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Latent Period of *Pseudomonas aeruginosa* in Diary Product (Yogurt and Pasteurized Milk)

[1]Noura K. M/Salih, [2]Nura Abdullahi, [3]Hayyan Al Taweel
[1,2]Infrastructure University Kuala Lumpur, Jlnikram/uniten, Kajang, 43000, Selangor Darul El Ehsan, Malaysia
[3]Department of Clinical Laboratory Science, Mohammad Al- Mana Collage for Health Sciences, Al- Khobar-KSA
Corresponding Author: Noura K. M/Salih,

Abstract: *Study was conducted in the Infrastructure University Kuala Lumpur to assess and determine the effect of psychotropic bacteria in dairy product.Six samples of dairy products which included Yogurt and Pasteurized milk were used in this research, the two samples (AL-Safi yogurt & Dutch lady pure farm UHT pasteurized milk) were brought from Kajang Arab shop, the second sample (Al-Khwaja& Nestle profesional pack full cream milk UHT) were brought from Serdang mines while the last sample (Al-Sharq& Nestle low fat milk UHT) were brought from DeCentrum city mall, Super Seven) and incubated at 4^oC for three weeks for bacterial identification. Following the incubation and isolation of samples, 99% of Gram negative bacillus bacteria which is Pseudomonas aeruginosa was isolated from the samples on the 20th day and 1% of Gram positive cocci bacteria (Staphylococci) was detected by using primary and secondary biochemical tests which were Gram staining techniques, morphology observation, methyl red, oxidase, catalase and indole test. The colony counting was also performed using Nutrient broth agar which was also incubated at 37^oC for 24 hours. After 24 hours of incubation using Mile's and Misra method and the bacterial growth curve was plotted using excel.*

As conclusion, The results from the group provides important input in storing assessment perspectives of milk and yoghurt in the fridge (4^o C) for more than three weeks as it enhances the growth of the psychotropic bacteria which may contain the presence of highly dangerous toxins.

Key words: *Latent period, Pseudomonas aeruginosa, Yogurt, Pasteurized milk*

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I. Introduction

The term Pychrotrophs (Psychrotolerant) refers to microorganisms that have the ability to grow at low temperatures but have optimal and maximal growth temperatures above 15 and 20 °C, respectively (Moyer and Morita, 2007).

The quality of raw milk and of dairy products has been considerably improved by the refrigeration. Unfortunately, the current practices for the collection and storage of the raw milk favored the growth of Psychotropic bacteria, able to grow below 7^{o}C, regardless of their optimal growth temperature, include both Gram-negative and Gram-positive bacteria: Psychrotrophic bacteria from numerous genera have been isolated from milk; they comprise representatives of *Pseudomonas, Aeromonas,Acinetobacter, Alcaligenes, Achromobacter, Enterobacter, Flavobacterium, Klebsiella, Bacillus, Clostridium, Corynebacterium, Microbacterium, Micrococcus* (that are both psychrotrophic and thermoduric), coliforms like *Escherichia, Enterobacter, Citrobacter and Klebsiella* (Hayes & Boor, 2001).

Pseudomonas is the most common organisms in raw or pasteurized milk at the time of the spoilage (Mc Phee& Griffiths, 2002). They comprise *P. fluorescens, P. putida, P. fragi*, and *P. putrefaciens*, less frequently *P. aeruginosa*. Besides their rapid growth ability in refrigerated milk, Psychrotrophs produce heat stable extracellular proteases, lipases and phospholipases: some enzymes can survive pasteurization and even UHT heat treatments. Pseudomonas is the primary concern with regard to lipolytic degradation of milk (Mc Phee& Griffiths, 2002).

It was found that sixteen raw milk samples representative of different Chinese farms were evaluated for psychrotrophic bacteria, 480 isolates were clustered into 85 groups based on RAPD profiles, *Pseudomonas* was the most dominant genus followed by *Acinetobacter* and *Flavobacterium*.

Pseudomonas fluorescens was the most dominant species followed by *Pseudomonas fragi* and *Pseudomonas psychrophila*. 26 species belonging to 14 genera proved to be previously unreported psychrotrophic contaminants in raw milk. (Lei et al., 2018, Michael Lu et al., 2017)

Lag phase represents the earliest and most poorly understood stage of the bacterial growth cycle. During batch culture, a typical bacterial growth curve shows five distinct phases of growth: lag phase, the delay before the start of exponential growth; exponential phase, where cell division proceeds at a constant rate; stationary phase, when conditions become unfavorable for growth and bacteria stop replicating. (Baranyi J, Roberts TA. 2000); death phase is when cells lose viability; and finally, long-term stationary phase, which can be extended for years (Finkel SE 2006).

Psychrotrophic bacteria isolated from cooled milk belong to Gram-negative and Gram-positive genera and are taxonomically classified into seven classes. *Gammaprotobacteria*, Bacilli and *Actinobacteria*are the dominant classes containing between 19 and 21 species, while *Alphaproteobacteria*, *Betaproteobacteria*, *Flavobacteria* and *Sphingobacteria* are the four less significant classes (HantsisZacharov& Halpern, 2007).

According to Mcphee and Griffiths (2011), the cultivable psychrotrophic bacteria in milk are represented predominantly by Gram-negative genera including *Pseudomonas, Achromobacter*, *Aeromonas, Serratia*, *Alcaligenes, Chromobacterium* and *Flavobacterium*, and at much lower numbers by Gram-positive genera including *Bacillus, Clostridium, Corynebacterium, Streptococcus, Lactobacillus* and *Microbacterium*spp.

Staphylococcus aureus is a Gram-positive bacterium that often resides harmlessly in a wide range of niches from environmental samples to the skin and mucosa of humans and other animals. However some strains are highly pathogenic and are frequently implicated as the causative agent of both human and animal disease. In humans *S.aureus* is a major contributor to food poisoning, pneumonia and post-operative wounds (Tong SYC, et al., 2015). *Staphylococcus aureus*, a facultative anaerobic Gram-positive coccus, is an important cause of bovine mastitis and one of the most cost-intensive diseases in the dairy industry (Dufour et al., 2012). *Staphylococcus aureus* (also known as golden staph) is a Gram-positive, round-shaped bacterium that is a member of the *Firmicutes*, and is frequently found in the nose, respiratory tract, and on the skin. It is often positive for catalase and nitrate reduction and is a facultative anaerobe that can grow without the need for oxygen. (Masalha M. et al., 2001).

Milk contains 87.0% water, 3.9% fat, 4.9% lactose, 3.5% proteins, 0.7% ash, and a trace amount of vitamins. Milk therefore is an optimum medium for bacterial growth. Raw milk is pasteurized to lower the total number of bacteria and eliminate pathogenic bacteria. Some bacteria are present in milk which grow at refrigeration temperatures and spoil the product. Microbial growth and metabolism shorten the shelf life of milk by producing undesirable changes in aroma and taste attributes that influence consumer acceptability of the products (Fromm & Boor, 2004).

Pseudomonas aeruginosa is a Gram negative, aerobic, rod shaped bacterium with unipolar motility (Ryan et al., 2004). It is a common bacterium which can cause disease in animals and humans, found in soil, water, and most man-made environments throughout the world.

P. aeruginosa is a slender Gram negative bacillus, 1.5-3µm x 0.5µm, actively motile by a polar flagellum. It is non-capsulated but many strains have a mucoid slime layer. Mucoid strains, particularly isolates from cystic fibrosis patients have an abundance of extracellular polysaccharides composed of alginate polymers. This forms a loose capsule in which micro colonies of the bacillus are enmeshed and protected from host defense's (Ananthanarayan et al., 2000). Four different pigments have been described in *P. aeruginosa:* pyocyanin, fluorescein, pyorubrin and pyomelanin. Pyocyanin is a water soluble blue-green phenazine pigment produced by active cultures of *P. aeruginosa*. Pyocyanin has antibiotic activity against bacteria, fungi and protozoa, but is of little therapeutic value because it is quite toxic to eukaryotic cells (Baron et al., 1981).

P. aeruginosa is typically an opportunistic pathogen that seldom causes disease in healthy subjects. Normally, for an infection to occur, some disruption of the physical barriers (skin or mucous membranes), or by-passing of them (e.g., by urinary catheters, endotracheal tubes or other invasive devices), and /or an underlying dysfunction of the immune defense mechanisms, such as neutropenia, is necessary. As a consequence, *P. aeruginosa* is mostly a nosocomial pathogen. (NNIS, 1999).

This research aimed to follow the growth pattern of the dominant psychotropic bacteria to identify the latent phase in dairy products (yogurt and pasteurized milk), so as to improve preservation and usage of yogurt and post-pasteurization cold milk.

II. Material And Methods

Isolation of microorganisms:

Three different brands of yoghurt drinks which are Al-Sharq, Al-Khwaja and Al-Safi yogurt. And three different pasteurized milk which are: Dutch lady pure farm UHT recombined milk, Nestle low fat UHT and Nestle full cream milk UHT on arrival to the laboratory were visually observed for packaging conditions and color which is clear, normal and these was recorded accordingly, microbiological investigations were carried out on them while they were incubated in fridge at 4oC as follows: Pour plate techniques were used for isolation of bacteria. One ml of each yogurt and pasteurized milk sample were drawn aseptically using a sterile needle and syringe and was transferred into each test tubes which was labelled as Y-S1, Y-S2, Y-S3 and PM-S1, PM-S2,

PM-S3. Two plates of media were used for each sample for the isolation of bacteria using pour plate method in week 1, 2, 3 & 4 in a sterile laminar flow. The media was incubated at 4^{o}C for 7 to 10 days by checking the plates every day for bacterial growth to rule out slower growing microorganisms. After the end of week 3 (20 days) of incubation some growth was observed in some samples and was recorded and was checked using primary and secondary biochemical tests. During the simulation experiments, all milk samples were conducted for microbial analysis according to (Quinn P.J. et al., 2011 (Fawole and Oso 2007) (Prescott et al., 2008) Gram Staining and catalase test (slide method) were done as a primary biochemical test. Then oxidase test (strip method), Methyl Red, Indole test (filter paper method) were done as a secondary biochemical tests.

Bacterial colony counting:

1. 0.8 g of N broth was dissolved into 100 ml of distilled water
2. The inoculum was autoclaved together with 400 ml of distilled water, 25 test tubes and pipettes at 121^{o} C for 15 minutes.
3. After autoclaving, 1 ml of stock was transferred into tube 1 containing 9 ml of distilled water, another 1 ml from tube one was transferred into test-tube two containing 9 ml of distilled water till test-tube four which is serial dilution.
4. A drop of the stock was transferred into the nutrient agar medium which is first hour and incubated at 37^{o} C for 1 day. This procedure was continuously repeated after each 1d, 4d, 8d, 12d, 16d, 20d, 24d and 34ds.Then the growth was counted and calculated using Mile's and Misra method for colony counting. (Quinn P.J. et al, 2011 (Fawole and Oso 2007) (Prescott et al., 2008)

III. Result

The bacterial isolates in (Fig. 1, 2, & 3) were characterized and identified based on colonial and cellular morphological features as well as biochemical tests as presented in table 1 below.

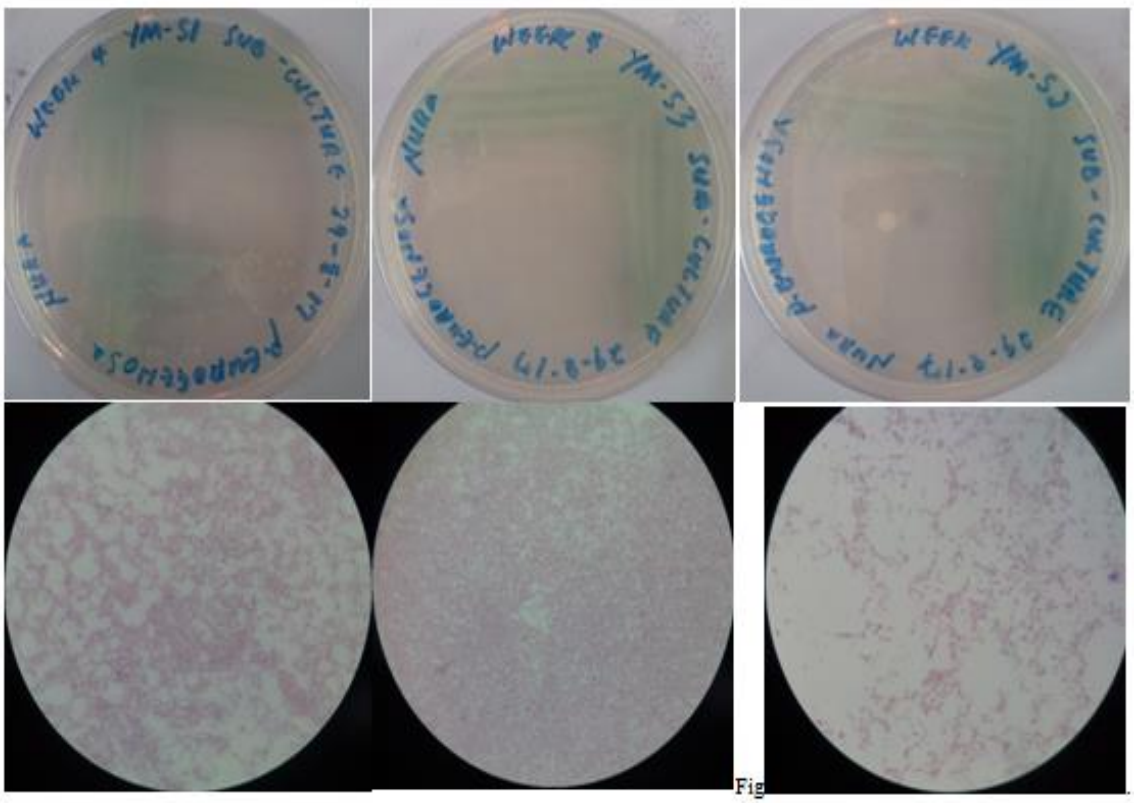

Fig. 1: Colonies morphology & Gram stain of yogurt samples 1,2&3 cultured in Nutrient agar, incubated at 4^{o}C for 28 days (Microscopy: total magnification at ocular magnification x objective lens magnification=10x100=1000, Gram negative bacilli has been isolated).

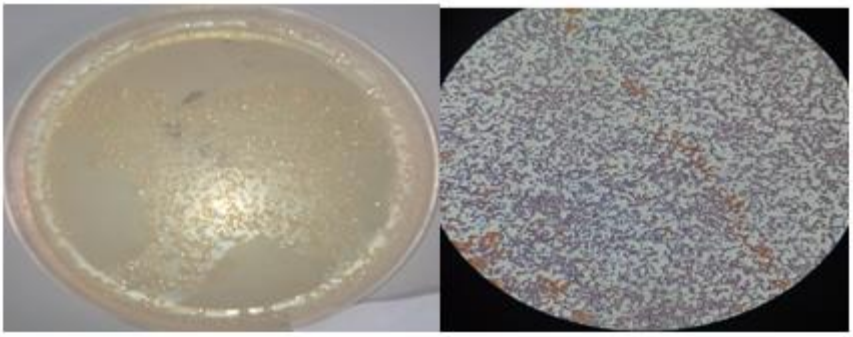

Fig. 2: Day 24 Pasteurized milk sample 1cultured in nutrient agar medium and incubated at 4^{o}C & Gram staining (Microscopy: total magnification at ocular magnification x objective lens magnification=10x100=1000 Gram positve cocci has been isolated).

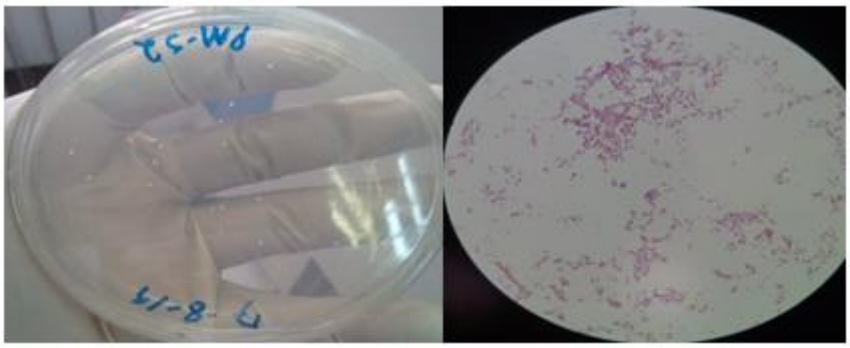

Fig: 3 : Day 20 (Pasteurized milk sample 2 cultured in Nutrient agar medium and incubated at 4^{o}C & Gram staining (Microscopy: total magnification at ocular magnification x objective lens magnification=10x100=1000, Gram positve cocci has been isolated).

Table 1 shows the colonial morphology (macroscopic observation of colony on plates) and the cellular morphology (microscopic characteristics) of the bacteria isolated from the yoghurt and pasteurized milk samples.

Table 1: Colonial and cellular morphology of the bacterial isolates from three different Yoghurt & Pasteurized milk samples.

Isolates	Colony Shape	Colony size	Elevation	Edge	Optical characteristics	Consistency	Pigmentation	Colony surface	Odor	Gram stain	Cellular morphology
Y-1	Round/ irregular	0.1 - 0.5µm	Flat	Entire	Opaque	soft	Blue green/milky	Smouth	Musty	-	rod
Y-2	Round	0.1 - 0.3µm	Flat	Entire	Opaque	soft	Blue green/milky	Smooth	Musty	-	rod
Y-3	Round/ irregular	0.1 - 0.4µm	Flat	Entire	Opaque	soft	Blue green/milky	Smouth	Musty	-	rod
PM-1	Round/ irregular	0.1 - 0.4µm	Flat	Entire	Opaque	soft	Pink/Milky	Smouth	Musty	+ & -	Cocci & rod

PM-2	Round	0.1 - 0.2µm	Flat	Entire	Opaque	soft	Blue green/milky	Smouth	Musty	-	rod
PM-3	Round/ small	0.1 - 0.2µm	Flat	Entire	Opaque	soft	Blue green/milky	Smouth	Musty	-	rod

The bacteria were characterized based on their reactions to the various biochemical tests and subsequently identified. The reactions of the bacterial isolates to the various biochemical tests performed on them are recorded in table 2 below. All the three samples of yogurt and three samples of pasteurized milk are catalase, oxidase, citrate, nitrate and pigment production are positive, while the indole, methyl red, urease and coagulase are negative in all samples.

Table 2: Results of the biochemical tests from three different yogurt and pasteurized milk samples.

Isolate	**catalase**	**Indole**	**oxidase**	**Methyl red**	**Citrate**	**Urease**	**Nitrate Reductionn**	**Cougulase**	**Pigment**
YS-1	+(ve)*	-(ve)	+(ve)	-(ve)	+(ve)	-(ve)	+(ve)	-(ve)	+(ve)
YS-2	+(ve)	-(ve)	+(ve)	-(ve)	+(ve)	-(ve)	+(ve)	-(ve)	+(ve)
YS-3	+(ve)	-(ve)	+(ve)	-(ve)	+(ve)	-(ve)	+(ve)	-(ve)	+(ve)
PM-1	+(ve)	-(ve)	+(ve)	-(ve)	+(ve)	-(ve)	+(ve)	-(ve)	+(ve)
PM-2	+(ve)	-(ve)	+(ve)	-(ve)	+(ve)	-(ve)	+(ve)	-(ve)	+(ve)
PM-3	+(ve)	-(ve)	+(ve)	-(ve)	+(ve)	-(ve)	+(ve)	-(ve)	+(ve)

** +ve = Positivereaction, –ve = negative*

The results for both total aerobic plates' culture of milk and yoghurt samples that are depicted in Fig. 1, 2 & 3 for Day 28, 24 & 28 *Pseudomonas* and *staphylococci* were isolated.

The total aerobic plates count remained fairly stable in the first two weeks under optimal storage conditions the microbial growth was visible only after 3 weeks of storage after the box has been opened for use during which some were changes in consistency and odors start to. The outgrowth of bacteria was observed during use and storage; *Pseudomonas* members had already started growing within the fridge (optimal storage condition) and showed an enhanced outgrowth under further storage time. The viable bacteria was counted and calculated using Mile's and Misra method for colony counting to follow the bacterial growth rate/CFU and identify the starting point of the latent period (Table 3).

Table 3: Bacterial viable count (CFU)

Time /day	**Growth**
1	0.000^{0} CFU
4	0.000^{0} CFU
6	0.000^{0} CFU
12	0.000^{0} CFU
16	0.000^{0} CFU
20	$0.001x10^{11}$ CFU
24	$6.3x10^{11}$ CFU
28	$9.6x10^{11}$CFU

There is no growth observed in the first days (1, 4, 8, 12, and 16) as stated in table 3. After three weeks of incubation the growth was started more rapidly at day 20, 24 and 28 (Fig.4).

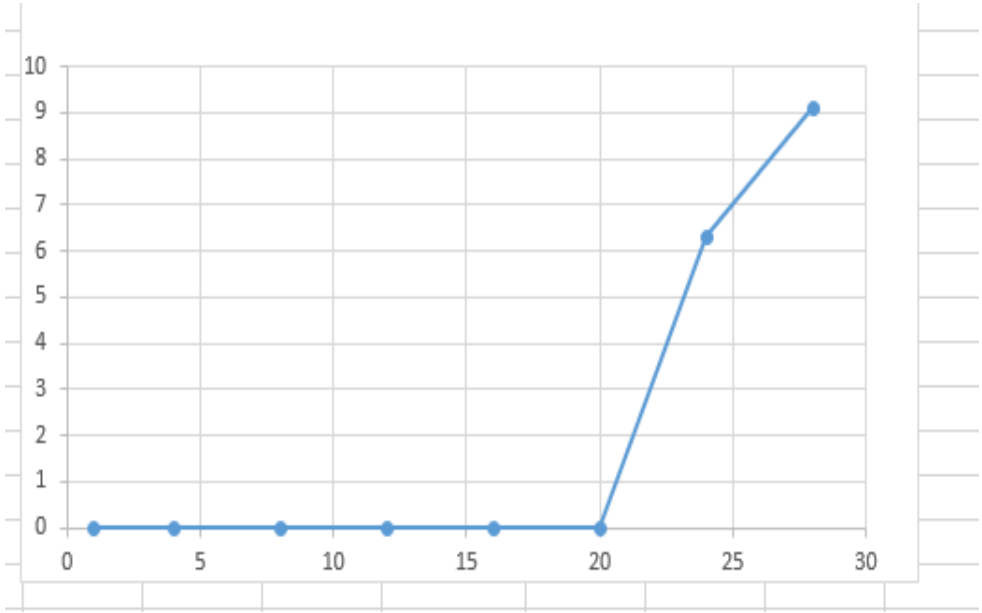

Figure 4: Bacterial latent phase ended after three weeks incubation in the fridge as the log phase started at day 20.

IV. Discussion

Dairy products are important components in the diet of human beings around the world. Their current consumption is relatively high and is expected to increase steadily during the next two decades (Gerosa and Skoet 2013). Psychrotrophs, particularly, *Pseudomonas* is known to be the main determinants of the shelf-life of pasteurized milk and refrigerated raw milk (Stevenson et al., 2003). The growth of *Pseudomonas* spp. is correlated with the occurrence of proteolytic activity in all food systems (Liu et al., 2006).

In this research six different samples of diary product were selected so as to improve control of post-pasteurization contamination in cold milk and to determine the effect of psychotropic bacteria in diary product which is yogurt and pasteurized milk. The samples were brought from three difference places.

Pseudomonas aeruginosa was isolated from 6 (six) different diary product samples by using nutrient agar which was promoted primarily based on characteristics colony morphology in nutrient agar after the incubation period. *Pseudomonas aeruginosa* produces circular mucoid smooth colonies with emits sweat grape and musty odor on nutrient agar. 99% Gram negative bacillus bacteria and 1% of Gram positive cocci was isolated from yogurt and pasteurized milk samples after the period of 28 days of incubation in 4^{o}C after opening the container, plus some primary and secondary biochemical tests, accordingly to (Quinn P.J. et al., 2011), who were detecting their bacteria by streaked the milk samples on sterile nutrient agar plates and overnight incubation at 37°C. Bacterial colonies with green color pigmentation were examined morphologically by Gram's staining method and all Gram-negative bacilli found were further cultured on sterile cetrimide agar plates for final purification, also found that *Pseudomonads* predominated in cold stored pasteurized milk at 10 and 5 days before expiration as well as on the expiration day.

In Table 1: Our results matching with Fawole and Oso (2007) who were first described and characterized the bacteria by their morphological appearances (i.e. colony shape, edge or margin, pigmentation, elevation, colony surface, consistency and optical characteristics) on the plate. In addition to the colonial characterization, cellular morphologies and biochemical characteristics as described in the Laboratory manual of microbiology and also used to characterize the bacteria.

The biochemical test was performed and the result were recorded in the table above which was showed the entire oxidase test were positive, this test indicates the presence of cytochrome c oxidase that is able to reduce oxygen (O_2) and artificial electron acceptors (Table 2).

Lastly, the bacterial colony was counted using Mile's and Misra method. Estimation of colony forming units (CFU) through serial dilution plating on a nutrient medium forms the most widely accepted method for monitoring cultivable bacteria and yeasts in different spheres of microbiology (R.K. Robinson, 2000), The viable cell density was approximately 10 8 CFU/ml. all serial dilutions were performed using sterile distilled water (standard methods for the examination of waste water, 19th ed.); The plate was incubated at 37^{o}C for 24 hrs. In 0 to 16 days milk and yoghurt samples, the plates were found with no growth while in the 20th day the total bacterial colony was observed and calculated as 0.001x10 11 CFU, at 24th day as 6.3x1011 CFU and 28th

day which was 9.6 x 1011 CFU. Then the growth curve was plotted using the calculated total numbers of bacterial colonies (Figure 1).

V. Conclusion

Based on the results of this study, it can be concluded that the storage of milk in the fridge at 4^{o} C can be affected with *Pseudomonas sp*. and *Staphylococci* bacteria when it kept while using for more than three weeks. In this study, the latent phase of milk incubated in cold environment ends in three weeks' time even if the expiry date is not near.

The psychrotrophs from refrigerated milk include both Gram-negative and Gram positive bacteria, *Pseudomonas* are the most common species in raw or pasteurized milk at the time of the spoilage; they constitute the predominant microorganisms limiting the shelf life of processed fluid milk at 4^{o}C. So it concluded that 99% in this research were Gram negative bacillus (*P. aeruginosa*) and 1% was Gram positive cocci after the biochemical tests of the microbial loads in the samples and following of the growth pattern.

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