultures of the *Staphylococcus aureus* and *Staphylococcus epidermidis* colonies - suspected colonies were prepared on nutrient agar (Appendix I). The latex reagents were allowed to warm at room temperature before used. The reagent particles then were resuspended by shaking the reagent bottle in to the vortex. One drop of the latex test reagent was dispensed on to the reaction card and mixed well with the suspected culture by using a sterile loop or stick for emulsification. The card was rocked thoroughly (but not vigorously) for 10 – 20 seconds and the clumping was examined (Fig. 2-17). The result of Gram staining is shown in Figure 2-18.

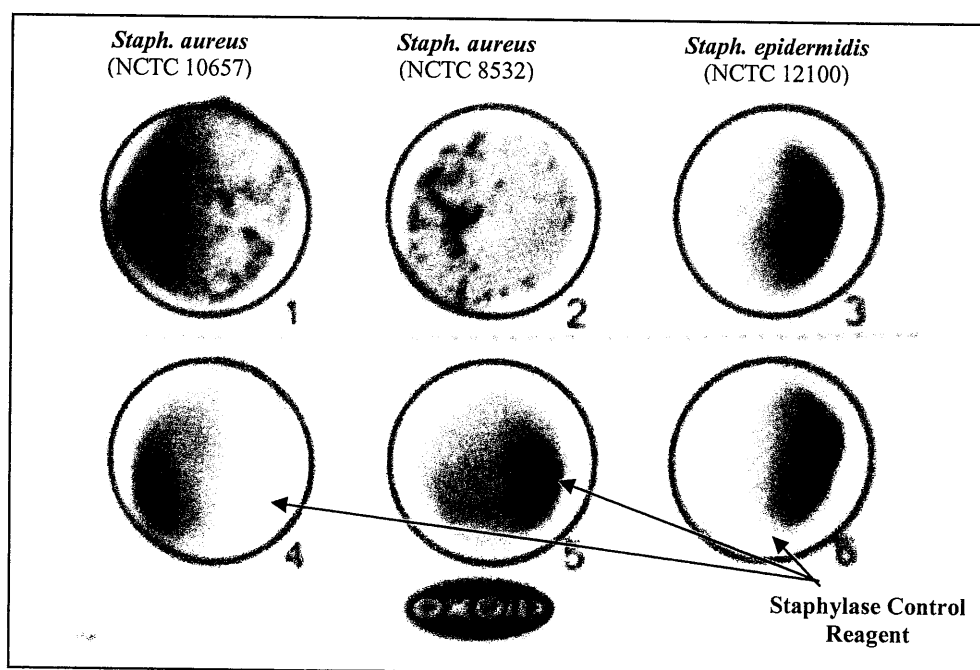


Figure 2-17 Staphylase test results. On the left (1 & 2), agglutination of rabbit plasma by *Staph. aureus* (NCTC 10657 and 8532) after the addition of staphylase test reagent indicate a positive result for coagulase. The smooth emulsion on the right (*Staph. epidermidis*) is negative for Coagulase.

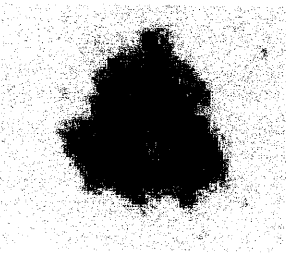
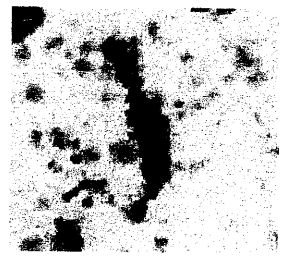
<i>Staphylococcus aureus</i> NCTC 8532	<i>Staphylococcus aureus</i> NCTC 10657	<i>Staphylococcus epidermidis</i> NCTC 8532
		

Figure 2-18 Gram stain results (Gram + ve) of the 3 examined strains (*Staphylococcus aureus* NCTC 10657, *Staphylococcus aureus* NCTC 8532, and *Staphylococcus epidermidis* NCTC 8532).

2.10 Statistical analysis

All experimental results were analyzed using the using Statistical Package for the Social Sciences (SPSS), version 14.0 edition for windows.

The colony counts and the difference in growth rate each strain during the three incubation days on three different temperatures was compared using t – test.

CHAPTER THREE

DISCUSSION AND CONCLUSION

3.5 Discussions and conclusions

3.6 Experiment 2: Evaluation of the effect of different storage times and temperatures on the growth of *Staphylococcus aureus* - NCTC 10657, *Staphylococcus aureus* - NCTC 8532, and *Staphylococcus epidermidis* - NCTC 12100.

Little research have been done on the effect of intrinsic and extrinsic factors on growth and enterotoxin production of the enterotoxigenic coagulase – negative staphylococci, though several studies emphasise its toxigenic capacity and indicate there potential hazard as foodborne intoxication (Breckinridge & Bergdoll, 1971; Bergdoll, 1982; Olsvik *et al.*, 1982; Evans *et al.*, 1983; Crass & Bergdoll, 1986; Ewald, 1987; Aliu & Bergdoll, 1988; Baustista *et al.*, 1988; Gomez-Lucia *et al.*, 1989; Valle *et al.*, 1990; Bennett, 1996). The detection of the enterotoxins genes in these strains validates and confirms their toxigenic capacity (Cunha *et al.*, 2005). In this study the effect of one extrinsic growth factor (storage temperature) on the growth and subsequent enterotoxin production of coagulase positive and negative – enterotoxigenic staphylococci representative strains was studied over a day trial, the 3 storage common shelf life of most ready to eat foods. The aim of the work was to determine the relationship between growth and enterotoxin production under different storage time between 0 and 3 days, and temperature (5°C, 30°C and 44°C) for three *Staphylococcus* species.

Three different temperatures (5°C, 30°C and 44°C) were examined considering the food product which is likely to encounter under the anticipated duration and storage (5°C), the storage temperature to which the product may be subjected by retailer to consumer (30°C) and the temperature (44°C - the optimum temperature for toxin production and the average summer temperature in Bahrain) at which the product may subjected to extremes of temperature (by retailer or consumer) that supporting the growth and enterotoxin production by enterotoxigenic staphylococci.

The lowest temperature was observed to be (5°C) the best for ready to eat food (e.g. custard) storage at which the growth of the 3 examined strains showed no significant differences in the original cell number during the 3 storage days. Staphylococcal growth was limited / restricted; however, the cells were not destroyed (Fig. 2-3, 2-4, and 2-5). This suppression explained by many scientists that the low temperature slowed the most growth and toxin production of foodborne microorganisms – metabolic rate and activities due to reduced in protein and enzyme's synthesis (Jay, 1992). In addition, Baird – Parker (2000) reported that *Staphylococcus aureus* species can also survive well in freezing temperature (-18°C) at least for 6 months where the cell number remains constant or slightly lower.

Although the maximum growth temperature for *Staphylococcus aureus* is range between 45°C to 47°C, it can still grow with a significant increase in cell counts at 44°C during the 3 storage temperature. This growth occurred due to high heat resistant properties of this species among the non-spore forming bacteria. It was reported with D value at 60°C this bacterium can survive from 2 – 50 min (Bremer *et al.*, 2004). Moreover, Baird – Parker (2000) reported that the resistance of *Staphylococcus aureus* to high temperature increased in low water activity food (e.g. D_{60°C} of this in milk of 5.3 min increased to 42.3 min by addition of 57% sucrose in milk). Therefore, the examined custard in this study (high sugar content) support the growth of both *Staphylococcus aureus* strains as well as *Staphylococcus epidermidis*.

The optimum growth temperature for *Staphylococcus aureus* is range between 30°C to 37°C (Bremer *et al.*, 2004) and between 35°C to 40°C for most staphylococci (Cohen, 1972; Baird – Parker, 2000), therefore the growth at 30°C is increased gradually within the 3 incubation days of the 3 strains. However, the growth of *Staphylococcus aureus* species is higher than *Staphylococcus epidermidis* by more than 1.2 log (cfu/ml) units at the end of the third incubation day.

It was found in this study that the growth rate of *Staphylococcus epidermidis* is slower than the other *Staphylococcus aureus* species, and this demonstrate the increasing in their growth at the end of the third incubation day (no decline in the growth curve). In addition, the SEC that produced mainly in the stationary phase (Bergdoll, 1989; Baird – Parker, 1990) was not produced in the first and the second incubation days in both 30°C and 44°C which indicate slow growth of this bacterium.

Wide ranges of foodstuff that consist of the essential nutritious elements support the growth of staphylococci and the subsequent enterotoxins production under optimal growth conditions. Rich protein content is highly associated with staphylococcal food poisoning intoxication (Bremer *et al.*, 2004). In this study custard which mainly consists of milk and eggs (rich in protein content) was used

3.1.1 Conclusion:

Low storage temperature (5°C) found to be the best for controlling the staphylococcal growth as well as their enterotoxins production.

The optimum growth temperature for the examined staphylococci species was at 30°C therefore, long storage of foodstuff at this Staphylococcal - critical growth temperature encourages the growth and the subsequent enterotoxins production. However, the optimum enterotoxins production temperature was at 44°C where the growth rate of *Staphylococcus* species is fast due to high metabolic activity. The growth rate of *Staphylococcus epidermidis* was found to be slower than the other examined *Staphylococcus aureus* species therefore no enterotoxins was detected in the first and second storage days at both 30°C and 44°C though the bacterial count was more than 10⁷CFU/ml. However, SEC was detected in the third day at 30°C and 44°C with an agglutination degree of (+2) and (+3) respectively.

3.7 Experiment 3: Evaluation of the effect of different storage times and temperatures on the staphylococcal enterotoxins production in the spiked custard samples by *Staphylococcus aureus*-NCTC 10657 and *Staphylococcus epidermidis*-NCTC 12100.

Extensive growth (multiplication) of staphylococci is required for enterotoxins production in food samples (Bremer *et al.*, 2004). Growth can be affected by several extrinsic and intrinsic factors. The present study deals with the influence of the various storage temperatures (5°C, 30°C and 44°C) found to be critical in staphylococcal growth and enterotoxin production during the common shelf life (3 days) of ready to eat foods.

No enterotoxins were detected at 5°C for both enterotoxigenic strains. Enterotoxins produced under the temperatures that permit the cell growth, therefore it produced by *Staphylococcus epidermidis* and *Staphylococcus aureus* at 44°C and 30°C and not 5°C. The maximum enterotoxins (SEA & SEB) concentration produced by *Staphylococcus aureus* (NCTC 10657) in the first incubation day were observed at 44°C. High agglutination pattern (+3) obtained in the first day of incubation (Fig.3.3.3.3) where the bacterial colony count was 2.2×10^7 CFU/ml. This production was due to the increasing of the growth rate at high temperature than the low temperature.

The concentration of SEA at 44°C remains constant at the second and third incubation days in which the agglutination did not exceed the forth microtiter's well. Comparing this result with the strain's growth during the second and third days indicates that this microorganism was in the stationary phase at the second day and started the death phase at the third incubation day where this staphylococcal enterotoxins as illustrated by Bergdoll (1989) and Baird-Parker (2000) synthesis dose not take place in these phase.

On the other hand, the concentration of SEA at 30°C during the 3 incubation days was increased gradually. The minimum agglutination of SEA (+1) was detected at

the third micrototer's well at the first incubation day which then increased and detected at the sixth and seventh micrototer's well in the second and third incubation day respectively. Comparing this result with the strain's growth during these days illustrate the increasing of SEA where the bacteria were in their log phase and the total cell counts were increased gradually in number.

SEB produced by this strain is higher than SEA at 30°C during the first incubation day though SEA produced in the earlier growth phase (Log phase) whereas SEB reported to be produced at the end of stationary phase (Bergdoll, 1989; Baird – Parker, 2000). It was demonstrated by Spero *et al.* (1988) that the production level of enterotoxins affected by the media and strain types.

Rich protein content food is highly associated with enterotoxins production (Bremer *et al.*, 2004). The representative ready to eat food in this study was ready to served custard that consists mainly of rich protein ingredients (eggs and milk). Custard has been considered by Peterson et al (1962) to be a highly favourable and selective culture medium for staphylococci.

SEC was detected by *Staphylococcus epidermidis* - NCTC 12100 only in the third day at 30°C and 44°C with an agglutination degree of (+2) and (+3) respectively. By comparing this result with the strain's growth during the 3 incubation days, it was found that this organism was generally increased in number (log phase) and the SEC as described by Bergdoll (1989) and Baird-Parker (2000) produced in the stationary growth phase. However, at the third incubation day there was no significant difference in the total cell counts between the second and third days for both 30°C and 44°C incubation temperature. The difference in the total cells number was only 0.3 and 0.18 log units at the temperature 30°C and 44°C respectively, which also explain the degree of agglutination at both temperatures.

Comparing with *Staphylococcus aureus*, *Staphylococcus epidermidis* have slow growth rate and therefore slow and low staphylococcal enterotoxins production obtained by *Staphylococcus epidermidis*.

Different methods have been developed for staphylococcal enterotoxin detection in food. In this study, reversed passive latex-based agglutination immunoassay (SET – RPLA diagnostic kit) was used to detect staphylococcal enterotoxins in the spikes custard sample and showed rapid (18 hours), simple and sensitive (0.5ng/ml) enterotoxin detections for the 3 examined strains.

3.2.1 Conclusion:

Staphylococcal enterotoxins are produced under the temperatures that permit cell growth, therefore it was produced by *Staphylococcus epidermidis* (NCTC 12100) and *Staphylococcus aureus* (NCTC 10657) at 44°C and 30°C but not at 5°C. There was a good correlation between growth and enterotoxin production by *Staphylococcus aureus* (NCTC 10657) at 44°C during the first day due to increasing in the growth rate at high temperature. And this demonstrates the inhibition of enterotoxins production at low temperature.

Staphylococcus epidermidis (NCTC 12100) and other CNS have been approved by several studies to produce the enterotoxins in foods, they must be considered (beside CPS) as a potential cause of staphylococcal food poisoning.

As the staphylococcal enterotoxins are highly resistant molecules, it is easily to control or limit the enterotoxigenic bacterial growth rather than destroy or inactivate them.

Since the most of *Staphylococcus aureus* that isolated from food are not enterotoxin producer and other *Staphylococcus* species are capable to produced enterotoxins (Loir, 2003), the most reliable test for enterotoxigenicity is the direct detection of these enterotoxins directly in food.

3.8 Experiment 4: Evaluation of the Effect of changing of the sample pH on the Growth and Staphylococcal enterotoxins production of *Staphylococcus aureus* - NCTC 10657, *Staphylococcus aureus* - NCTC 8532, and *Staphylococcus epidermidis* - NCTC 12100.

In general, staphylococci are capable to grow over a wide range of pH value that range between 4.5 – 9.3 (Cohen, 1972) and *Staphylococcus aureus* in particular is able to grow over a pH range between 4 – 10 (Baird – Parker, 2000). There was no direct effect of changing of pH value on the staphylococcal enterotoxin production during the 3 incubation day. The drop in pH value due to microbial growth decreases the rate of growth and then the subsequent enterotoxin production.

3.3.1 Conclusion:

Staphylococcal growth and enterotoxin production can not be controlled by single factor and the effectiveness of the storage temperature on the growth and the subsequent and enterotoxin production by staphylococci influenced by the effectiveness of other growth factors such as time, pH, water activity ...etc

3.9 Experiment 5: Detection of mannitol fermentation

Varity of studies carried on to find a correlation between biochemical and physiological prosperities of *Staphylococcus aureus* with toxin production (Bennett, 1986). For instance, TNase and coagulase activity have been reported to correlate with *Staphylococcus aureus* enterotoxigenicity (Tatini *et al.*, 1976; Bennett, 1986; Jay, 1992). However, other studies have been shown that these enzymes are also produced by most of the non toxigenic strains (Bennett, 1986). The coagulase result of *Staphylococcus aureus* - NCTC 8532 in this study confirm this fact in which the non – enterotoxigenic *Staphylococcus aureus* strain was

capable to produce the coagulase enzyme whereas the enterotoxigenic *Staphylococcus epidermidis* was not (Fig. 3.1.3).

Mannitol salt agar is a selective medium due to the high salt concentration (7.5%) In addition, this medium is also differential due to mannitol and phenol red (pH indicator) that indicate any drop in pH due to acidic products formation (Johnson, 2006). *Staphylococcus aureus* (NCTC 8532 and 10657) ferment the mannitol and produce acid that change the media colour to bright yellow, however, *Staphylococcus epidermidis* (NCTC 12100) is non-mannitol fomenter, therefore, no alteration in media colour was observed and the colonies appear red with reddish purple zones. By addition of 5% v/v egg yolk emulsion, the selectivity of this media increased.. The tellurite reduced by *Staphylococcus aureus* and form black shiny colonies after 36 hours incubation at 37°C. Both *Staphylococcus aureus* strains produced yellow opaque zones around their colonies due to lipase activity (Oxoid, 2001). These zones were obtained after 48 hours incubation at 37°C.

MSA can be utilized by most enterotoxigenic CPS and CNS strains (Table 1), and it was described by Bautista *et al.* (1987) as the best test for the differentiation between enterotoxigenic and non- enterotoxigenic Staphylococci. The use of mannitol utilization activity can be useful to detect potentially enterotoxigenic *Staphylococcus* species However, this test are not reliable for all enterotoxigenic staphylococci such as *Staph. epidermidis* (Fig. 3.2.2) and other non-mannitol fomenter enterotoxigenic *Staphylococcus* species. For example, the coagulase positive *Staph. hyicus* that produced SEC (Adesiyn *et al*, 1984; Jay, 1992) and the coagulase negative *Staph. hominis* that produced SEA (Valle *et al.*, 1990; Cunha *et al.*, 2005) are non-mannitol fomenter (Jay, 1992; Baird-Parker, 2000).

3.4.1 Conclusion

There was no correlation between mannitol fermentation nor coagulase production and the enterotoxigenicity of *Staphylococcus aureus* (NCTC 8532) and *Staphylococcus epidermidis* (NCTC 12100).

Neither mannitol fermentation nor coagulase activities appeared to be specific for enterotoxigenicity of all *Staphylococcus* species, however, both tests can be useful to differentiate between these species and detect the potentially enterotoxigenic *Staph. aureus* and other (CNS and CPS) strains. Mannitol salt agar could be use as a differential selective media for isolation and quantification of enterotoxigenic CPS as well as CNS since their ability to ferment mannitol was approved (Bautista *et al.*, 1987; Jay, 1992; Baird-Parker, 2000)

CHAPTER FOUR

FURTHER WORK AND RECOMMENDATIONS

4.1 Further works

4.1.4 Further work on coagulase negative *Staphylococcus* species

Further studies are required for the identification of new enterotoxigenic staphylococci strains as well as new enterotoxins in various food matrices since 5% of staphylococcal food poisoning outbreaks are caused by unidentified SEs (Wieneke *et al.*, 1993; Pimbley and Patel, 1998).

Further studies are suggested towards CNS and their enterotoxigenic capacity under different extrinsic and intrinsic growth factors.

Other option is to study the effect of other bacteria affecting or repression the growth and the SEs production are by CNS. It was found that *Lactobacillus* species can inhibit the growth and the enterotoxins production by *Staphylococcus aureus* (Mossel and Nutten, 1990; Sameshima *et al.*, 1998). Looking for the effect of these bacteria on other enterotoxigenic CNS is important to reduce their potential cause of food poisoning.

4.1.5 Further work on the factors effect the growth and enterotoxin production by enterotoxigenic strains of *Staphylococcus* species

More research effort is required to understand the correlation between the enterotoxigenic staphylococci strains and the staphylococcal enterotoxins production mechanisms in variety of food.

Looking for the effect of these factors on the growth and subsequent enterotoxin production using the newly discovered enterotoxigenic staphylococcal strains which implicated in several food poisoning outbreaks could be useful for better control and reduction of staphylococcal food poisoning outbreaks.

4.1.6 Further work on other staphylococcal detection methods

Further studies using new, rapid, sensitive and specific methods such as the real time - polymerase chain reaction (RT-PCR) method for identification of the enterotoxigenic *Staphylococci* strains and detection of the staphylococcal enterotoxins directly in foods are suggested on different food matrices to evaluate the performance of such method for routine laboratory work.

Other option is to compare different immunological – staphylococcal enterotoxin diagnostic kits such as BOMMELI, RIDASCREEN, TECRA, TRANSIA and VIDAS in term of sensitivity, specificity and practicality using different food matrices as well as different enterotoxigenic - *Staphylococcus* species

4.2 Recommendations

Staphylococcal food poisoning remains a significant cause of foodborne intoxication in wide range of the world (Baird-Parker, 2000).

The routine laboratory detection of the enterotoxigenic *Staphylococcus* strains in Public health laboratory - Bahrain is required to be developed/improved by using the recent developed systems such as the advanced immunological assays (e.g. VIDAS staphylococcal enterotoxin – SET 2 assay) and the DNA-based assays such as the Real – Time PCR that detect the enterotoxigenic genes rapidly and directly from the food samples.

Although CPS are the main enterotoxin producer, several CNS have been involved in plenty of food poisoning outbreaks and several studies emphasise its toxigenic capacity (Breckinridge & Bergdoll, 1971; Bergdoll, 1982; Olsvik *et al.*, 1982; Evans *et al.*, 1983; Crass & Bergdoll, 1986; Ewald, 1987; Aliu & Bergdoll, 1988; Baustista *et al.*, 1988; Gomez-Lucia *et al.*, 1989; Valle *et al.*, 1990; Bennett, 1996; Cunha *et al.*, 2005). This capacity could be a significant potential cause of food poisoning outbreaks; therefore it is recommended that during the

investigation of the suspected staphylococcal food poisoning outbreaks, these strains should not be ignored in the routine food microbiology laboratories.

Since the staphylococcal food poisoning outbreaks are caused mainly by human - *Staphylococcus aureus* strains (Baird-Parker, 2000), it is strongly recommended to increase the application of the HACCP system in the local food factories, restaurants and all food businesses in Bahrain.

CHAPTER FIVE

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APPENDICES I

APPENDIX I: MICROBIOLOGICAL METHODS (MEDIA)

BAIRD – PARKER AGAR BASE (BP)

(Oxiod Code CM275)

Formula	gm/liter
Tryptone	10.0
'Lab-Lemco' powder	5.0
Yeast extract	1.0
Sodium pyruvate	10.0
Glycine	12.0
Lithium chloride	5.0
Agar	20.0
pH 6.8 ± 0.2	

Directions

63 g of Baird – parker agar (Oxiod-CM275) was Suspend in to one liter of distilled water and boiled to dissolve the medium. The medium sterilized by autoclaving at 121°C for 15 minutes and Cooled in water bath to 50°C. 50 ml of Egg Yolk Tellurite Emulsion (Oxoid SR0054) was added aseptically and mixed very well before pouring into sterile Petri Dishes (15 – 20 ml/plate).

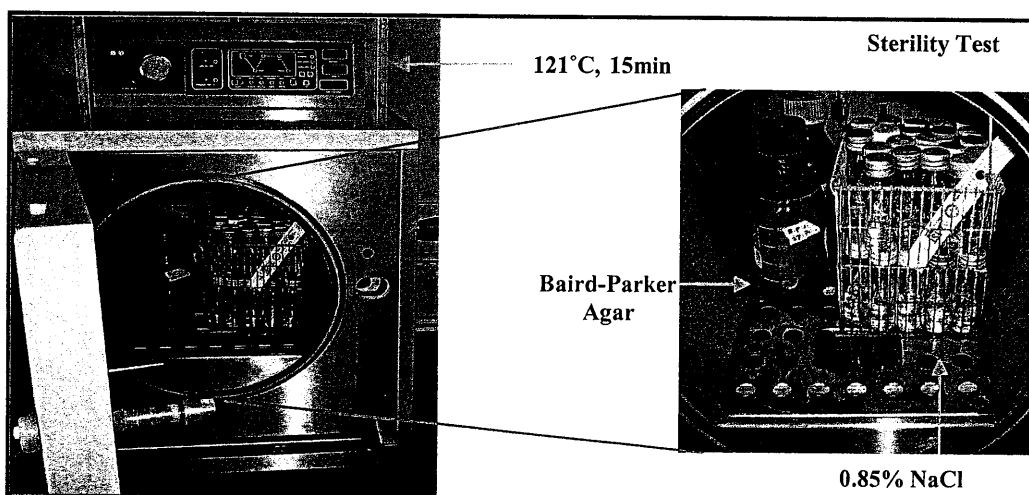


Figure. Baird – parker agar medium and 0.85%NaCl solution before sterilization inside the Autoclave (NRI, 2007).

MANNITOL SALT AGAR

Code: CM0085

Formula	gm/litre
Lab-Lemco powder	1.0
Peptone	10.0
Mannitol	10.0
Sodium chloride	75.0
Phenol red	0.025
Agar	15.0

pH 7.5 ± 0.2

Directions

111g was suspended in one litre of distilled water and dissolved completely by boiling. The media was sterilised by autoclaving at 121°C for 15 minutes.

MAXIMUM RECOVERY DILUENT (MRD)

(Oxoid Code: CM0733)

Formula	gm/litre
Peptone	1.0
Sodium chloride	8.5

pH 7.0 ± 0.2

Directions

9.5 g of (MRD) was dissolved in 1 litre of distilled water. 9ml of the suspension was dispensed into glass screw cap containers and sterilised by autoclaving at 121°C for 15 minutes.

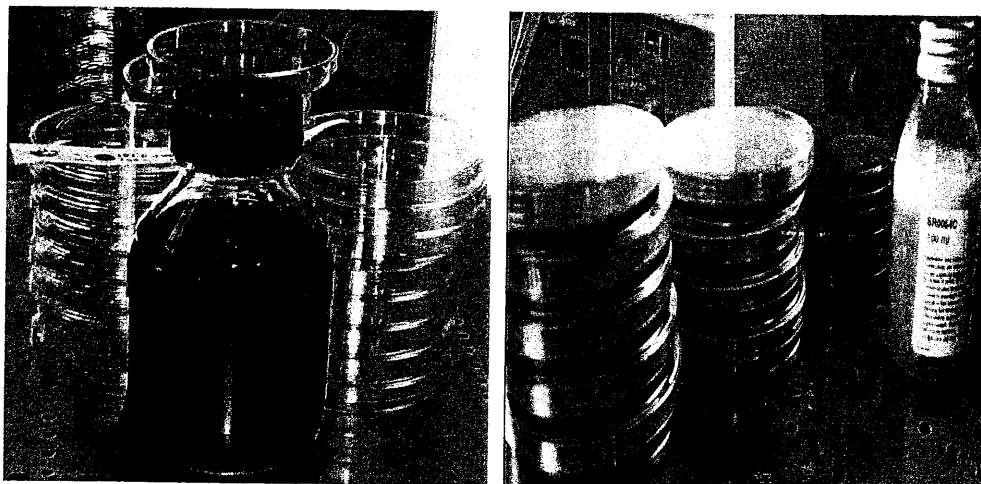


Figure 2.3. Mannitol salt agar media. On the right, Manittol agar with 5% v/v egg yolk tellurite emulsion.

NUTRIENT AGAR

(Oxoid Code: CM0003)

Formula	gm/litre
Lab-Lemco powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0

pH 7.4 ± 0.2

Directions

28g was suspended in to one litre of distilled water and dissolved completely by boiling. The suspension was sterilised by autoclaving at 121°C for 15minutes.

SODIUM CHLORIDE SOLUTION (0.85%)

Formula	gm/litre
NaCl	8.5
pH 5.64	

Directions

8.5 g of NaCl was dissolved in one liter of distilled water. Each 10ml of the sodium chloride solution was distributed into glass screw cup container. The solution was sterilised by autoclaving at 121°C for 15 minutes and stored at 5°C until used for toxin extraction from the food matrices.

TRYPTONE SOYA BROTH (TSB)

(Oxoid Code: CM0876)

Formula	gm/litre
Pancreatic digest of casein	17.0
Papaic digest of soybean meal	3.0
Sodium chloride	5.0
Dibasic potassium phosphate	2.5
Glucose	2.5

pH 7.3 ± 0.2

Directions

30 g of TSB dehydrated media was suspended in to one litre of distilled water and each 10ml of the suspension was distributed into glass screw cup container. The media was sterilised by autoclaving at 121°C for 15 minutes.

APPENDICES II

Appendix II: Results and Statistical analysis

Staphylococcus aureus (NCTC 10657):

Table 3-1 Evaluation of the initial inoculum of *Staphylococcus aureus* (NCTC 10657) using spread plate method on Baird – Parker agar. ⁻¹

Dilutions	CFU/0.1ml (dilution)	CFU/ml (dilution)	CFU/ml (Original Sample)	Average CFU/ml (dilution)	Average CFU/ml (Original Sample)
10^{-1}	1- TNTC	1- TNTC	1- TNTC	TNTC	TNTC
	2- TNTC	2- TNTC	2- TNTC		
	3- TNTC	3- TNTC	3- TNTC		
10^{-2}	1- TNTC	1- TNTC	1- TNTC	TNTC	TNTC
	2- TNTC	2- TNTC	2- TNTC		
	3- TNTC	3- TNTC	3- TNTC		
10^{-3}	1- TNTC	1- TNTC	1- TNTC	TNTC	TNTC
	2- TNTC	2- TNTC	2- TNTC		
	3- TNTC	3- TNTC	3- TNTC		
10^{-4}	1- TNTC	1- TNTC	1- TNTC	TNTC	TNTC
	2- TNTC	2- TNTC	2- TNTC		
	3- TNTC	3- TNTC	3- TNTC		
10^{-5}	1- 585	1- 5.9×10^3	1- 5.9×10^8	6.5×10^3	6.5×10^8
	2- 636	2- 6.4×10^3	2- 6.4×10^8		
	3- 715	3- 7.2×10^3	3- 7.2×10^8		
10^{-6}	1- 76	1- 7.6×10^2	1- 7.6×10^8	7.4×10^2	7.4×10^8
	2- 70	2- 7.0×10^2	2- 7.0×10^8		
	3- 77	3- 7.7×10^2	3- 7.7×10^8		
10^{-7}	1- 7	1- 70	1- 7.0×10^8	50	5.0×10^8
	2- 2	2- 20	2- 2.0×10^8		
	3- 6	3- 60	3- 6.0×10^8		

TNTC: Too numerous to count.

Calculation:

One millilitres of the dilution 10^{-6} gave $7.4 \text{ e}^2 \text{cfu/ml}$, so it requires approximately to add 1.4 ml from the dilution 10^{-3} to every 100ml of custard to give an initial level of approximately 1.0e^4 c.f.u/ml of spiked custard.

Table 3-2 Evaluation of the initial inoculums of *Staphylococcus aureus* (NCTC 10657) on the spiked custard (1.4 ml of 10^{-3} dilution per 100ml custard).

Dilution	Count/0.1 ml	C.F.U/ml	Average	Total Average count
Un diluted	1- 987	1- 9.9E ³	9.7 E ³	9.7 E ³
	2- 975	2- 9.8 E ³		
	3- 933	3- 9.3 E ³		
10 ⁻¹	1- 105	1- 1.1 E ⁴	9.7 E ³	
	2- 104	2- 1.0 E ⁴		
	3- 80	3- 8.0 E ³		
10 ⁻²	1- 16	1- 1.6 E ⁴	1.3 E ⁴	
	2- 13	2- 1.3 E ⁴		
	3- 10	3- 1.0 E ⁴		
10 ⁻³	1- 1	1- 1.0 E ⁴	1.0 E ⁴	
	2- 1	2- 1.0 E ⁴		
	3- 1	3-1.0 E ⁴		

Bacterial Growth in Different Storage time and Temperatures

Table 3-3 Evaluation of bacteria viable count at 5°C - (First Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average cfu/ml cfu/ml
10^{-1}	1.2×10^2	1.7×10^2	1.7×10^2	
10^{-1}	1.5×10^2	2.1×10^2	98	
Average cfu/ml	1.4×10^4	1.9×10^4	1.3×10^4	1.6×10^4
10^{-2}	17	18	20	
10^{-2}	10	20	14	
Average cfu/ml	1.4×10^4	1.9×10^4	1.7×10^4	1.7×10^4
10^{-3}	4	5	7	
10^{-3}	2	3	3	
Average cfu/ml	3.0×10^4	4.0×10^4	5.0×10^4	4.0×10^4

Statistics

The number of colony forming unit per ml of spiked sample stored at 5°C (day 1)

N	Valid Missing	6 3
Mean		16000.00000
Median		15500.00000
Std. Deviation		2683.281573
Variance		7200000.000
Minimum		13000.000
Maximum		19000.000

Average cfu/ml: 1.6×10^4

Descriptive Statistics

Log CFU per ml of spiked sample stored at 5°C (day 1)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	4.11	4.23	4.1633	0.06110

Table 3-4 Evaluation of bacteria viable count at 5°C - (Second Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average cfu/ml
10 ⁻¹	114	202	100	1.4 x 10 ⁴
10 ⁻¹	109	180	140	
Average cfu/ml	1.1 x 10⁴	1.9 x 10⁴	1.2 x 10⁴	
10 ⁻²	22	21	20	1.9 x 10 ⁴
10 ⁻²	11	22	15	
Average cfu/ml	1.7 x 10⁴	2.2 x 10⁴	1.8 x 10⁴	
10 ⁻³	2	1	1	2.3 x 10 ⁴
10 ⁻³	3	2	3	
Average cfu/ml	3.0 x 10⁴	2.0 x 10⁴	2.0 x 10⁴	

Statistics

The number of colony forming unit per ml of spiked sample stored at 5°C (day 2)

N	Valid Missing	3 6
Mean		14000.00
Median		12000.00
Std. Deviation		4358.899
Variance		19000000.000
Minimum		11000
Maximum		19000

Average cfu/ml: 1.4E⁴

Descriptive Statistics

Log CFU per ml of spiked sample stored at 5°C (day 2)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	4.04	4.23	4.1233	0.09713

Table 3-5 Evaluation of bacteria viable count at 5°C - (Third Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average cfu/ml
10 ⁻¹	112	154	171	
10 ⁻¹	109	176	134	
Average cfu/ml	1.1 x 10⁴	1.7 x 10⁴	1.5 x 10⁴	1.4 x 10⁴
10 ⁻²	10	20	20	
10 ⁻²	17	23	15	
Average cfu/ml	1.4 x 10⁴	2.2 x 10⁴	1.8 x 10⁴	1.8 x 10⁴
10 ⁻³	2	4	3	
10 ⁻³	5	4	7	
Average cfu/ml	4.0 x 10⁴	4.0 x 10⁴	5.0 x 10⁴	4.3 x 10⁴

Statistics

The number of colony forming unit per ml of spiked sample stored at 5°C (day 3)

N	Valid Missing	3 6
Mean		14333.3333
Median		15000.0000
Std. Deviation		3055.05046
Variance		9333333.333
Minimum		11000.00
Maximum		17000.00

Average cfu/ml: 1.4 E⁴

Descriptive Statistics

Log CFU per ml of spiked sample stored at 30°C (day 1)

	N	Minimum	Maximum	Mean	Std. Deviation
	3	4.04	4.23	4.1500	0.09849

Table 3-6 Evaluation of bacteria viable count at 30°C - (First Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average CFU/ml
10 ⁻¹	TNTC	TNTC	TNTC	
10 ⁻¹	TNTC	TNTC	TNTC	
Average cfu/ml	TNTC	TNTC	TNTC	TNTC
10 ⁻²	TNTC	TNTC	TNTC	
10 ⁻²	TNTC	TNTC	TNTC	
Average cfu/ml	TNTC	TNTC	TNTC	TNTC
10 ⁻³	2.3 x 10 ³	2.0 x 10 ³	1.8 x 10 ³	
10 ⁻³	1.8 x 10 ³	2.3 x 10 ³	2.9 x 10 ³	
Average cfu/ml	2.1x 10⁷	2.2 x 10⁷	2.4 x 10⁷	2.2 x 10⁷

Statistics

The number of colony forming unit per ml of spiked sample stored at 30°C (day 1)

N	Valid Missing	3 6
Mean		22333333.3333
Median		22000000.0000
Std. Deviation		1527525.23165
Variance		233333333333.334
Minimum		21000000.00
Maximum		24000000.00

Average CFU/ml: 2.2 E⁷

Descriptive Statistics

Log CFU per ml of spiked sample stored at 30°C (day 1)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	7.32	7.38	7.3467	0.03055

Table 3-7 Evaluation of bacteria viable count at 30°C - (Second Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average cfu/ml
10 ⁻²	TNTC	TNTC	TNTC	
10 ⁻²	TNTC	TNTC	TNTC	
Average cfu/ml	TNTC	TNTC	TNTC	TNTC
10 ⁻³	TNTC	TNTC	TNTC	
10 ⁻³	TNTC	TNTC	TNTC	
Average cfu/ml	TNTC	TNTC	TNTC	TNTC
10 ⁻⁴	5.2 x 10 ³	3.7 x 10 ³	3.0 x 10 ³	
10 ⁻⁴	4.2 x 10 ³	4.7 x 10 ³	4.6 x 10 ³	
Average cfu/ml	4.7 x 10⁸	4.2 x 10⁸	3.8 x 10⁸	4.2 x 10⁸

Statistics

The number of colony forming unit per ml of spiked sample stored at 30°C (day 2)

N	Valid Missing	3 0
Mean		423333333.33
Median		420000000.00
Std. Deviation		45092497.528
Variance		203333333333333.000
Minimum		380000000
Maximum		470000000

Average CFU/ml: 4.2E⁸

Descriptive Statistics

Log CFU per ml of spiked sample stored at 30°C (day 2)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	8.58	8.67	8.6233	0.04509

Table 3-8 Evaluation of bacteria viable count at 30°C - (Third Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average cfu/ml
10 ⁻⁴	TNTC	TNTC	TNTC	
10 ⁻⁴	TNTC	TNTC	TNTC	
Average cfu/ml	TNTC	TNTC	TNTC	TNTC
10 ⁻⁵	3.4 x 10 ³	3.0 x 10 ³	2.4 x 10 ³	
10 ⁻⁵	3.3 x 10 ³	2.5 x 10 ³	3.4 x 10 ³	
Average cfu/ml	3.4 x 10³	2.8 x 10³	2.9 x 10³	3.0 x 10³
10 ⁻⁶	715	650	500	
10 ⁻⁶	910	921	715	
Average cfu/ml	8.1 x 10³	7.9 x 10³	6.1 x 10³	7.4 x 10³

Statistics

The number of colony forming unit per ml of spiked sample stored at 30°C (day 3)

N	Valid Missing	3 0
Mean		7366666666.67
Median		7900000000.00
Std. Deviation		1101514109.457
Variance		1213333333333333000.00
Minimum		6100000000
Maximum		8100000000

Average CFU/ml: 7.4 E⁹

Descriptive Statistics

Log CFU per ml of spiked sample stored at 30°C (day 3)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	9.79	9.91	9.8667	0.06658

Table 3-9 Evaluation of bacteria viable count at 44°C - (First Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average cfu/ml
10 ⁻¹	TNTC	TNTC	TNTC	
10 ⁻¹	TNTC	TNTC	TNTC	
Average cfu/ml	TNTC	TNTC	TNTC	TNTC
10 ⁻²	TNTC	TNTC	TNTC	
10 ⁻²	TNTC	TNTC	TNTC	
Average cfu/ml	TNTC	TNTC	TNTC	TNTC
10 ⁻³	1.2 x 10 ³	1.2 x 10 ³	3.4 x 10 ³	
10 ⁻³	1.4 x 10 ³	1.6 x 10 ³	1.4 x 10 ³	
Average cfu/ml	1.3 x 10⁷	1.4 x 10⁷	2.4 x 10⁷	1.7 x 10⁷

Statistics

The number of colony forming unit per ml of spiked sample stored at 44°C (Day1)

N	Valid Missing	3 6
Mean		17000000.00
Median		14000000.00
Std. Deviation		6082762.530
Variance		37000000000000.000
Minimum		13000000
Maximum		24000000

Average CFU/ml: 1.7 E⁷

Descriptive Statistics

Log CFU per ml of spiked sample stored at 44°C (day 1)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	7.11	7.40	7.2200	0.15716

Table 3-10 Evaluation of bacteria viable count at 44°C - (Second Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average cfu/ml
10 ⁻²	TNTC	TNTC	TNTC	
10 ⁻²	TNTC	TNTC	TNTC	
Average cfu/ml	TNTC	TNTC	TNTC	TNTC
10 ⁻³	TNTC	TNTC	TNTC	
10 ⁻³	TNTC	TNTC	TNTC	
Average cfu/ml	TNTC	TNTC	TNTC	TNTC
10 ⁻⁴	410	340	480	
10 ⁻⁴	540	444	350	
Average cfu/ml	4.8 x 10⁷	3.9 x 10⁷	4.2 x 10⁷	4.3 x 10⁷

Statistics

The number of colony forming unit per ml of spiked sample stored at 44°C (Day2)

N	Valid Missing	3 0
Mean		43000000.0000
Median		42000000.0000
Std. Deviation		4582575.69496
Variance		21000000000000.000
Minimum		39000000.00
Maximum		48000000.00

Average CFU/ml: 4.3 E⁷

Descriptive Statistics

Log CFU per ml of spiked sample stored at 44°C (day 2)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	7.60	7.68	7.6333	0.04163

Table 3-11 Evaluation of bacteria viable count at 44°C - (Third Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average cfu/ml
10 ⁻⁴	235	159	156	
10 ⁻⁴	168	213	230	
Average cfu/ml	2.0 x 10⁷	1.9 x 10⁷	1.9 x 10⁷	1.9 x 10⁷
10 ⁻⁵	24	11	20	
10 ⁻⁵	16	9	22	
Average cfu/ml	2.0 x 10⁷	1.0 x 10⁷	2.1 x 10⁷	1.7 x 10⁷
10 ⁻⁶	<1	3	<1	
10 ⁻⁶	<1	1	<1	
Average cfu/ml	< 10⁶	2.0 x 10⁷	< 10⁶	2.0 x 10⁷

Statistics

The number of colony forming unit per ml of spiked sample stored at 44°C (Day3)

N	Valid Missing	3 0
Mean		19333333.33
Median		19000000.00
Std. Deviation		577350.269
Variance		333333333333.334
Minimum		19000000
Maximum		20000000

Average CFU/ml: 1.9 E⁷

Descriptive Statistics

Log CFU per ml of spiked sample stored at 44°C (day 3)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	7.28	7.30	7.2867	0.01155

Staphylococcal Enterotoxins Production:

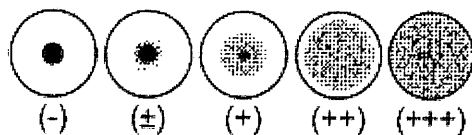


Table 3-12 Results of Staphylococcal Enterotoxins Production by *Staphylococcus aureus* (NCTC 10657) in culture fluid (TSB) using SET – REPLA Diagnostic Kit after 18 hours incubation at 37°C.

Enterotoxins	Replicate # 1	Replicate # 2	Replicate # 3	Average	Interpretation
SEA	+3	+3	+3	+3	Positive (> 0.5ng/ml)
AEB	+3	+3	+3	+3	Positive (> 0.5ng/ml)
SEC	—	—	—	—	Negative
SED	—	—	—	—	Negative

Table 3-13 Results of Staphylococcal Enterotoxins Production by *Staphylococcus aureus* (NCTC 10657) in ready to served custard at 5°C During 3 incubations days using SET – REPLA Diagnostic Kit.

Storage Day	Replicate # 1	Replicate # 2	Replicate # 3	Average	Interpretation
First Day	SEA: —	SEA: —	SEA: —	SEA: —	Negative
	SEB: —	SEB: —	SEB: —	SEB: —	
	SEC: —	SEC: —	SEC: —	SEC: —	
	SED: —	SED: —	SED: —	SED: —	
Second Day	SEA: —	SEA: —	SEA: —	SEA: —	Negative
	SEB: —	SEB: —	SEB: —	SEB: —	
	SEC: —	SEC: —	SEC: —	SEC: —	
	SED: —	SED: —	SED: —	SED: —	
Third Day	SEA: —	SEA: —	SEA: —	SEA: —	Negative
	SEB: —	SEB: —	SEB: —	SEB: —	
	SEC: —	SEC: —	SEC: —	SEC: —	
	SED: —	SED: —	SED: —	SED: —	

Table 3-14 Results of Staphylococcal Enterotoxins Production by *Staphylococcus aureus* (NCTC 10657) in ready to served custard at 30°C During 3 incubations days using SET – REPLA Diagnostic Kit.

Storage Day	Replicate # 1	Replicate # 2	Replicate # 3	Average	Interpretation
First Day	SEA: +2	SEA: +2	SEA: +2	SEA: +2	Positive (> 0.5ng/ml)
	SEB: +3	SEB: +3	SEB: +3	SEB: +3	Positive (> 0.5ng/ml)
	SEC: –	SEC: –	SEC: –	SEC: –	Negative
	SED: –	SED: –	SED: –	SED: –	Negative
Second Day	SEA: +3	SEA: +3	SEA: +3	SEA: +3	Positive (> 0.5ng/ml)
	SEB: +3	SEB: +3	SEB: +3	SEB: +3	Positive (> 0.5ng/ml)
	SEC: –	SEC: –	SEC: –	SEC: –	Negative
	SED: –	SED: –	SED: –	SED: –	Negative
Third Day	SEA: +3	SEA: +3	SEA: +3	SEA: +3	Positive (> 0.5ng/ml)
	SEB: +3	SEB: +3	SEB: +3	SEB: +3	Positive (> 0.5ng/ml)
	SEC: –	SEC: –	SEC: –	SEC: –	Negative
	SED: –	SED: –	SED: –	SED: –	Negative

Table 3-15 Results of staphylococcal enterotoxins Produced by *Staphylococcus aureus* (NCTC 10657) in ready to served custard at 44°C during 3 incubations days using SET – REPLA diagnostic kit.

Storage Day	Replicate # 1	Replicate # 2	Replicate # 3	Average	Interpretation
First Day	SEA: +3	SEA: +3	SEA: +3	SEA: +3	Positive (> 0.5ng/ml)
	SEB: +3	SEB: +3	SEB: +3	SEB: +3	Positive (> 0.5ng/ml)
	SEC: –	SEC: –	SEC: –	SEC: –	Negative
	SED: –	SED: –	SED: –	SED: –	Negative
Second Day	SEA: +3	SEA: +3	SEA: +3	SEA: +3	Positive (> 0.5ng/ml)
	SEB: +3	SEB: +3	SEB: +3	SEB: +3	Positive (> 0.5ng/ml)
	SEC: –	SEC: –	SEC: –	SEC: –	Negative
	SED: –	SED: –	SED: –	SED: –	Negative
Third Day	SEA: +3	SEA: +3	SEA: +3	SEA: +3	Positive (> 0.5ng/ml)
	SEB: +3	SEB: +3	SEB: +3	SEB: +3	Positive (> 0.5ng/ml)
	SEC: –	SEC: –	SEC: –	SEC: –	Negative
	SED: –	SED: –	SED: –	SED: –	Negative

Table 3-16 Changing in pH

Storage Day	Storage Temperatures	Replicate # 1	Replicate # 2	Replicate # 3	Average pH	Control
First Day	5°C	6.86	6.85	6.86	6.86	6.86
	30°C	6.32	6.32	6.32	6.32	6.84
	44°C	5.85	5.84	5.85	5.85	6.80
Second Day	5°C	6.86	6.86	6.86	6.86	6.86
	30°C	5.26	5.26	5.26	5.26	6.84
	44°C	5.16	5.16	5.16	5.16	6.80
Third Day	5°C	6.86	6.86	6.86	6.86	6.86
	30°C	5.29	5.12	5.26	5.22	6.84
	44°C	4.24	4.70	4.26	4.40	6.80

Table 3-17 Average growth of *Staphylococcus aureus* (NCTC 10657) at different storage times and temperatures.

Storage Day	Average CFU/ml at 5°C	Log of CFU/ml at 5°C	Average CFU/ml at 30°C	Log of CFU/ml at 30°C	Average CFU/ml at 44°C	Log of CFU/ml at 44°C
Day 0	1.0 E ⁴	4.00	1.0 E ⁴	4.00	1.0 E ⁴	4.00
Day 1	1.6 E ⁴	4.20	2.2 E ⁷	7.34	1.7 E ⁷	7.23
Day2	1.4 E ⁴	4.12	4.2 E ⁸	8.62	4.3 E ⁷	7.63
Day3	1.4 E ⁴	4.12	7.4 E ⁹	9.87	1.9 E ⁷	7.29

Table 3-18 Descriptive Statistics of the effect of storage temperature on the growth rate of *Staphylococcus aureus* (NCTC 10657) over 3 storage days

	N	Minimum	Maximum	Mean	Std. Deviation
Growth log (CFU/ml) at 5°C	4	4.00	4.20	4.1100	0.08246
Growth log (CFU/ml) at 30°C	4	4.00	9.90	7.4650	2.53542
Growth log (CFU/ml) at 44°C	4	4.00	7.63	6.5375	1.70081

***Staphylococcus aureus* (NCTC 8532):**

Table 3-19 Evaluation the initial inoculum of *Staphylococcus aureus* (NCTC 8532) using spread plate method on Baird – Parker agar.

Dilutions	CFU/0.1ml (dilution)	CFU/ml (dilution)	CFU/ml (Original Sample)	Average CFU/ml (dilution)	Average CFU/ml (Original Sample)
10^{-1}	1- TNTC 2- TNTC 3- TNTC	1- TNTC 2- TNTC 3- TNTC	1- TNTC 2- TNTC 3- TNTC	TNTC	TNTC
10^{-2}	1- TNTC 2- TNTC 3- TNTC	1- TNTC 2- TNTC 3- TNTC	1- TNTC 2- TNTC 3- TNTC	TNTC	TNTC
10^{-3}	1- TNTC 2- TNTC 3- TNTC	1- TNTC 2- TNTC 3- TNTC	1- TNTC 2- TNTC 3- TNTC	TNTC	TNTC
10^{-4}	1- TNTC 2- TNTC 3- TNTC	1- TNTC 2- TNTC 3- TNTC	1- TNTC 2- TNTC 3- TNTC	TNTC	TNTC
10^{-5}	1- 715 2- 910 3- 780	1- 7.2×10^3 2- 9.1×10^3 3- 7.8×10^3	1- 7.2×10^8 2- 9.1×10^8 3- 7.8×10^8	8.0×10^3	8.0×10^8
10^{-6}	1- 72 2- 93 3- 63	1- 7.2×10^2 2- 9.3×10^2 3- 6.3×10^2	1- 7.2×10^8 2- 9.3×10^8 3- 6.3×10^8	7.6×10^2	7.6×10^8
10^{-7}	1- 7 2- 6 3- 8	1- 70 2- 60 3- 80	1- 7.0×10^8 2- 6.0×10^8 3- 8.0×10^8	70	7.0×10^8

Calculation:

One millilitres of the dilution 10^{-6} gave 7.6×10^2 , so it requires approximately adding 1.3ml from the dilution 10^{-3} to every 100ml of custard to give an initial level of approximately 1.0×10^4 c.f.u/ml of spiked custard.

Table 3-20 Evaluation of the initial inoculums of *Staphylococcus aureus* (NCTC 10657) on the spiked custard (1.3 ml of 10^{-3} dilution per 100ml custard).

Dilution	C.F.U/0.1 ml	C.F.U/ml	Average	Total Average count
Un diluted	1- 980	1- 9.8E ³	9.5 E ³	9.7 E ³
	2- 975	2- 9.8 E ³		
	3- 900	3- 9.0 E ³		
10 ⁻¹	1- 108	1- 1.1 E ⁴	9.7 E ³	
	2- 101	2- 1.0 E ⁴		
	3- 81	3- 8.1 E ³		
10 ⁻²	1- 13	1- 1.3 E ⁴	1.1 E ⁴	
	2- 11	2- 1.1 E ⁴		
	3- 10	3- 1.0 E ⁴		
10 ⁻³	1- 3	1- 3.0 E ⁴	1.3 E ⁴	
	2- 1	2- 1.0 E ⁴		
	3- <1	3- <1.0 E ⁴		

Bacterial Growth in Different Storage time and Temperatures

Table 3-21 Evaluation of bacteria viable count at 5°C - (First Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average cfu/ml
10 ⁻¹	108	98	103	
10 ⁻¹	105	100	95	
Average cfu/ml	1.1 x 10⁴	9.9 x 10³	99 x 10³	1.0 x 10⁴
10 ⁻²	20	10	20	
10 ⁻²	10	10	30	
Average cfu/ml	2.0 x 10⁴	1.0 x 10⁴	3.0 x 10⁴	2.0 x 10⁴
10 ⁻³	3	1	<1	
10 ⁻³	1	1	<1	
Average cfu/ml	2.0 x 10⁴	1.0 x 10⁴	<10⁴	1.0 x 10⁴

Statistics

The number of colony forming unit per ml of spiked sample stored at 5°C (day 1)

N	Valid Missing	3 6
Mean		10266.67
Median		9900.00
Std. Deviation		635.085
Variance		403333.333
Minimum		9900
Maximum		11000

Average CFU/ml: 1.0 E⁴

Descriptive Statistics

Log CFU per ml of spiked sample stored at 5°C (day 1)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU / ml	3	4.00	4.04	4.0133	0.02309

Table 3-22 Evaluation of bacteria viable count at 5°C - (Second Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average cfu/ml
10 ⁻¹	104	99	107	
10 ⁻¹	98	93	104	
Average cfu/ml	1.0 x 10⁴	9.6 x 10³	1.1 x 10⁴	1.0 x 10⁴
10 ⁻²	10	8	10	
10 ⁻²	10	11	9	
Average cfu/ml	1.0 x 10⁴	1.0 x 10⁴	1.0 x 10⁴	1.0 x 10⁴
10 ⁻³	<1	1	2	
10 ⁻³	<1	<1	1	
Average CFU/ml	< 10⁴	1.0 x 10⁴	2.0 x 10⁴	1.0 x 10⁴

Statistics

The number of colony forming unit per ml of spiked sample stored at 5°C (day 2)

N	Valid Missing	3 6
Mean		10200.0000
Median		10000.0000
Std. Deviation		721.11026
Variance		520000.000
Minimum		9600.00
Maximum		11000.00

Average CFU/ml: 1.0 E⁴

Descriptive Statistics

Log CFU per ml of spiked sample stored at 5°C (day 2)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	3.98	4.04	4.0067	0.03055

Table 3-24 Evaluation of bacteria viable count at 5°C - (Third Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average cfu/ml
10 ⁻¹	130	100	100	
10 ⁻¹	101	100	90	
Average cfu/ml	1.2 x 10⁴	1.0 x 10⁴	1.0 x 10⁴	1.1 x 10⁴
10 ⁻²	20	10	10	
10 ⁻²	10	7	10	
Average CFU/ml	1.5 x 10⁴	9.0 x 10³	1.0 x 10⁴	1.1 x 10⁴
10 ⁻³	<1	<1	<1	
10 ⁻³	<1	<1	<1	
Average CFU/ml	<10⁴	<10⁴	<10⁴	<10⁴

Statistics

The number of colony forming unit per ml of spiked sample stored at 5°C (day 3)

N	Valid Missing	3 6
Mean		10666.6667
Median		10000.0000
Std. Deviation		1154.70054
Variance		1333333.333
Minimum		10000.00
Maximum		12000.00

Average CFU/ml: 1.1 E⁴

Descriptive Statistics

Log CFU per ml of spiked sample stored at 5°C (day 3)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	4.00	4.10	4.0333	0.05774

Table 3-25 Evaluation of bacteria viable count at 30°C - (First Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average cfu/ml
10 ⁻³	TNTC	TNTC	TNTC	
10 ⁻³	TNTC	TNTC	TNTC	
Average cfu/ml	TNTC	TNTC	TNTC	TNTC
10 ⁻⁴	2.2 x 10 ³	2.1 x 10 ³	3.1 x 10 ³	
10 ⁻⁴	2.0 x 10 ³	2.2 x 10 ³	2.5 x 10 ³	
Average cfu/ml	2.1 x 10³	2.2 x 10³	2.8 x 10³	2.4 x 10³
10 ⁻⁵	515	567	460	
10 ⁻⁵	455	676	520	
Average cfu/ml	4.9 x 10²	6.2 x 10²	4.9 x 10²	5.3 x 10²

Statistics

The number of colony forming unit per ml of spiked sample stored at 30°C (day 1)

N	Valid Missing	3 6
Mean		533333333.33
Median		490000000.00
Std. Deviation		75055534.995
Variance		5633333333333330.000
Minimum		490000000
Maximum		620000000

Average CFU/ml: 5.3 E²

Descriptive Statistics

Log CFU per ml of spiked sample stored at 30°C (day 1)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	8.70	8.80	8.7333	0.05774

Table 3-26 Evaluation of bacteria viable count at 30°C - (Second Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average cfu/ml
10 ⁻⁴	TNTC	TNTC	TNTC	
10 ⁻⁴	TNTC	TNTC	TNTC	
Average cfu/ml	TNTC	TNTC	TNTC	TNTC
10 ⁻⁵	740	790	929	
10 ⁻⁵	600	598	751	
Average cfu/ml	6.7 x 10⁸	6.9 x 10⁸	8.4 x 10⁸	7.3 x 10⁸
10 ⁻⁶	97	78	79	
10 ⁻⁶	83	80	76	
Average CFU/ml	9.0 x 10⁸	7.9 x 10⁸	7.8 x 10⁸	8.2 x 10⁸

Statistics

The number of colony forming unit per ml of spiked sample stored at 30°C (day 2)

N	Valid Missing	3 6
Mean		823333333.3334
Median		790000000.0000
Std. Deviation		66583281.18480
Variance		44333333333333.000
Minimum		780000000.00
Maximum		900000000.00

Average CFU/ml: 8.2 E⁸

Descriptive Statistics

Log CFU per ml of spiked sample stored at 30°C (day 2)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	8.90	8.95	8.9167	0.02887

Table 3-27 Evaluation of bacteria viable count at 30°C - (Third Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average cfu/ml
10 ⁻⁴	TNTC	TNTC	TNTC	
10 ⁻⁴	TNTC	TNTC	TNTC	
Average CFU/ml	TNTC	TNTC	TNTC	TNTC
10 ⁻⁵	TNTC	TNTC	TNTC	
10 ⁻⁵	TNTC	TNTC	TNTC	
Average CFU/ml	TNTC	TNTC	TNTC	TNTC
10 ⁻⁶	215	157	168	
10 ⁻⁶	168	222	170	
Average CFU/ml	1.9 x 10⁹	1.9 x 10⁹	1.7 x 10⁹	1.8 x 10⁹

Statistics

The number of colony forming unit per ml of spiked sample stored at 30°C (day 3)

N	Valid Missing	3 6
Mean		1833333333.3334
Median		1900000000.0000
Std. Deviation		115470053.83793
Variance		1333333333333330.000
Minimum		1700000000.00
Maximum		1900000000.00

Average CFU/ml: 1.8 E⁹

Descriptive Statistics

Log CFU per ml of spiked sample stored at 30°C (day 3)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	9.23	9.28	9.2633	0.02887

Table 3-28 Evaluation of bacteria viable count at 44°C - (First Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average cfu/ml
10 ⁻³	2.0 x 10 ³	1.3 x 10 ³	2.1 x 10 ³	
10 ⁻³	1.1 x 10 ³	1.0 x 10 ³	2.1 x 10 ³	
Average cfu/ml	1.9 x 10⁷	1.2 x 10⁷	2.1 x 10⁷	1.7 x 10⁷
10 ⁻⁴	921	740	780	
10 ⁻⁴	806	680	758	
Average cfu/ml	8.6 x 10⁷	7.1 x 10³	7.7 x 10⁷	7.8x 10⁷
10 ⁻⁵	78	62	79	
10 ⁻⁵	82	80	81	
Average cfu/ml	8.0 x 10⁷	7.1 x 10⁷	8.0 x 10⁷	7.7 x 10⁷

Statistics

The number of colony forming unit per ml of spiked sample stored at 44°C (day 1)

N	Valid Missing	3 1
Mean		77000000.0000
Median		80000000.0000
Std. Deviation		5196152.42271
Variance		27000000000000.000
Minimum		71000000.00
Maximum		80000000.00

Average CFU/ml: 7.7 E⁷

Descriptive Statistics

Log CFU per ml of spiked sample stored at 44°C (day 1)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU / ml	3	7.85	7.90	7.8833	0.02887

Table 3-29 Evaluation of bacteria viable count at 44°C - (Second Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average cfu/ml
10 ⁻⁴	284	335	420	
10 ⁻⁴	302	100	298	
Average cfu/ml	2.9 x 10⁷	2.2 x 10⁷	3.6 x 10⁷	2.9 x 10⁷
10 ⁻⁵	31	36	40	
10 ⁻⁵	35	32	34	
Average cfu/ml	3.3 x 10⁷	3.4 x 10⁷	3.7 x 10⁷	3.5 x 10⁷
10 ⁻⁶	4	3	2	
10 ⁻⁶	2	3	<1	
Average cfu/ml	3.0 x 10⁷	3.0 x 10⁷	1.0 x 10⁷	2.0 x 10⁷

Statistics

The number of colony forming unit per ml of spiked sample stored at 44°C (day 2)

N	Valid Missing	3 6
Mean		34666666.6667
Median		34000000.0000
Std. Deviation		2081665.99947
Variance		433333333333.333
Minimum		33000000.00
Maximum		37000000.00

Average CFU/ml: 3.5 E⁷

Descriptive Statistics

Log CFU per ml of spiked sample stored at 44°C (day 2)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	7.51	7.57	7.5367	0.03055

Table 3-30 Evaluation of bacteria viable count at 44°C - (Third Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average cfu/ml
10 ⁻⁴	291	295	520	
10 ⁻⁴	302	300	308	
Average cfu/ml	3.0 x 10⁷	3.0 x 10⁷	4.1 x 10⁷	3.4 x 10⁷
10 ⁻⁵	34	33	24	
10 ⁻⁵	30	31	26	
Average cfu/ml	3.2 x 10⁷	3.2 x 10⁷	2.5 x 10⁷	3.0 x 10⁷
10 ⁻⁶	<1	3	2	
10 ⁻⁶	2	3	3	
Average cfu/ml	1.0 x 10⁷	3.0 x 10⁸	3.0 x 10⁷	2.3 x 10⁷

Statistics

The number of colony forming unit per ml of spiked sample stored at 44°C (day 3)

N	Valid	3
	Missing	6
Mean		29666666.6667
Median		32000000.0000
Std. Deviation		4041451.88433
Variance		16333333333333.330
Minimum		25000000.00
Maximum		32000000.00

Average CFU/ml: 3.0 E⁷

Descriptive Statistics

Log CFU per ml of spiked sample stored at 44°C (day 3)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	7.40	7.50	7.4667	0.05774

Table 3-33 Results of Staphylococcal Enterotoxins Production by *Staphylococcus aureus* (NCTC 8532) in ready to served custard at 30°C During 3 incubations days using SET – REPLA Diagnostic Kit.

Storage Day	Replicate # 1	Replicate # 2	Replicate # 3	Average	Interpretation
First Day	SEA: –	SEA: –	SEA: –	SEA: –	Negative
	SEB: –	SEB: –	SEB: –	SEB: –	
	SEC: –	SEC: –	SEC: –	SEC: –	
	SED: –	SED: –	SED: –	SED: –	
Second Day	SEA: –	SEA: –	SEA: –	SEA: –	Negative
	SEB: –	SEB: –	SEB: –	SEB: –	
	SEC: –	SEC: –	SEC: –	SEC: –	
	SED: –	SED: –	SED: –	SED: –	
Third Day	SEA: –	SEA: –	SEA: –	SEA: –	Negative
	SEB: –	SEB: –	SEB: –	SEB: –	
	SEC: –	SEC: –	SEC: –	SEC: –	
	SED: –	SED: –	SED: –	SED: –	

Table 3-34 Results of Staphylococcal Enterotoxins Production by *Staphylococcus aureus* (NCTC 8532) in ready to served custard at 44°C During 3 incubations days using SET – REPLA Diagnostic Kit.

Storage Day	Replicate # 1	Replicate # 2	Replicate # 3	Average	Interpretation
First Day	SEA: –	SEA: –	SEA: –	SEA: –	Negative
	SEB: –	SEB: –	SEB: –	SEB: –	
	SEC: –	SEC: –	SEC: –	SEC: –	
	SED: –	SED: –	SED: –	SED: –	
Second Day	SEA: –	SEA: –	SEA: –	SEA: –	Negative
	SEB: –	SEB: –	SEB: –	SEB: –	
	SEC: –	SEC: –	SEC: –	SEC: –	
	SED: –	SED: –	SED: –	SED: –	
Third Day	SEA: –	SEA: –	SEA: –	SEA: –	Negative
	SEB: –	SEB: –	SEB: –	SEB: –	
	SEC: –	SEC: –	SEC: –	SEC: –	
	SED: –	SED: –	SED: –	SED: –	

Table 3-35 Changing in pH

Storage Day	Storage Temperatures	Replicate # 1	Replicate # 2	Replicate # 3	Average pH	Control
First Day	5°C	6.87	6.87	6.84	6.86	6.87
	30°C	6.23	6.20	6.32	6.28	6.81
	44°C	6.23	6.43	6.25	6.30	6.80
Second Day	5°C	6.86	6.86	6.86	6.86	6.86
	30°C	5.33	5.35	5.33	5.32	6.81
	44°C	4.24	4.24	4.26	4.25	6.68
Third Day	5°C	6.71	6.70	6.68	6.70	6.80
	30°C	5.29	5.12	5.26	5.32	6.37
	44°C	4.44	4.25	4.26	4.25	6.34

Table 3-36 Average growth of *Staphylococcus aureus* (NCTC 8532) at different storage times and temperatures.

Storage Day	Average CFU/ml at 5°C	Log of CFU/ml at 5°C	Average CFU/ml at 30°C	Log of CFU/ml at 30°C	Average CFU/ml at 44°C	Log of CFU/ml at 44°C
Day 0	1.0 E ⁴	4.00	1.0 E ⁴	4.00	1.0 E ⁴	4.00
Day 1	1.0 E ⁴	4.00	3.5 E8	8.73	7.7 E7	7.89
Day2	1.0 E ⁴	4.00	8.2 E8	8.91	3.5 E7	7.53
Day3	1.1 E ⁴	4.04	1.8 E9	9.26	3.0 E7	7.47

Table 3-37 Descriptive Statistics of the effect of storage temperature on the growth rate of *Staphylococcus aureus* (NCTC 8532) over 3 storage days

	N	Minimum	Maximum	Mean	Std. Deviation
Growth log (CFU/ml) at 5°C	4	4.00	4.04	4.0100	0.02000
Growth log (CFU/ml) at 30°C	4	4.00	9.26	7.7225	2.49172
Growth log (CFU/ml) at 44°C	4	4.00	7.90	6.7300	1.82943

***Staphylococcus epidermidis* (NCTC 12100):**

Table 3-38 Evaluation the initial inoculum of *Staphylococcus epidermidis* (NCTC 12100) using spread plate method on Baird – Parker agar.

Dilutions	CFU/0.1ml (diluted sample)	CFU/ml (diluted sample)	CFU/ml (Original Sample)	Average CFU/ml (diluted sample)	Average CFU/ml (Original Sample)
10^{-1}	1- TNTC	1- TNTC	1- TNTC	TNTC	TNTC
	2- TNTC	2- TNTC	2- TNTC		
	3- TNTC	3- TNTC	3- TNTC		
10^{-2}	1- TNTC	1- TNTC	1- TNTC	TNTC	TNTC
	2- TNTC	2- TNTC	2- TNTC		
	3- TNTC	3- TNTC	3- TNTC		
10^{-3}	1- TNTC	1- TNTC	1- TNTC	TNTC	TNTC
	2- TNTC	2- TNTC	2- TNTC		
	3- TNTC	3- TNTC	3- TNTC		
10^{-4}	1- TNTC	1- TNTC	1- TNTC	TNTC	TNTC
	2- TNTC	2- TNTC	2- TNTC		
	3- TNTC	3- TNTC	3- TNTC		
10^{-5}	1- TNTC	1- TNTC	1- TNTC	TNTC	TNTC
	2- TNTC	2- TNTC	2- TNTC		
	3- TNTC	3- TNTC	3- TNTC		
10^{-6}	1- 111	1- 1.1×10^3	1- 1.1×10^9	9.9×10^2	9.9×10^8
	2- 96	2- 9.6×10^2	2- 9.6×10^8		
	3- 90	3- 9.0×10^2	3- 9.0×10^8		
10^{-7}	1- 8	1- 80	1- 8.0×10^8	80	8.0×10^8
	2- 7	2- 70	2- 7.0×10^8		
	3- 9	3- 90	3- 9.0×10^8		

Calculation:

One millilitres of the dilution 10^{-6} gave 9.9×10^2 , so it requires approximately adding **one** ml from the dilution 10^{-3} to every 100ml of custard to give an initial level of approximately 1.0×10^4 CFU/ml of spiked custard.

Table 3-39 Evaluation of the initial inoculums of *Staphylococcus epidermidis* (NCTC 12100) on the spiked custard (1.0 ml of 10^{-3} dilution per 100ml custard).

Dilution	C.F.U/0.1 ml	C.F.U/ml	Average	Total Average count
Un diluted	1- TNTC 2- TNTC 3- TNTC	1- TNTC 2- TNTC 3- TNTC	TNTC	1.0 E ⁴
10⁻¹	1- 101 2- 103 3- 112	1- 1.0 E ⁴ 2- 1.0 E ⁴ 3- 1.1 E ⁴	1.0 E ⁴	
10⁻²	1- 10 2- 11 3- 14	1- 1.0 E ⁴ 2- 1.1 E ⁴ 3- 1.4 E ⁴	1.2 E ⁴	
10⁻³	1- 1 2- 1 3- <1	1- 1.0 E ⁴ 2- 1.0 E ⁴ 3- <1.0 E ⁴	1.0 E ⁴	

Bacterial Growth in Different Storage time and Temperatures

Table 3-40 Evaluation of bacteria viable count at 5°C - (First Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average CFU/ml
10 ⁻¹	103	94	88	
Average CFU/ml	1.0 x 10⁴	9.4 x 10³	8.8 x 10³	9.4 x 10³
10 ⁻²	20	20	10	
Average CFU/ml	2.0 x 10⁴	2.0 x 10⁴	1.0 x 10⁴	1.7 x 10⁴
10 ⁻³	<1	<1	<1	
Average CFU/ml	< 10⁴	< 10⁴	<10⁴	< 10⁴

Statistics

The number of colony forming unit per ml of spiked sample stored at 5°C (day 1)

N	Valid Missing	3 6
Mean		9400.00
Median		9400.00
Std. Deviation		600.000
Variance		360000.000
Minimum		8800
Maximum		10000

Average CFU/ml: 9.4 E³

Descriptive Statistics

Log CFU per ml of spiked sample stored at 5°C (day 1)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	3.94	4.00	3.9700	0.03000

Table 3-41 Evaluation of bacteria viable count at 5°C - (Second Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average CFU/ml
10 ⁻¹	89	99	107	
Average CFU/ml	8.9 x 10³	9.9 x 10³	1.4 x 10⁴	1.1 x 10⁴
10 ⁻²	10	8	12	
Average CFU/ml	1.0 x 10⁴	8.0 x 10³	1.2 x 10⁴	1.0 x 10⁴
10 ⁻³	1	1	2	
Average CFU/ml	1.0 x 10⁴	1.0 x 10⁴	2.0 x 10⁴	1.3 x 10⁴

Statistics

The number of colony forming unit per ml of spiked sample stored at 5°C (day 2)

N	Valid Missing	3 6
Mean		10933.3333
Median		9900.0000
Std. Deviation		2702.46801
Variance		7303333.333
Minimum		8900.00
Maximum		14000.00

Average CFU/ml: 1.1E⁴

Descriptive Statistics

Log CFU per ml of spiked sample stored at 5°C (day 2)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	3.95	4.15	4.0333	0.10408

Table 3-42 Evaluation of bacteria viable count at 5°C - (Third Day)

Dilution	Replicate 1 CFU/0.1ml	Replicate 2 CFU/0.1ml	Replicate 3 CFU/0.1ml	Average CFU/ml
10 ⁻¹	120	98	99	
Average CFU/ml	1.2 x 10⁴	9.8 x 10³	9.9 x 10³	1.1 x 10⁴
10 ⁻²	7	9	10	
Average CFU/ml	7.0 x 10³	9.0 x 10³	1.0 x 10⁴	8.6 x 10³
10 ⁻³	<1	<1	1	
Average CFU/ml	<10⁴	<10⁴	1.0 x 10⁴	1.0 x 10⁴

Statistics

The number of colony forming unit per ml of spiked sample stored at 5°C (day 3)

N	Valid	3
	Missing	0
Mean		10566.6667
Median		9900.0000
Std. Deviation		1242.30968
Variance		1543333.333
Minimum		9800.00
Maximum		12000.00

Average CFU/ml: 1.1 E⁴

Descriptive Statistics

Log CFU per ml of spiked sample stored at 5°C (day 3)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	4.00	4.10	4.0333	0.05774

Table 3-43 Evaluation of bacteria viable count at 30°C - (First Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average CFU/ml
10 ⁻⁴	395	390	384	
Average CFU/ml	4.0 x 10 ⁷	3.9 x 10 ⁷	3.8 x 10 ⁷	3.9 x 10 ⁷
10 ⁻⁵	46	59	56	
Average CFU/ml	4.6 x 10 ⁷	5.9 x 10 ⁷	5.6 x 10 ⁷	5.4 x 10 ⁷
10 ⁻⁶	5	4	5	
Average CFU/ml	5.0 x 10 ⁷	4.0 x 10 ⁷	5.0 x 10 ⁷	4.7 x 10 ⁷

Statistics

The number of colony forming unit per ml of spiked sample stored at 30°C (day 1)

N	Valid	3
	Missing	6
Mean		53666666.6667
Median		56000000.0000
Std. Deviation		6806859.28555
Variance		46333333333333.300
Minimum		46000000.00
Maximum		59000000.00

Average CFU/ml: 5.4 E⁷

Descriptive Statistics

Log CFU per ml of spiked sample stored at 30°C (day 1)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	4.00	4.10	4.0333	0.05774

Table 3-44 Evaluation of bacteria viable count at 30°C - (Second Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average CFU/ml
10 ⁻⁴	TNTC	TNTC	TNTC	
Average CFU/ml	TNTC	TNTC	TNTC	TNTC
10 ⁻⁵	237	204	200	
Average CFU/ml	2.4 x 10⁸	2.0 x 10⁸	2.0 x 10⁸	2.1 x 10⁸
10 ⁻⁶	22	17	23	
Average CFU/ml	2.2 x 10⁸	1.7 x 10⁸	2.3 x 10⁸	2.1 x 10⁸

Statistics

The number of colony forming unit per ml of spiked sample stored at 30°C (day 2)

N	Valid Missing	3 6
Mean		213333333.3334
Median		200000000.0000
Std. Deviation		23094010.76759
Variance		533333333333333.000
Minimum		200000000.00
Maximum		240000000.00

Average CFU/ml: 2.1 E⁸

Descriptive Statistics

Log CFU per ml of spiked sample stored at 30°C (day 2)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	8.30	8.38	8.3267	0.04619

Table 3-45 Evaluation of bacteria viable count at 30°C - (Third Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average CFU/ml
10 ⁻⁴	TNTC	TNTC	TNTC	
Average CFU/ml	TNTC	TNTC	TNTC	TNTC
10 ⁻⁵	366	297	396	
Average CFU/ml	3.7 x 10⁸	3.0 x 10⁸	4.0 x 10⁸	3.6 x 10⁸
10 ⁻⁶	41	40	45	
Average CFU/ml	4.1 x 10⁸	4.0 x 10⁸	4.5 x 10⁸	4.2 x 10⁸

Statistics

The number of colony forming unit per ml of spiked sample stored at 30°C (day 3)

N	Valid Missing	3 6
Mean		420000000.0000
Median		410000000.0000
Std. Deviation		26457513.11065
Variance		700000000000000.00
Minimum		0
Maximum		400000000.00 450000000.00

Average CFU/ml: 4.2 E⁸

Descriptive Statistics

Log CFU per ml of spiked sample stored at 30°C (day 3)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	8.60	8.65	8.6200	0.02646

Table 3-46 Evaluation of bacteria viable count at 44°C - (First Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average CFU/ml
10 ⁻⁴	240	341	220	
Average CFU/ml	2.4 x 10 ⁷	3.4 x 10 ⁷	2.2 x 10 ⁷	2.7 x 10 ⁷
10 ⁻⁵	31	27	23	
Average CFU/ml	3.1 x 10 ⁷	2.7 x 10 ⁷	2.3 x 10 ⁷	2.7 x 10 ⁷
10 ⁻⁶	3	4	1	
Average CFU/ml	3.0 x 10 ⁷	4.0 x 10 ⁷	1.0 x 10 ⁷	2.7 x 10 ⁷

Statistics

The number of colony forming unit per ml of spiked sample stored at 44°C (day 1)

N	Valid Missing	3 6
Mean		26666666.6667
Median		24000000.0000
Std. Deviation		6429100.50733
Variance		41333333333333.330
Minimum		22000000.00
Maximum		34000000.00

Average CFU/ml: 2.7 E⁷

Descriptive Statistics

Log CFU per ml of spiked sample stored at 44°C (day 1)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	7.34	7.53	7.4167	0.10017

Table 3-47 Evaluation of bacteria viable count at 44°C - (Second Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average CFU/ml
10 ⁻⁴	360	361	457	
Average CFU/ml	3.6 x 10 ⁷	3.6 x 10 ⁷	4.6 x 10 ⁷	3.9 x 10 ⁷
10 ⁻⁵	33	34	44	
Average CFU/ml	3.3 x 10 ⁷	3.4 x 10 ⁷	4.4 x 10 ⁷	3.7 x 10 ⁷
10 ⁻⁶	5	3	3	
Average CFU/ml	5.0 x 10 ⁷	3.0 x 10 ⁷	3.0 x 10 ⁷	3.7 x 10 ⁷

Statistics

The number of colony forming unit per ml of spiked sample stored at 44°C (day 2)

N	Valid	3
	Missing	6
Mean		37000000.0000
Median		34000000.0000
Std. Deviation		6082762.53030
Variance		37000000000000.000
Minimum		33000000.00
Maximum		44000000.00

Average CFU/ml: 3.7 E⁷

Descriptive Statistics

Log CFU per ml of spiked sample stored at 44°C (day 2)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	7.52	7.64	7.5633	0.06658

Table 3-48 Evaluation of bacteria viable count at 44°C - (Third Day)

Dilution	Replicate 1 cfu/0.1ml	Replicate 2 cfu/0.1ml	Replicate 3 cfu/0.1ml	Average cfu/ml
10 ⁻³	TNTC	TNTC	TNTC	
Average CFU/ml	TNTC	TNTC	TNTC	TNTC
10 ⁻⁴	TNTC	TNTC	TNTC	
Average CFU/ml	TNTC	TNTC	TNTC	TNTC
10 ⁻⁵	52	62	54	
Average CFU/ml	5.2 x 10⁷	6.2 x 10⁷	5.4 x 10⁷	5.6 x 10⁷

Statistics

The number of colony forming unit per ml of spiked sample stored at 44°C (day 3)

N	Valid Missing	3 6
Mean		56000000.0000
Median		54000000.0000
Std. Deviation		5291502.62213
Variance		28000000000000.000
Minimum		52000000.00
Maximum		62000000.00

Average CFU/ml: 5.6 E⁷

Descriptive Statistics

Log CFU per ml of spiked sample stored at 44°C (day 3)

	N	Minimum	Maximum	Mean	Std. Deviation
Log of CFU/ml	3	7.72	7.80	7.7500	0.04359

Staphylococcal enterotoxins production:

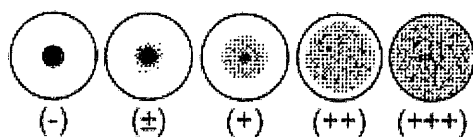


Table 3-49 Results of Staphylococcal Enterotoxins Production by *Staphylococcus epidermidis* (NCTC 12100) in culture fluid (TSB) using SET – REPLA Diagnostic Kit after 18 hours incubation at 37°C.

Enterotoxins	Replicate # 1	Replicate # 2	Replicate # 3	Average	Interpretation
SEA	—	—	—	—	Negative
AEB	—	—	—	—	Negative
SEC	+ 3	+ 3	+ 3	+ 3	Positive (> 0.5ng/ml)
SED	—	—	—	—	Negative

Table 3-50 Results of Staphylococcal Enterotoxins Production by *Staphylococcus epidermidis* (NCTC 12100) in ready to served custard at 5°C During 3 incubations days using SET – REPLA Diagnostic Kit.

Storage Day	Replicate # 1	Replicate # 2	Replicate # 3	Average	Interpretation
First Day	SEA: —	SEA: —	SEA: —	SEA: —	Negative
	SEB: —	SEB: —	SEB: —	SEB: —	
	SEC: —	SEC: —	SEC: —	SEC: —	
	SED: —	SED: —	SED: —	SED: —	
Second Day	SEA: —	SEA: —	SEA: —	SEA: —	Negative
	SEB: —	SEB: —	SEB: —	SEB: —	
	SEC: —	SEC: —	SEC: —	SEC: —	
	SED: —	SED: —	SED: —	SED: —	
Third Day	SEA: —	SEA: —	SEA: —	SEA: —	Negative
	SEB: —	SEB: —	SEB: —	SEB: —	
	SEC: —	SEC: —	SEC: —	SEC: —	
	SED: —	SED: —	SED: —	SED: —	

Table 3- 51 Results of Staphylococcal Enterotoxins Production by *Staphylococcus epidermidis* (NCTC 12100) in ready to served custard at 30°C During 3 incubations days using SET – REPLA Diagnostic Kit.

Storage Day	Replicate # 1	Replicate # 2	Replicate # 3	Average	Interpretation
First Day	SEA: –	SEA: –	SEA: –	SEA: –	Negative
	SEB: –	SEB: –	SEB: –	SEB: –	
	SEC: –	SEC: –	SEC: –	SEC: –	
	SED: –	SED: –	SED: –	SED: –	
Second Day	SEA: –	SEA: –	SEA: –	SEA: –	Negative
	SEB: –	SEB: –	SEB: –	SEB: –	
	SEC: –	SEC: –	SEC: –	SEC: –	
	SED: –	SED: –	SED: –	SED: –	
Third Day	SEA: –	SEA: –	SEA: –	SEA: –	Negative Negative Positive (> 0.5 ng/ml) Negative
	SEB: –	SEB: –	SEB: –	SEB: –	
	SEC: + 2	SEC: +2	SEC: +2	SEC: +2	
	SED: –	SED: –	SED: –	SED: –	

Table 3-52 Results of Staphylococcal Enterotoxins Production by *Staphylococcus epidermidis* (NCTC 12100) in ready to served custard at 44°C During 3 incubations days using SET – REPLA Diagnostic Kit.

Storage Day	Replicate # 1	Replicate # 2	Replicate # 3	Average	Interpretation
First Day	SEA: –	SEA: –	SEA: –	SEA: –	Negative
	SEB: –	SEB: –	SEB: –	SEB: –	
	SEC: –	SEC: –	SEC: –	SEC: –	
	SED: –	SED: –	SED: –	SED: –	
Second Day	SEA: –	SEA: –	SEA: –	SEA: –	Negative
	SEB: –	SEB: –	SEB: –	SEB: –	
	SEC: –	SEC: –	SEC: –	SEC: –	
	SED: –	SED: –	SED: –	SED: –	
Third Day	SEA: –	SEA: –	SEA: –	SEA: –	Negative Negative Positive (> 0.5 ng/ml) Negative
	SEB: –	SEB: –	SEB: –	SEB: –	
	SEC: +3	SEC: +3	SEC: +3	SEC: +3	
	SED: –	SED: –	SED: –	SED: –	

Table 3-53 Changing in pH

Storage Day	Storage Temperatures	Replicate # 1	Replicate # 2	Replicate # 3	Average pH	Control
First Day	5°C	6.57	6.58	6.57	6.57	6.57
	30°C	6.51	6.51	6.51	6.51	6.57
	44°C	6.38	6.40	6.38	6.39	6.54
Second Day	5°C	6.57	6.57	6.57	6.57	6.57
	30°C	6.32	6.32	6.12	6.25	6.51
	44°C	6.13	6.13	6.13	6.13	6.52
Third Day	5°C	6.56	6.56	6.57	6.57	6.57
	30°C	5.42	5.53	5.46	5.47	6.54
	44°C	5.85	5.84	5.82	5.84	6.46

Table 3-54 Average growth of *Staphylococcus epidermidis* (NCTC 12100) at different storage times and temperatures.

Storage Day	Average CFU/ml at 5°C	Log of CFU/ml at 5°C	Average CFU/ml at 30°C	Log of CFU/ml at 30°C	Average CFU/ml at 44°C	Log of CFU/ml at 44°C
Day 0	1.0 E ⁴	4.00	1.0 E ⁴	4.00	1.0 E ⁴	4.00
Day 1	9.4 E ³	3.43	5.4 E ⁷	7.73	2.7 E ⁷	7.43
Day2	1.1 E ⁴	4.10	2.1 E ⁸	8.32	3.7 E ⁷	7.57
Day3	1.1 E ⁴	4.04	4.2 E ⁸	8.62	5.6 E ⁷	7.75

Table 3-55 Descriptive Statistics of the effect of storage temperature on the growth rate of *Staphylococcus epidermidis* (NCTC 12100) over 3 storage days

	N	Minimum	Maximum	Mean	Std. Deviation
Growth log (CFU/ml) at 5°C	4	4.00	4.04	4.0200	0.02309
Growth log (CFU/ml) at 30°C	4	4.00	8.62	7.1675	2.14379
Growth log (CFU/ml) at 44°C	4	4.00	7.75	6.6875	1.79645