Effects of Sublethal Temperature Stresses on the Culturability and Percentage Injury of *Escherichia coli* grown in the Laboratory Medium

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Abstract- Microorganism may adapt with environmental stresses by altering metabolisms and structures of their physiology. Temperature is a major environmental factor that can be used to inhibit microorganisms. However, sublethal temperature stress will not kill microorganism effectively and yet microorganisms are injured. The objectives of the present study were to determine the culturability and percentage injury of *Escherichia coli* ATCC 25922 grown in the laboratory medium when shifting from 37°C to sublethal temperature stresses temperatures of 45°C, 40°C and 20°C at log and stationary phases. Initially, the *E. coli* culture was grown in Tryptic Soy Broth at 37°C for 6 hours and 18 hours to reach log phase and stationary phase, respectively before the culture was transferred to three sublethal temperature stresses and culturability were determined by using two-plating systems; TSA with and without 4% NaCl (TSAS). Results showed that cells of *E. coli* at stationary phase were more resistant compared to log phase at both temperatures of 40°C and 45°C, however, stationary phase cells of *E. coli* at 20°C was less resistant compared to log phase. At log phase, the percentage of injured cells of *E. coli* at three sublethal temperatures of 45°C, 40°C and 20°C were ranged between 9.43%-20.40%, 0.95%-19.66% and 1.42%-7.83%, whereas the percentage injuries of *E. coli* at stationary phase for 45°C, 40°C and 20°C were ranged between 3.57%-25.65%, 0.36%-11.16% and 3.16%-13.54%, respectively. Results concluded that the culturability and injury of *E. coli* were affected by the type of sublethal temperature stress and its bacterial phase.

Index Terms- culturability, *Escherichia coli*, laboratory medium, percentage injury, temperature stress

I. INTRODUCTION

All living microorganisms are exposed to variety of environmental stress conditions and their responses and adaptation are much influenced by the severity of the environmental stress exposures. The changes of organisms through stress adaptation may affect the ability of these pathogens to survive in foods [1].

Escherichia coli was first isolated by Theodor Escherich in 1884. It is one of five recognised species of *Escherichia* and the member of family *Enterobacteriaceae*. *E. coli* is a Gram-negative rod which can ferment lactose within 48 hours and also can produce metallic green sheen on agar such EMB agar [2]. The natural habitat of *E. coli* is the large intestine of animals including humans which is in temperature 37ºC. The presence of *E. coli* in environment is considered as contaminant because many fecal-borne microorganisms are pathogenic in animals and humans. It can contribute to human diseases such as infant diarrhea and infections of the urinary tracts [3].

Temperature is one of the most influential environmental factors among microorganisms and it is used to control the growth of microorganism in foods and enhances the storage life and safety of food products. Temperature stress strongly influences the potential for survival and rate of growth of bacteria [4]. All *E. coli* strains can grow at a wide range of temperature between 8ºC to 48ºC [2].

Foodborne bacteria are exposed to variety of stresses in environment. Following exposure to a sublethal stress, a bacterial population have three different physiology which are uninjured or normal cells, sublethally injured cells (or injured cells) and lethally injured cells (or dead cells) [5]. Cell injury is defined as any damage to the components of cells itself by any stresses which weaken the ability of cells to survive or multiply and will increase the sensitivity of cells to any harmful factors [6]. One of the characteristics which show that the cells injured are they increased in sensitivity to many compounds where many normal cells are resistant. When expose and keep the cells at critical environment, the injured cells can not multiply and eventually die. In a non-selective and nutritionally medium, the cells are able to repair their injury which can extend 1 hour to 6 hour depending on the nature of stress and degree of injury. After repair, the cells will regain their normal characteristics and initiate multiplication [7].

When cells are exposed to stress, there are some cell components are damaged or injured but there are also some components which could be harmful due to specific stresses only. Some structural and components which are identified to be damaged or injured by sublethal stresses are the cell wall (or outer membrane), cytoplasmic membrane (or inner membrane), ribosomal RNA (rRNA) and DNA including some enzymes. The damages in the cell wall and cytoplasmic membrane usually because of freezing and drying while injured in ribosomal RNA are caused of heat treatment whereas damaging in DNA following the radiation of cells [7].

Bacteria are considered to be injured when treated with sublethal stress if they are not able to grow and produce colonies when plated on agar media containing selective agents [8]. Selective agents such as bile salts, organic dyes, antibiotics and surfactants are often used in media which may kill the injured

cells [7] by inhibiting the repair process of injured cells. But, on non-selective agars such as Tryptic Soy Agar (TSA), both injured and non-injured cells can grow and form colonies. For this study, the selecting agent used is sodium chloride, NaCl. Therefore, by plating agar containing NaCl (4%) which added to TSA, the percentage injury of cells can be measured.

The aim of this study was to determine the effects of *E. coli* when exposed to selected temperature stresses (20°C, 40°C and 45°C). The growth of bacteria at 20°C was selected because it was slightly below ambient temperature (lower optimal temperature). Temperature of 40°C was chosen because it is a mild heat treatment for most of microorganisms and it is reflected the temperature abuse during food handling or storage. Whereas, 45°C is considered as severe temperature stress for *E. coli* as it is near to the maximum limit for its growth.

Therefore, this study was conducted to determine the culturability and percentage injury of *E. coli* when exposed to sublethal temperature stresses after grown at optimal temperature. The finding of this study would provide new scientific evidence on microbial adaptation of *E. coli* under environmental stress conditions. This information is important to improve understanding about how stress can be implemented as preservation strategy.

II. EXPERIMENTAL METHOD

A. Source of Microorganism

Escherichia coli (ATCC 25922) used in this study was obtained from American Type Culture Collection (ATCC) which provided by the School of Food Science and Technology, Universiti Malaysia Terengganu (UMT).

B. Culture Media

The bacteriological media which used on this study were Tryptic Soy Broth (MERCK, Germany), Tryptic Soy Agar (MERCK, Germany), Tryptic Soy Agar with 4% NaCl, 0.1% Peptone water (OXOID, UK). The media were prepared and sterilized using an autoclave as specified on manufacturer's direction. The prepared agars were poured in sterile Petri dishes and were leaved and cooled before they were used for the analysis.

C. Confirmation of E. coli Isolate

Morphological and biochemical tests were performed to confirm the purity of *E. coli* that was used during this study. On IMViC tests, positive results of *E. coli* were showed on Indole Test and Methyl Red tests whereby negative result were determined on Voges-Proskauer and citrate tests. Isolates that showed positive result on lactose fermentation, motility, glucose fermentation, arabinose, glycerol, maltose, mannitol, rhamnose, xylose and negative result on oxidase, grew in the presence of potassium cyanide (KCN) was considered *E. coli* [2]. The strain also was confirmed using API 20E (BioMérieux, France).

D. Comparison between Static versus Agitated Cultivation Method for E. coli

Preparation of test suspension began with inoculum from a stock culture of *E. coli* strain. The inoculum was streaked onto Tryptic Soy Agar (TSA) and the plate was incubated at 37°C for 24 hours in order to obtain the single colony. After incubation, the fresh isolated single colony of the strain was picked off using a sterile loop and dispersed into a flask containing 100 ml of sterile TSB. Following incubation at 37°C for 30 minutes, 1 ml was transferred into 300 ml of TSB. The procedure provided an initial cell concentration of approximately 1 x 10^3 CFU/ml. The flask containing *E. coli* in 300 ml Tryptic Soy Broth (TSB) was incubated in a static incubator or in a shaker incubator. 1 ml of bacterial culture on the flask was diluted with 9 ml of 0.1 % peptone water and the 0.1 ml of desired dilution factor was transferred into TSA plates for a spread plate method in duplicates. Then, the plates were incubated for 24 hours at 37°C. The plates were enumerated and transformed to $\log_{10}$ CFU/ml and growth curve was constructed. The growth curve of *E. coli* at 37°C was established under agitated and static cultivation methods.

E. Survival and culturability of E. coli under temperature stresses

After incubation for 24 hour at 37°C, a single isolated colony on Tryptic Soy Agar (TSA) was transferred in 100 ml sterile Tryptic Soy Broth (TSB) in conical flask which later was incubated at 37°C for 30 minutes for an initial growth. After that, 1 ml of broth was taken and was transferred to 300 ml sterile TSB. The bacterium was grown at 37°C until it reached middle log phase (6 hours) and middle stationary phase (18 hours) before subjected to the selected temperature stresses (20°C, 40°C and 45°C). The samplings of bacterial culture shifted from 37°C to 20°C, 40°C and 45°C were done for every 1 hour for 9 hours at sublethal temperature stresses by duplicate the plates on TSA (without 4% NaCl) and TSAS (with 4% NaCl). After the samples were serial diluted at the desired serial dilution, 0.1 ml dilution from 9 ml of 0.1 % of peptone water was transferred to TSA and TSAS by using spread plate method. All plates were incubated for 24 hours at 37°C.

F. Determination of Percentage Injury of E. coli

Tryptic Soy Agar is a non-selective medium that support the growth of both injured and uninjured *E. coli*, whereas TSAS is a selective medium that support the growth of uninjured cells. The results from the culturability of cells on plate were used to determine the percentage injury of *E. coli*. The percentage injury (%) of cell was calculated by using the equation below [8]:

Percentage Injury (%) =

$$1 - \left(\frac{\text{Count on selective media (TSAS)}}{\text{Count on non selective media (TSA)}} \times 100 \right)$$

III. RESULTS AND DISCUSSIONS

A. Agitation Versus Static Cultivation method for E. coli

Figure 1 shows the growth curve of *E. coli* at 37°C using static and agitated cultivation methods when logarithmic of bacterial population was plotted against time. Although the agitated and static cultivation methods were not significantly different, the static cultivation method was chosen to grow *E. coli* in order to mimic the growth culture of *E. coli* in food which is static.

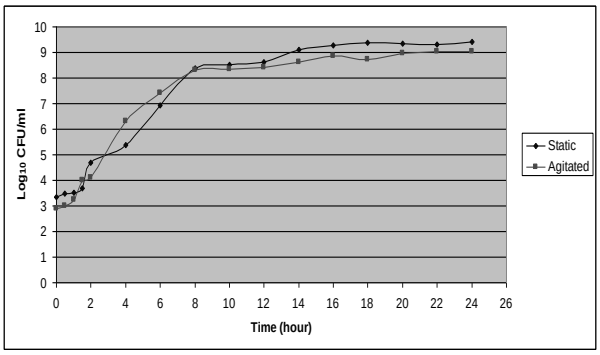

Figure 1: Growth curve of *E. coli* at 37°C using static and agitated cultivation method

From the growth curve of *E. coli*, the time for log and stationary phase were obtained. The results showed that the time taken for log and stationary phase of *E. coli* were 6 and 18 hour respectively. The time for stationary phase was similar with the previous study by [9], where the *E. coli* was grown for 18 hour indicated the stationary phase cells of *E. coli*.

B. Survival and culturability of E. coli under temperature stress

The exposure of the culture that was grown at 37°C to the sublethal temperature stresses (20°C, 40°C, and 45°C) by using TSA media at log phase is shown in Figure 2.

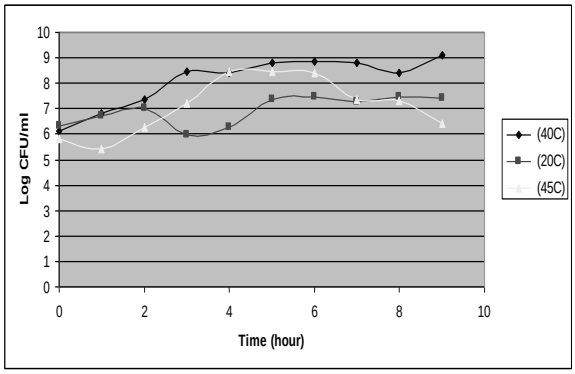

Figure 2: Survival curve of *E. coli* on exposure to temperature stresses at log phase using TSA media.

For the log phase, the cells were grown first at 37°C until reached log phase for 8 hours and it was then shifted to 20°C, 40°C and 45°C. During log phase, the population growth at 40°C was more resistant compared to the other two temperatures (20°C and 45°C). This is because since 40°C is near to the optimum temperature, the population growth was not exactly influenced by sudden shifting of temperature where the cells still can easily adapt the new temperature (40°C).

In Figure 2, results clearly demonstrated that sudden shifting of *E. coli* from 37°C to 20°C reduced the growth rate of *E. coli*. The growth was inhibited for the first 3 hours where cells of *E. coli* were adapted to a new temperature of 20°C and slowly after that *E. coli* was gradually increased the growth with one $\log_{10}$ lower than cells grown at 40°C. During cell growth at 45°C, at the first hour the growth of *E. coli* was decreased because the bacterium was still adapting with the new temperature (45°C) but after the second hour, the cells started to grow and adapted with temperature of 45°C until 6 hour before started to decline. The decline of *E. coli* at 45°C after 6 hours was due to the limitations of nutrient in the broth and due to exposure of high temperature at 45°C continuously.

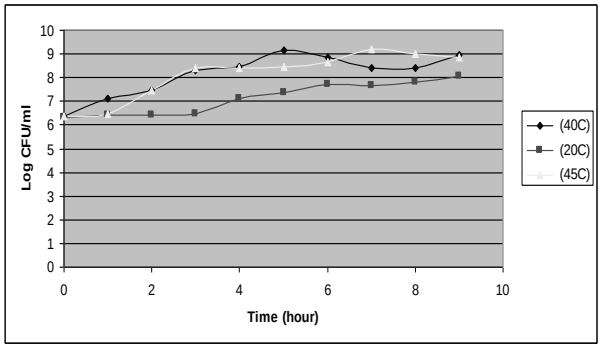

Figure 3: Survival curve of *E. coli* on exposure to temperature stresses at stationary phase using TSA media

The exposures of the culture that were grown at 37°C to the sublethal temperature stresses (20°C, 40°C, and 45°C) by using TSA media at stationary phases were shown in Figure 3. Cells were grown first at 37°C until reached stationary phase for 18 hours and it was then shifted separately at 20°C, 40°C and 45°C. From Figure 3, the population growth at 45°C is more resistant compared to viable cells at 40°C and 20°C. At 45°C, the cells grew rapidly between 1 to 3 hours while the two temperatures of 20°C and 40°C were still adjusting with the new environment and the developments of cells were continued increasingly until 7th hours before the cells started to decline. The ability of cells to delay the multiplication in a new environment illustrates the resistances of cells towards stresses. Meanwhile, the population growth at 20°C was more susceptible compared to the other two stresses temperature and it reduced the growth of *E. coli*. This is due to the lack of growth of *E. coli* cells compared to the growth at 40°C and 45°C. Mild cold shifted from 37°C to 20°C caused a significant decrease in the growth rate of *E. coli* cells [10].

From the Figures 2 and 3, it is concluded that the population growths of *E. coli* at stationary phase were more resistant compare to the log phase. For microorganisms like *Salmonella* and *E. coli*, cells in exponential are more sensitive to the variety of stresses compare to the cells at stationary phase because at this stage the cells are rapidly dividing. The differentiation in resistance between two phases can be explained by the differential expression of biosynthetic pathways, gene regulators and associated enzyme systems that supply an adaptive advantage to the stationary phase cells [11]. Cells at stationary phase are more resistant to numerous of stresses including heat [7]. This is due to the production of alternative sigma factor, σ^s or *RpoS* where these sigma factors is induced in early stationary phase and thus influence the growth of population cells at this phase [7].

C. Percentage Injury of E. coli

The effects of sublethal temperature stresses on sublethal injury for each hour are shown in Tables 1 and 2 for log and stationary phases, respectively. From Table 1, the percentage of injured cells at log phase at three different sublethal temperature 45°C, 40°C and 20°C were ranged between 9.43%-20.40%,

0.95%-19.66% and 1.42%-7.83%. The percentage injury cells at 45°C are the highest at log phase due to the severe temperature because 45°C is near to the maximum temperature of *E. coli*. However, the slowest rate of injury developed at 20°C because 20°C is still in the range of growth of *E. coli*. Therefore, temperature 20°C is not effective to inhibit *E. coli*. The fluctuation of injury was caused by the presences of injured and uninjured cells in the broth were not consistent because the lag duration was varied and the temperatures used were different.

Meanwhile, the percentage injuries (%) at stationary phase for 45°C, 40°C and 20°C were ranged between 3.57%-25.65%, 0.36%-11.16% and 3.16%-13.54% respectively. The highest rate for injured cells in stationary was at 45°C and the slowest rate was at 40°C. The slowest rate at 40°C can be justified because this temperature is near to the optimum temperature which is 37°C where the population of normal cells is high and thus the rate of injury are low. At 45°C, the percentage injury was higher compared to the other two temperatures (20°C and 40°C) with the highest value 25.65% at 9th hour.

Table 1: Percentage sublethal injury cells of *E. coli* at log phase under different temperature stresses

Time (h)	45°C Log	40°C Log	20°C Log
0	0.0%	0.0%	0.0%
1	9.43%	2.64%	5.48%
2	10.69%	4.90%	2.30%
3	16.76%	4.14%	1.68%
4	14.57%	6.76%	1.42%
5	18.09%	7.89%	2.72%
6	20.40%	19.66%	1.61%
7	12.81%	6.00%	6.04%
8	17.62%	0.95%	5.50%
9	10.00%	9.22%	7.83%

Table 2: Percentage sublethal injury cells of *E. coli* at stationary phase under different temperature stresses

Time (h)	45°C Stat	40°C Stat	20°C Stat
0	0.0%	0.0%	0.0%
1	8.04%	9.85%	8.54%
2	17.61%	3.35%	6.70%
3	18.05%	11.16%	4.17%
4	13.76%	2.72%	10.55%
5	14.45%	2.20%	12.62%
6	3.57%	4.77%	6.91%
7	13.14%	0.36%	7.69%
8	13.87%	5.00%	7.28%
9	25.65%	8.93%	13.54%

The results showed that sublethal injury cells at 40°C and 45°C were developed rapidly at log phase than in stationary phase. Expression of specific proteins was higher at stationary phase, and thus, it might influence the susceptibility of cells in log phase which the cells suffer greater damages due to the active metabolism at this phase [12]. That is why the injured cells were higher in log phase compared to stationary phase.

The percentage injuries (%) for *E. coli* cells subjected to 45°C were the highest both in exponential and stationary phases. The study also demonstrated that there were distinct differentiations between 45°C and 20°C or 40°C. Since 45°C is a highest temperature for the growth of *E. coli* and known as severe temperature for this bacterium, the percentage injuries of *E. coli* were higher. Maybe most of the cells that form the colony on TSA not only injured but were poorly recovered on the selective media (TSA + 4% NaCl).

But, it was different with the survival of *E. coli* at 20°C, where the percentage injury log phase is lower than in stationary phase. At log phase for temperature below than 20°C, *E. coli* expresses a number of proteins which are higher compare to the cells that are growing at advanced temperature [13]. The expression of proteins will increase the resistances of cells through stresses [12] and the percentage of injury in cells became lower. For the stationary phase, the lack expression of protein will raise the susceptibility of cells on stresses. Thus, the percentage injury for stationary phase was lower compared to log phase with average 4.013 % at log and 8.116 % at stationary phase. The results suggested that the percentage injury were affected by the bacteria phase and the formation of Heat Shock Proteins (*HSPs*).

IV. CONCLUSIONS

It was found that when shifting *E. coli* from optimal temperature to three sublethal temperature stresses (20°C, 40°C and 45°C), stationary phase of *E. coli* was more resistant to the sublethal temperature stress compared to the population growth of *E. coli* at log phase. Higher percentage injury was obtained during the log phase for 40°C and 45°C. But for 20°C, the higher percentage injury was obtained during stationary phase. Further study is required to investigate the mechanism of stress adaptation by foodborne pathogenic bacteria.

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