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Viability of Clinical *Staphylococcus aureus* and *Staphylococcus aureus* ATCC 25923 Under Different Preservation Methods

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ABSTRACT

In this study, four methods of bacterial preservation methods: sterile soil, sterile distilled water, agar overlaid with mineral oil and lyophilisation were employed for maintaining clinical Staphylococcus aureus and Staphylococcus aureus ATCC 25923 for a period of six months (180 days). The morphology, Gram staining properties, biochemical characteristics and viability were monitored at regular intervals of one month (30 days) for six months (180 days).The spread plate colony count method was used to investigate the rate of recovery or viability of all the four organisms from equally inoculated bacterial suspensions (2.0 McFarland equivalent to 8.8 log_{10} cfu/ml) in differently prepared stock culture of each preservation method. Agar overlaid with mineral oil was found to be the best method for maintenance and preservation of both clinical Staphylococcus aureus and Staphylococcus aureus ATCC 25923 organisms.

Keywords: *Staphylococcus aureus, Culture, Preservation, Lyophilisation, Viability*

INTRODUCTION

Microbial culture collections have existed since bacteriologists were first able to isolate and cultivate micro organisms and have been essential aspect of microbiology. They supply rich source of micro organisms that are of present, past and potential future interest. They are usually

considered to be a means to preserve micro-organisms (Caktu and Turkoglu, 2011).

The uniqueness of microorganisms and their often unpredictable nature and biosynthetic capabilities, given a specific set of environmental and cultural conditions, has made them likely candidates for solving particularly difficult problems in the life sciences and other fields as well. The various ways in which microorganisms have been used over the past 50 years to advance medical technology, human and animal health, food processing, food safety and quality, genetic engineering, environmental protection, agricultural biotechnology, and more effective treatment of agricultural and municipal wastes provide a most impressive record of achievement. Many of these technological advances would not have been possible using straightforward chemical and physical engineering methods, or if they were, they would not have been practically or economically feasible (Higa and Parr, 1994).

All practicing microbiologists have felt the need to preserve the viability of microorganisms with which they work. It is desirable, oftentimes essential, for laboratories to maintain bacterial strains in culture for extended periods of time for research, teaching and quality control purposes. In addition, all the cultural characteristics of a culture, as they were at the time of preservation, must be conserved. The nature of work being done will determine whether the preservation requirement is only very short-term or for an unlimited time period. Long-term preservation of a culture is required if a culture is to be deposited in one of the service culture collections with a view to preserving something of scientific value "for perpetuity". Many methods of preservation for microorganisms have been developed. Here, it is to be noted that there exist different types of microorganisms: bacteria, viruses, algae, protozoa, yeasts and moulds (Anonymous, 2009).

Common methods employed include storage in agar slant, under mineral oil, lyophilization, water culture storage, repeated sub-culturing, storage at -80°F in defibrinated rabbit blood or freezing in liquid nitrogen. There is a vast amount of literature available detailing different long-term storage methods for microorganisms (Siberry *et al*., 2001), though much of this literature is species-specific (Obara *et al*., 1981), and several bacterial species are historically difficult to maintain long-term (Murray *et al*., 2003).

Genus Staphylococcus is the cause of many different disease in human and animals, furthermore it is one of the most important bacteria in food hygiene and the serious problem caused by Staphylococcus species is food intoxication to be created with enterotoxins of this organisms (Jay *et al*., 2005). *Staphylococcus aureus* is a Gram positive, non-motile, cocci and facultative anaerobe usually in clusters (Prescott *et al*., 2007). It causes boils, pustules, impetigo and other diseases. Besides its clinical relevance, it also a very interesting model used to study the mechanism of cell division and of antibiotic resistance to cell wall targeting antibiotics (Anonymous, 2012). It can grow between 7 ºC and 45 ºC. Its optimal temperature range is around 35-39 ºC. Generally the organism grows best at a neutral pH; however it can grow between a range

of pH 4-9. It can grow in both aerobic and anaerobic conditions (better in the presence of oxygen) and can survive in highly desiccated environments (Shafiei *et al.,* 2011).

In this study, viability of maintenance cultures of *Staphylococcus aureus* was followed for six months, with the objective of describing a simple and cost effective method for storing them.

MATERIALS AND METHODS

Collection of Isolates

Isolates of *Staphylococcus aureus* from clinical specimens were obtained from University Health Service Clinic (Sickbay), Ahmadu Bello University, Zaria, Nigeria. The reference standard strain of *Staphylococcus aureus* ATCC 25923 was obtained from the National Institute for Pharmaceutical Research and Development, Idu, Abuja, Nigeria.

Identification of Isolates

Macroscopy (morphological examination of culture and purification)

Staphylococcus aureus which appeared yellow to cream or white on blood agar or chocolate agar was 1-2mm in diameter and was subcultured on to Mannitol Salt Agar (Cheesebrough, 2004). This was to purify the isolate. *Staphylococcus aureus* appeared golden yellow on Mannitol Salt Agar.

Microscopy (Gram staining)

This was carried out by Gram staining of the isolates. A colony was emulsified in sterile distilled water and a thin preparation was made on a slide. This was allowed to air dry. The slide was then covered with crystal violet stain for 30-60 seconds and was rinsed rapidly with sterile distilled water. Lugol's iodine was then applied for 30-60 seconds and was also rinsed with sterile distilled water. The smear was decolourised by applying acetone for a few seconds and was rinsed immediately with sterile distilled water. It was finally covered with neutral red for one minute, rinsed with sterile distilled water and air dried. Microscopic examination under oil immersion objective was carried out (Cheesebrough, 2004).

Biochemical characterization of pure culture

Catalse and coagulase test was used to identify *Staphylococcus aureus.*

Catalase Test

The isolate was emulsified in 2-3 drops of 3% hydrogen peroxide on a clean glass slide, active bubbling indicated positive test for *Staphylococcus aureus* (Murray *et al.,* 2007).

Coagulase Test

The isolate was emulsified in 2-3 drops of human plasma on a clean glass slide. After 10seconds clumping indicated positive test for *Staphylococcus aureus* (Murray *et al.,* 2007).

Standardisation of Inoculum

The inoculum of the organism was prepared by inoculating a colony of the organism into sterile distilled water. The turbidity was compared to match 2.0 McFarland standard. The McFarland standard was prepared by dilution of 1ml of concentrated sulphuric acid with 99ml of sterile distilled water, 1.175g of Barium chloride was

diluted with 100ml of sterile distilled water. Exactly 98ml of sulphuric acid solution and 2ml of barium chloride solution was mixed to obtain 1% barium sulphate (precipitate), a standard of 2.0 Mcfarland solution (Murray *et al.*, 2007).

Preservation of Pure Culture

Pure culture of *Staphylococcus aureus* was preserved by **four** different methods:

Agar Slant Overlaid with Mineral Oil

Tryptic soy agar of about 10mls was prepared in tubes and slanted. 0.1ml of pure culture of the standardized inoculum was grown on the agar slant. When good growth was seen after incubation at 37°C for 24hours, sterile mineral oil was added about 1cm above the tip of the slant. It was stored at room temperature. When subculture was needed, the growth from under the oil will be scrapped (WHO, 1991).

Sterile Soil

Soil stocks were prepared by mixing soil (20 %), sand (78%) and calcium carbonate (2%) and distributed into tubes (about 5grams per tube). They were sterilized using dry heat in an hot air oven for 8-15 hours at 130°C and then cooled. A suspension of the soil was made and 0.1ml of the suspension was cultured on tryptone soy agar in order to check the sterility of the soil. In the absence of growth, exactly 0.1ml of actively growing pure culture which was standardized was added to the sterile soil and incubated at 37ºC for 24 hours. Then the soil stock was stored at room temperature with cotton plugs or screw caps to protect it from dust. When subculture was needed, a suspension of the soil stock was made in sterile distilled water (Patil *et al.*, 2008).

Sterile Distilled Water

10ml of sterile distilled water was inoculated with 0.1ml of standardized inoculum in a screw cap tube. The tubes were sealed with parafilm membrane and stored in the dark at room temperature (Liao and Shollenberger, 2003).

Lyophilisation (Freeze- Drying)

Labels were prepared, cut to size and pasted on each ampoule. Each ampoule was plugged with cotton wool. The ampoules were packed in an autoclave bag and were sterilised by autoclaving at 121ºC for 15 minutes. An isolate of the bacteria was cultured on Tryptic soy agar incubated at 37°C for 24 hours. Liquid culture was prepared using nutrient broth to match 2.0 Mc farland standard. The cells were harvested by asceptic centrifugation for 30 minutes at 4000 rpm in eppendorf tubes. The supernatant was decanted. A dense suspension of the culture was prepared in a suitable suspending fluid such as 5% bacteriological peptone. One drop of the bacterial suspension was placed at the bottom of an ampoule using a sterile Pasteur pipette. The cotton wool plug was then replaced and the prepared ampoule will be placed in a rack. It was then dried using a vacuum pump and a dessicator containing silica gel (Malik 1990). The ampoules were sealed with a Bunsen flame while under vacuum (Goszczynska *et al.*, 2000). During recovery, the contents of the freeze dried ampoule was

dissolved in 1ml of bacteriological peptone water and incubated at 37 ºC for 24 hours (Malik, 1990).

Quantification of Specimen Viability

Initial quantification: A representative inoculum from each of the organism preserved by various methods was quantified on tryptic soy agar plates; 1 ml bacterial suspension was diluted in 9 ml of sterile physiological saline (0.85 % NaCl w/v, pH 7.0), and 0.1ml of the diluted suspension was spread on tryptic soy agar plates and incubated at 37 °C for 24 hours.

$$\frac{\text{The number of colonies on the plate}}{\text{The volume plated (0.1ml)}} \times \text{dilution factor} = \text{Colony Forming Unit/ml}$$

(Siberry *et al*., 2001)

Quantification at Monthly Interval

The viability of preserved strain of the organism was checked on day 0 and on monthly interval (30days) by subculturing them on tryptic soy agar, 1 ml of thawed suspension was diluted in 9ml sterile physiological saline (0.85%NaCl w/v, pH 7.0) and 0.1ml of this was spread on Tryptic soy agar. Colonies were counted after overnight incubation at 37°C for 24hours.

$$\frac{\text{The number of colonies on the plate}}{\text{The volume plated (0.1ml)}} \times \text{dilution factor} = \text{Colony Forming Unit/ml}$$

(Siberry *et al*., 2001)

Checking for the morphology and biochemical characteristics of the preserved organisms.

The morphology and biochemical characteristics of the preserved organisms were monitored from day 0 and on monthly interval (30days) for six months following the procedure above.

Analysis of Results

Variations between counts of clinical *Staphylococcus aureus and Staphylococcus aureus* ATCC 25923 was compared using student's t-test while variation in the counts of each bacterium over the study period of six months was compared using analysis of variance (ANOVA) with level of significance set at 0.05(i.e. $p \leq 0.05$)

RESULT

Morphology and gram staining ability of the preserved micro organisms -

It was observed that there was no change in the morphological and Gram staining ability of both clinical *Staphylococcus aureus* and *Staphylococcus aureus* ATCC 25923 after six months (180 days). Although those preserved in sterile soil loss their viability after two months (60 days). After six months (180 days), the two *Staphylococcus aureus* isolates were Gram positive cocci in clusters with golden yellow and circular pin head colonies on Tryptone Soy Agar (Table 1).

Table 1: Cultural and morphological characteristics of clinical *Staphylococcus aureus* and *Staphylococcus aureus* ATCC 25923 before storage

Organism	Growth Medium	Morphology
Staphylococcus aureus (clinical)	Mannitol Salt Agar	Gram positive cocci in clusters; golden yellow colonies
Staphylococcus aureus (clinical)	Tryptone Soy Agar	Gram positive cocci in clusters; golden yellow with circular pin head colonies
Staphylococcus aureus ATCC 25923	Mannitol Salt Agar	Gram positive cocci in clusters; golden yellow colonies
Staphylococcus aureus ATCC 25923	Tryptone Soy Agar	Gram positive cocci in clusters; golden yellow with circular pin head colonies

Table 2: Cultural and morphological characteristics of clinical *Staphylococcus aureus* and *Staphylococcus aureus* ATCC 25923 after storage

Organism	Growth Medium	Morphology
Staphylococcus aureus (clinical)	Tryptone Soy Agar	Gram positive cocci in clusters; golden yellow with circular pin head colonies
Staphylococcus aureus ATCC 25923	Tryptone Soy Agar	Gram positive cocci in clusters; golden yellow with circular pin head colonies

Biochemical characteristics of test organisms

It was observed that there was no change in the biochemical characteristics of clinical *Staphylococcus aureus* and *Staphylococcus aureus* ATCC 25923 after six months (180 days). Although those preserved in sterile soil lost their viability after two months (60 days). After six months (180 days), clinical *Staphylococcus aureus* and *Staphylococcus aureus* ATCC 25923 isolates were coagulase and catalase positive. (Table 3)

Table 3: Results indicating biochemical test recorded for both clinical *Staphylococcus aureus* and *Staphylococcus aureus* ATCC 25923 before and after storage

Experimental procedure	Observation	Results
Catalase Test	Bubbling and frothing	Positive
Coagulase Test	Clumping of cells	Positive

VIABLE COUNTS OF PRESERVED ORGANISMS AFTER STORAGE

Four bacteria preservation methods (i.e. sterile soil, sterile distilled water, agar slant overlaid with mineral oil and lyophilisation) were employed to preserve both clinical and standard strains of *Staphylococcus aureus*. The isolates were preserved for a period of six months (180days).

At month zero the mean bacterial count for *Staphylococcus aureus* was compared using analysis of variance (ANOVA) and it was found that there was no significant difference between the isolates and the preservation methods ($F=0.291$, $p=0.393$).

At one month (30 days), there was significant difference between the isolates and the preservation methods ($F=1493.110$, $p<0.001$). The preservation of clinical *Staphylococcus aureus* and *Staphylococcus aureus* ATCC 25923 in agar overlaid with mineral oil had the highest mean bacterial count of $\log_{10}$ 8.89 and $\log_{10}$ 8.90 respectively while clinical *Staphylococcus aureus* preserved in sterile distilled water had the least mean bacterial count of 6.93 (Table 4). Though, clinical *Staphylococcus aureus* preserved in sterile distilled water ($\log_{10}$ 6.93) and *Staphylococcus aureus* ATCC 25923 preserved in sterile distilled water ($\log_{10}$ 6.90) were not significantly different ($p=0.955$).

At two months (60 days), there was significant difference between the isolates and the preservation methods ($F=3012.330$, $p<0.001$). The preservation of *Staphylococcus aureus* ATCC 25923 preserved in agar overlaid with mineral oil had the highest mean bacterial count of $\log_{10}$ 8.89 while clinical *Staphylococcus aureus* preserved in sterile distilled water had the least mean bacterial count of $\log_{10}$ 5.34 (Table 4). Though, *Staphylococcus aureus* ATCC 25923 preserved in sterile distilled water ($\log_{10}$ 6.77), clinical *Staphylococcus aureus* preserved in sterile soil ($\log_{10}$ 5.49) and clinical *Staphylococcus aureus* preserved in sterile distilled water ($\log_{10}$ 5.34) were not significantly different ($p= 0.472$).

At three months (90 days), there was significant difference between the isolates and the preservation methods ($F=1070.367$, $p<0.001$) The preservation of *Staphylococcus aureus* ATCC 25923 in agar overlaid with mineral oil had the highest mean bacterial count of $\log_{10}$ 8.90 while clinical *Staphylococcus aureus* and *Staphylococcus aureus* ATCC 25923 preserved in sterile soil had the mean least bacterial count of $\log_{10}$ 0 (Table 4). Though, clinical *Staphylococcus aureus* preserved by lyophilisation ($\log_{10}$ 6.75),

Staphylococcus aureus ATCC 25923 preserved by lyophilisation (log_{10} 6.53), *Staphylococcus aureus* ATCC 25923 preserved in sterile distilled water (log_{10} 5.30), clinical *Staphylococcus aureus* preserved in sterile soil (log_{10} 0) and *Staphylococcus aureus* ATCC 25923 preserved in sterile soil (log_{10} 0) were not significantly different (p=0.787).

Table 4: Log_{10} of mean bacterial counts for clinical *Staphylococcus aureus* and *Staphylococcus aureus* ATCC 25923 in cfu/ml ± SD

Organism & method of preservation	0 month (0days)	1 month (30days)	2 months (60days)	3months (90days)	4 months (120days)	5 months (150days)	6 months (180days)
SASS	8.79±7.00	7.80±6.78 d	6.54±5.70d	0±0c	0±0c	0±0d	0±0c
SASDW	8.79±7.00	6.90±5.17 e	6.77±5.00d	5.30±0c	4.69±3.00c	3.51±1.70d	3.47±0.00c
SAAM	8.79±7.00	8.90±6.70a	8.89±7.00a	8.90±0.00 a	7.93±6.65 a	7.92±0.00a	7.90±5.70a
SAL	8.79± 7.18	8.45±7.30 c	7.52±6.00c	6.53±4.70 c	6.40±5.00c	6.37±5.18c	6.31±4.70c
SCSS	8.79±6.70	7.63±6.00d	5.49±3.70d	0±0c	0±0c	0±0d	0±0c
SCSDW	8.78±0.00	6.93±5.74e	5.34±4.30d	5.06±4.17 c	4.34±4.26c	3.57±1.70 d	3.51±2.30c
SCAM	8.79±7.00	8.89±7.00a	8.46±7.00b	8.51±7.40 b	7.74±5.70b	7.59±5.70b	7.41±6.30b
SCL	8.78±6.70	8.51±6.70b	7.48±5.70c	6.75±5.00 c	6.40±5.48c	6.40±5.00c	6.30±5.30c

Variations are mean of duplicate counts. P-values less than or equals 0.05(i.e. $p \leq 0.05$) were considered significant. Values with different superscripts in the same column are significantly different. Mean values were separated (ranked) using Duncan's Multiple Range Test (DMRT).

KEY:

SASS- *Staphylococcus aureus* ATCC 25922 preserved in sterile soil

SASDW- *Staphylococcus aureus* ATCC 25922 preserved in sterile distilled water

SAAM- *Staphylococcus aureus* ATCC 25922 preserved in agar overlaid in mineral oil

SAL- *Staphylococcus aureus* ATCC 25922 preserved by lyophilisation

SCSS-*Staphylococcus aureus* clinical preserved in sterile soil

SCSDW-*Staphylococcus aureus* clinical preserved in sterile distilled water

SCAM- *Staphylococcus aureus* clinical preserved in agar overlaid with mineral oil

SCL- *Staphylococcus aureus* clinical preserved by lyophilisation

At four months (120 days), there was significant difference between the isolates and the preservation methods (F=425.086, p<0.001). The preservation of *Staphylococcus aureus* ATCC 25923 in agar overlaid with mineral oil had the highest mean bacterial count of log_{10} 7.93 while clinical *Staphylococcus aureus* and *Staphylococcus aureus* ATCC 25923 preserved in sterile soil had the least mean bacterial count of log_{10} 0 (Table 4). Though, clinical *Staphylococcus aureus* preserved by lyophilisation (log_{10} 6.40), *Staphylococcus aureus* ATCC 25923 preserved by lyophilisation (log_{10} 6.40), *Staphylococcus aureus* ATCC 25923 preserved in sterile distilled water (log_{10} 4.69), clinical *Staphylococcus aureus* preserved in sterile distilled water (log_{10} 4.34) , clinical *Staphylococcus aureus* preserved in sterile soil (log_{10} 0) and *Staphylococcus aureus*

ATCC 25923 preserved in sterile soil (log_{10} 0) were not significantly different (p=0.333).

At five months (150 days), there was significant difference between the isolates and the preservation methods (F=26387.041, p<0.001). The preservation of *Staphylococcus aureus* ATCC 25923 in agar overlaid with mineral oil had the highest mean bacterial count of log_{10} 7.92 while clinical *Staphylococcus aureus* and *Staphylococcus aureus* ATCC 25923 preserved in sterile soil had the least mean bacterial count of log_{10} 0 (Table 4). Though, clinical *Staphylococcus aureus* preserved in sterile distilled water (log_{10} 3.57), *Staphylococcus aureus* ATCC 25923 preserved in sterile distilled water (log_{10} 3.51), clinical *Staphylococcus aureus* preserved in sterile soil (log_{10} 0) and *Staphylococcus aureus* ATCC 25923 preserved in sterile soil (log_{10} 0) were not significantly different (p=0.990).

At six months (180 days), there was significant difference between the isolates and the preservation methods (F=1500.721, p<0.001). The preservation of *Staphylococcus aureus* ATCC 25923 in agar overlaid with mineral oil had the highest mean bacterial count of log_{10} 7.90 while clinical *Staphylococcus aureus* and *Staphylococcus aureus* ATCC 25923 preserved in sterile soil had the least mean bacterial count of log_{10} 0 (Table 4). Though, *Staphylococcus aureus* ATCC 25923 preserved by lyophilisation (log_{10} 6.31), clinical *Staphylococcus aureus* preserved by lyophilisation (log_{10} 6.30), clinical *Staphylococcus aureus* preserved in sterile distilled water (log_{10} 3.51), *Staphylococcus aureus* ATCC 25923 preserved in sterile distilled water (log_{10} 3.47), clinical *Staphylococcus aureus* preserved in sterile soil (log_{10} 0) and *Staphylococcus aureus* ATCC 25923 preserved in sterile soil (log_{10} 0) were not significantly different(p=0.105)

Table 5: Initial and final log_{10} mean bacterial counts with t-test values for clinical *Staphylococcus aureus* and *Staphylococcus aureus* ATCC 25923

Organism and method of preservation	Initial count(cfu/ml)	Final count (cfu/ml)	T-test	p-value
SASS	8.79	6.54	57.762	0.011*
SASDW	8.79	3.47	62.995	0.010*
SAAM	8.79	7.90	50.429	0.013*
SAL	8.79	6.31	40.728	0.016*
SCSS	8.79	5.49	122.816	0.005*
SCSDW	8.78	3.51	2999983.500	<0.001*
SCAM	8.79	7.41	73.000	0.009*
SCL	8.78	6.30	125.625	0.005*

*- significant difference exists (p≤0.05)

KEY:

SASS- *Staphylococcus aureus* ATCC 25922 preserved in sterile soil

SASDW- *Staphylococcus aureus* ATCC 25922 preserved in sterile distilled water

SAAM- *Staphylococcus aureus* ATCC 25922 preserved in agar overlaid in mineral oil

SAL- *Staphylococcus aureus* ATCC 25922 preserved by lyophilisation

SCSS-*Staphylococcus aureus* clinical preserved in sterile soil

SCSDW-*Staphylococcus aureus* clinical preserved in sterile distilled water

SCAM- *Staphylococcus aureus* clinical preserved in agar overlaid with mineral oil

SCL- *Staphylococcus aureus* clinical preserved by lyophilisation

The difference between mean bacterial count (i.e. the initial and final count for each preservation method was also compared using t-test. It was found that there was statistical significant difference between the initial and final mean bacterial counts. For *Staphylococcus aureus* ATCC 25923 preserved in sterile soil (T=57.762, p=0.011), *Staphylococcus aureus* ATCC 25923 preserved in sterile distilled water (T=62.995, p=0.010), *Staphylococcus aureus* ATCC 25923 preserved in agar overlaid with mineral oil (T=50.429, p=0.013), *Staphylococcus aureus* ATCC 25923 preserved by lyophilisation (T=40.728, p=0.016), clinical *Staphylococcus aureus* preserved in sterile soil (T=122.816, p=0.005), clinical *Staphylococcus aureus* preserved in sterile distilled water (T=29999983.5, p<0.001), clinical *Staphylococcus aureus* preserved in agar overlaid with mineral oil (T=73.0, p=0.009), and *Staphylococcus aureus* clinical preserved by lyophilisation (T=125.625, p=0.005) (Table 5).

DISCUSSION

In this findings, the methods of preservation affected the bacterial counts of clinical *Staphylococcus aureus* and *Staphylococcus aureus* ATCC 25923 significantly ($p \leq 0.05$) over a period of six months.

It was generally noted that both organisms had the highest viable mean bacterial count for those preserved in agar with mineral oil, followed by lyophilisation and then sterile distilled water. Those preserved in sterile soil lost their viability before six months. This is in line with Patil *et al.*, (2008) who stated that sterile soil has found wide use for stock culture maintenance of microorganisms that form spores and this method is applied to spore forming organisms particularly fungi of which *Staphylococcus aureus* don't form. Also, Liao and Shollenberger, 2003 stated that almost all the bacteria tested which included *Staphylococcus aureus* survived in water for at least several months and up to 16 years and Gram positive bacteria *Staphylococcus aureus* and *Listeria monocytogenes* declined more rapidly in water than other bacteria. Inspite of the simplicity, preservation of bacteria in pure water has not been widely adopted in most of the microbiological laboratories possibly because of the lack of experimental data from multiple-year studies to validate the reliability of the method. Furthermore, only a small number of bacteria,

mainly phytopathogens have been tested in the past. It was not known prior to this study, if this method might be applied to store bacteria which are of clinical, environmental or industrial importance. In addition, more studies are needed to determine if non-spore forming gram positive bacteria other than *Staphylococcus aureus* can be preserved in water for years (Liao and Shollenberger, 2003).

Apart from these organisms' potential application for culture preservation, demonstration of the long-term survival of *Staphylococcus aureus* in water as described in this study further emphasizes the importance of sanitization treatments for water to be used for drinking or for cleaning of fresh and ready-to-eat produce.

Preservation of bacteria by overlaying cultures with mineral oil ensures the provision of anaerobic conditions and prevents dehydration thereby allowing the organism to remain in a dormant state. In line with this, Standburry and Whitaker (1990) stated that preservation of cultures on agar slopes may be extended to one year if the slopes are covered with mineral oil. Similarly, Zahoor *et al.,* (2003) concluded that *Lactobacillus bulgaricus* is best preserved under mineral oil for two months with no change in its morphology, Gram staining properties, biochemical characteristics and still retained its viability. Also, Stioanova and Arka'eva 2000 stated that immersion under mineral oil was effective and lyophilisation was the best method of preservation of *Lactobacillus lactis* subspecies *lactis*. Kirsop *et al.,* (1991) and Smith *et al.,* (1994) stated that major methods that give stable preservation are freeze-drying, L-drying (drying from the liquid state), cryopreservation (in the vapor phase of liquid nitrogen or in a deep freezer) and subculture under mineral oil.

Life and its reactions are dependent upon water. Nevertheless, dehydration to the point of total desiccation can be a good method to preserve live cells in a state of arrested metabolism (anhydrobiosis), which hopefully can be restarted following rehydration. Several microorganisms, invertebrates and plants naturally use anhydrobiosis for survival during periods of drought (Browne *et al.,* 2002). From this work lyophilisation was one of the good method of preservation that had a satisfactory result although it was not the best. However, this was because bacteria is said to be sensitive to freezing, by control of temperature, rates of cooling, and suspending media (Manzur *et al.*,1957) Most viability losses are considered to occur during the drying phase of the lyophilization process (Wagman and Weneck, 1963). For an organism to survive in harsh condition, many stress responses mechanisms are needed mechanism. Evolution has led to the development of *Staphylococcus aureus* to become a highly adaptable organism, as even subtle changes in environmental conditions affect its physiology (Chan and Foster, 1998).

The result stated above, shows that lyophilisation was one of the good methods of preservation but was not the best. This contradicts the work of Greig *et al.,* 1970 who observed that freeze drying (lyophilisation) was the best method of preservation. Similarly, the preservation in agar overlaying with mineral oil provides dissolved

oxygen, prevents drying of the agar and apparently decreases the metabolic activity of the cells to an almost negligible rate. However, genetic changes could occur in cultures stored in this manner (Patil *et al.*, 2008).

CONCLUSION

It should be kept in mind that different species and strains of bacteria exhibit great variability with respect to different preservation methods. The best method of preservation of clinical *Staphylococcus aureus* and *Staphylococcus aureus* ATCC 25923 for duration of six months (180 days) is agar overlaid with mineral oil. Lyophilisation and sterile distilled water are still good preservation methods although lyophilisation was the most expensive while sterile distilled water was the cheapest. However, sterile soil cannot be used to preserve these organisms for duration of six months (180 days) as they were found to have lost their viability after two months (60 days).

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