Bangladesh J Microbiol, Volume 36, Number 1, June 2019, pp 55-62

Short Communication

Impact of temperature on the growth of *Pseudomonas* spp. (SUBP01), *Bacillus* spp. (SUBB01) and *Salmonella* spp. (SUBS01) and determination of the critical growth temperature

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With a previous observation of the defensive response against elevated temperatures and nutrient variation in *Escherichia coli* in different culture media, present study further demonstrated the consequences of high temperature on the growth of *Pseudomonas* spp. (SUBP01) *Bacillus* spp. (SUBB01), and *Salmonella* spp. (SUBS01). Bacterial growth was measured through the enumeration of the viable and culturable cells on Luria-Bertani (LB) and nutrient agar (NA) plates up to 72 hours, microscopic experiments were conducted to monitor subsequent physiological changes, and finally the loss of culturability due to high temperature stress was further confirmed by the observation of growth incapability through spot tests. A slight reduction in the culturable cells of *Pseudomonas* spp. (SUBP01) was observed at 45°C after 24 to 72 hours of incubation in the LB media while no such inhibition of growth was noticed in the nutrient media. Notably, *Bacillus* spp. and *Salmonella* spp. remained uninfluenced up to 52°C in both media, suggestive of the existence of a stringent defense mechanism against heat shock in these bacterial cells. However, both *Bacillus* and *Salmonella* cells were found to lose their culturablility completely at 53°C, and hence the critical temperature were recorded to be 52°C both for *Bacillus* spp. (SUBB01) and *Salmonella* spp. (SUBS01) while 45°C for *Pseudomonas* spp. (SUBP01) cells. Interestingly, the recovery of cells was noticed upon supplementation of Mg^{2+} and ethylene diamine tetra acetic acid (EDTA) through the increase in the viable and culturable cells at heat stressed condition. Consequently, all 3 bacterial species were subjected to transient heat shock by temperature up-shifting from 37°C to 45°C and 30 °C to 47°C in order to justify the bacterial heat shock response in the phenotypic state, whereby *Pseudomonas* spp. (SUBP01) cells were found lose culturability by temperature up-shifting from 30°C to 47°C, while *Salmonella* spp. (SUBS01) and *Bacillus* spp. (SUBB01) cells remained uninfluenced.

Keywords:Bacterial growth; Heat stress; Bacillus spp; Pseudomonas spp; Salmonella spp.

Bacterial community possesses a surprising adaptability towards the adverse conditions both *ex situ* and *in situ*[1]. Variations in of the environmental factors may bring up several biochemical and metabolic changes within cells through the activation of a set of genes (like the heat shock genes encoding the heat shock proteins, HSPs, general stress response gene like *rpoS* and *rpoE*; the heat shock gene *rpoH*, etc.) turning on or off as and when necessary[1-5]. Indeed a range of study involving bacterial responses (especially in *Escherichia coli*) against stress stimuli including nutrient depletion, high and low temperatures, osmotic shock, oxidative stress, alternation in pH, ethanol concentration, and against the toxic chemicals have been reported so far[3,9-12]. The consequences of the environmental stress factors are generally noticed through the lack of formation of colonies (the colony forming units, CFUs) on the agar plates; increase in the defective and dead cells; and finally the lysis of the dead cells [3,15]. Other than *E. coli*, the study of bacterial stress response has also been investigated in *Pseudomonas* spp., *Bacillus* spp. and *Salmonella* spp. to some extent[19-21]. Survival even by the yeast strain against the environmental factors was also studied[33].

In response to exposure of the bacterial cells under different stress signals, a variety of physicochemical protective mechanisms have been reported within the cells too[4]. Indeed, the stress signals especially in *E. coli* are principally sensed by the increase in the outer membrane porin (OMP) precursors in the periplasm, which are in turn further transduced into the cytoplasm resulting in the activation of the genes essential for the cellular homeostasis[6,23]. The up-regulation of the genes encoding the HSPs during the increase of temperature has also been intensely investigated. To deal with high temperature stress, CspB and CspE are known to be induced in *Bacillus* cells, whereas in *Escherichia coli* and *Salmonella* spp., GroEL and DnaK protein amounts have been

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reported to be elevated[3,8,25]. While such chaperone mediated defense mechanisms varies along different bacterial cells, interestingly a nearly similar mode of cellular biochemical network has been reported in *Pseudomonas* spp. and *E. coli*[19,20].

Our earlier studies revealed the genetic regulation and the influence of the temperature up-shift on the generation of oxidative stress response in *E. coli*[13-15]. The physiological influence of the external and internal oxidative stress in different bacterial cell has been inquired extensively[8,11]. The variations in the nutritional composition in triggering the cellular growth have also been noticed[17]. In cohort with our earlier studies, *E. coli* (SUBE01) and *Pseudomonas* spp. (SUBP01) were found to be devoid of colony forming ability on the agar plates when exposed to oxidative stress which is often related to the raise of temperature[11].

Being inspired from these previous findings on the cellular activities against the oxidative stress, furthermore, this very study attempted to envisage on (1) the investigation of the heat-shock response in bacteria other than *E. coli*, i.e., in *Pseudomonas* spp. (SUBP01), *Bacillus* spp. (SUBB01) and *Salmonella* spp. (SUBS01), (2) distinguishing among the growth patterns of these bacteria in different culture media with variations in nutrient composition, (3) observation of the altered cell morphology and arrangements during heat shock, and (4) finally the demonstration of the growth temperature range (critical and optima) of *Pseudomonas* spp. (SUBP01), *Bacillus* spp. (SUBB01) and *Salmonella* spp. (SUBS01) based on their phenotypic response against a range of high temperatures

Conventional experiments measuring the bacterial growth (cell viability and cultivability) of the laboratory stock cultures of *Pseudomonas* spp. (SUBP01), *Salmonella* spp. (SUBS01) and *Bacillu*s spp. (SUBB01) were conducted on the LB agar, Nutrient broth (NB) and LB broth as described by the previous studies[4,10]. After 24 hour incubation on NA plates at 37°C, one loopful of each of the bacterial culture was introduced into 5 ml NA followed by static incubation at 37 °C for 4-6 hours (pre-culture). After adjusting optical density of the pre-culture at 600 nm (OD_{600}) to 0.1, 30 µL of each of the inoculum was introduced into 2 different sets of 30 ml of NA and LB broth and then incubated at 30°C, 37°C, 45°C, 46°C, 47°C, 50°C, 52°C, 53 °C and 54°C at static condition. For *Psedumonas* spp. the temperature range is limited because the critical temperature for *Psedumonas* spp. was recorded at 40°C[41]. At every 12 hours, the bacterial cell growth was monitored by measuring OD_{600}, and the formation of colony forming units (CFUs) were estimated by counting the colonies up to 72 hours at every 24 hour intervals[14]. All the experiments were performed three times independently. Results were statistically analyzed by determining the P value through t test. Standard deviations were also measured with the aid of statistical hypothesis testing [14].

In order to observe the cell morphology and arrangements in the prescribed conditions, simple staining (Crystal Violet, Hucker's Solution) was applied as previously done[4,24]. Spore staining (malachite green oxalate, safranin O) was conducted to differentiate the bacterial spores from vegetative cells following standard procedures[24]. An aliquot of 10 µl from each of the test bacterial culture suspension was removed at every 12 hour interval, and the shape and organization of the cells were observed under the light microscope (Optima Biological Microscope G206, manufactured in Taiwan) at 1000× magnification[4].

As described previously, each of the bacterial culture suspensions was serially diluted in 9 ml nutrient broth to obtain up to 10^{-4} fold dilution [4,11,14]. From each dilution, an aliquot of 5 µl was dropped over the NA and LB agar plates, allowed to dry off for approximately 15 minutes, and finally the plates were incubated at 37°C for 24 hours. Spotting on the agar was accomplished at every 12 hours of growth.

In both nutrient broth (NB) and nutrient agar (NA) media, no significant changes were observed in the cell turbidity (Figure. 1a, Figure. 1c and Figure. 1e) as well as in the numbers of colonies appeared on the respective plates (Figure. 2a, Figure. 2c and Figure. 2e). All three bacterial species were found to exhibit a steady state of growth at 30°C, 37°C and 45°C up to 72 hours; i.e., an exponential growth rate till 12 hours followed by stasis afterwards. Such a result is not unlikely as was observed in the earlier investigations, possibly due to the intrinsic heat resistance traits in the bacterial cells examined[14,15]. Interestingly, after 24 to 72 hours of incubation, a relatively sharp decline both in the cell turbidity and in the culturable cells was observed in case of *Pseudomonas* spp. (SUBP01) in both LB broth and LB agar at 30°C and 45°C (Figure. 1a, Figure. 2b). Lack of growth hindrance of *Pseudomonas* cells in nutrient media unlike the LB media could be due to the heterogeneous nutrient sources in the nutrient broth or agar LB media. On the contrary, the growth pattern of *Bacillus* spp. (SUBB01) and *Salmonella* spp. (SUBS01) remained unchanged regardless the temperature up-shifting and the length of incubation (Figure. 1d, Figure. 1f, Figure. 2d and Figure. 2f). Hence, the optimal growth temperature for *Pseudomonas* spp. (SUBP01), *Bacillus* spp. (SUBB01) and *Salmonella* spp. (SUBS01) was noted to be 37°C. All the data found in this study were estimated as significant (P = 0.05).

The relative resistance of *Bacillus* and *Salmonella* cells against high temperature might be due to the rapid physiological and genetic reorganizations capable of inducing several heat shock proteins, known to accelerate the development of the multi-stress resistant cells for extensive subsistence[21,23,27]. As stated earlier, *Salmonella* spp. may employ the chaperons GroEL and DnaK against elevated temperature[28]; whereas *Bacillus* spp. has so far been reported to possess 6 classes of heat shock proteins (HSPs) including HrcA and GroE chaperons of the class I category, RsbV, RsbR, RsbW and RsbX of the Class II category (regulated by the alternative sigma factor $\tilde{A}^B$ and $\tilde{A}^F$), Class III HSPs, HtpG of Class IV, HtrA and HtrB of Class V, and finally the VI HSps in order to survive against heat shock[23,26]. This is also to be

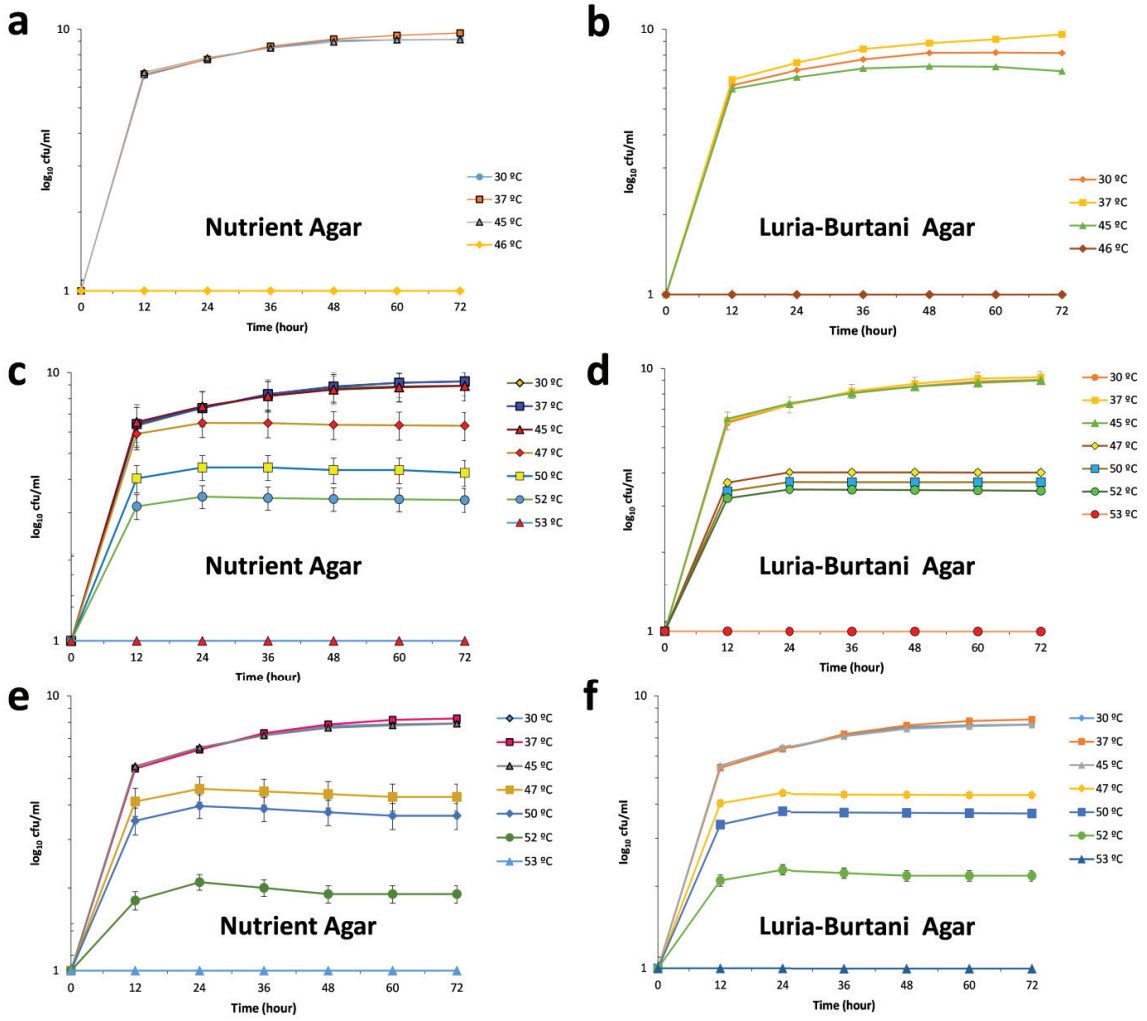

Figure 1. *Assessment of growth variations among Pseudomonas spp. (SUBP01), Bacillus spp. (SUBB01) and Salmonella (SUBS01) spp. at 30°C, 37°C, 45°C, 46°C, 47°C, 50°C, 52°C and 53°C in nutrient broth (NB) and Luria Burtani (LB) broth media. Cells were grown as stated in the Methods section. Growth was determined by estimating the optical density at 600 nm (OD_{600}). Standard deviations for all data have been indicated by error bars.*

mentioned that the heat resistant spores of *Bacillus* spp. are mostly regulated by the transcription of the $\tilde{A}^F$ regulon genes[27].

To get the mechanical insight into the decline in the culturable cells of *Pseudomonas* spp., microscopic studies were conducted to vivid the cellular morphology. No significant morphological changes were observed in case of *Bacillus* spp. and *Salmonella* cells when grown at 30 °C, 37 °C and 45 °C in LB broth and NB up to 72 hours, (Figure. 3). Although *Pseudomonas* cells exhibited regular cellular morphology when they were grown in LB broth at 37 °C; however, they were found to be slightly shrunken at 30 °C (Figure. 3a) and swollen at 45 °C (Figure. 3c). Elongated cells were also noticed in the nutrient broth of *Pseudomonas* spp. at 45 °C (Figure. 3j), while the cells remained unaffected at 30 °C and 37 °C (Figure. 3h and Figure. 3i). In consistent to the current findings under the simulation by heat stress, previously the aerobic bacterium *Pseudomonas* spp. (SUBP01) were found to be stretched to a greater extent on prolonged incubation under the oxidative stress, whereas the obligate aerobe *Bacillus* spp. (SUBB01) and the facultative anaerobic bacterium *Salmonella* spp. (SUBS01) were found to remain unaffected[11].

Under normal conditions, bacterial cell population undergoes stasis after the exponential growth usually after 10-12 hours, and maintains the equilibrium within the viable cells for many days[3,11]. In the current study, as stated earlier, after 72 hours of incubation, *Pseudomonas* cells were found to exhibit a decreased turbidity at 600 nm along with a dramatic reduction in the culturable cells at 30°C and 45°C in LB agar, whereas *Salmonella* spp. and *Bacillus* cells remained uninfluenced. Elimination of growth of

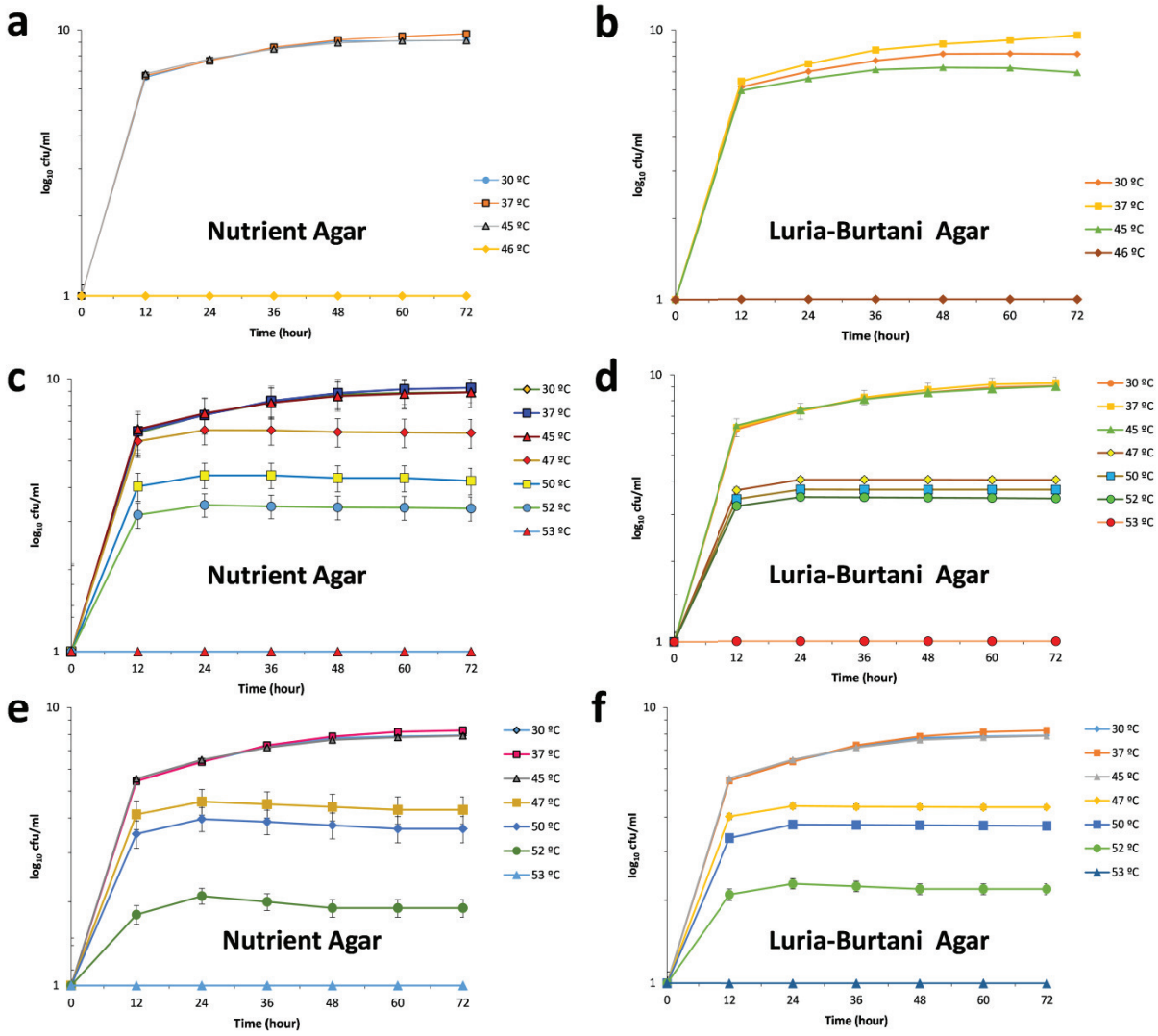

Figure 2. *Assessment of culturable cells in terms of the colony forming units (CFUs) of Pseudomonas spp. (SUBP01), Bacillus spp. (SUBB01) and Salmonella (SUBS01) spp. at 30°C, 37°C, 45C, 46°C, 47 °C, 50C, 52°C and 53°C in nutrient and Luria Burtani (LB) agar media. Cells were grown as stated in the Methods section. Culturable cells were quantified by estimating the CFUs on agar plates. The number of CFUs in Pseudomonas spp. (a, b) was found to be declined completely at 46°C in consistent with the cell turbidity in both agar media. However, Bacillus spp. (c, d) and Salmonella spp. (e, f) showed declined completely at 53°C in both agar media. Standard deviations for all data have been indicated by error bars.*

Pseudomonas cells was further supported by their defective cellular morphology as seen under the microscope. Based on these observations, to confirm such a loss of viability of *Pseudomonas* cells, all the experimental bacteria were subjected to spot tests (results not shown). Dilutions up to 10^{-4} of nutrient broth culture of all the three experimental bacterial species showed a steady growth on nutrient agar at 30°C, 37°C and 45°C (results not shown). In contrast, the growth reduction of *Pseudomonas* spp. was scored after 24 hours incubated in the LB broth in dilutions 10^{-3} and 10^{-4} as well as on the LB agar at 45 °C (results not shown).

The ability of growth of *Bacillus* spp. and *Salmonella* spp. at 45°C drew the interest to investigate the maximum threshold growth temperature for these two bacteria. Both species were found to lose the culturable cells completely at 53°C (Figure. 1 and Figure. 2) and However, sporulation was observed in case of *Bacillus* spp. (SUBB01), when cells were grown at 53°C in both Luria-Bertani and nutrient media (Figure. 3u and Figure. 3g¢). Hence the critical temperatures for both *Bacillus* spp. (SUBB01) and *Salmonella* spp. (SUBS01) were estimated to be 52°C. However, the critical temperature for the *Pseudomonas* spp. (SUBP01) was also determined which was found to be 45°C (Figure. 1 and Figure. 2). It has been demonstrated previously that the expression of the thermotolerant genes of *E. coli,* supporting the bacterial growth and survival at heat stress, might be affected by the cellular exposure to the critical high

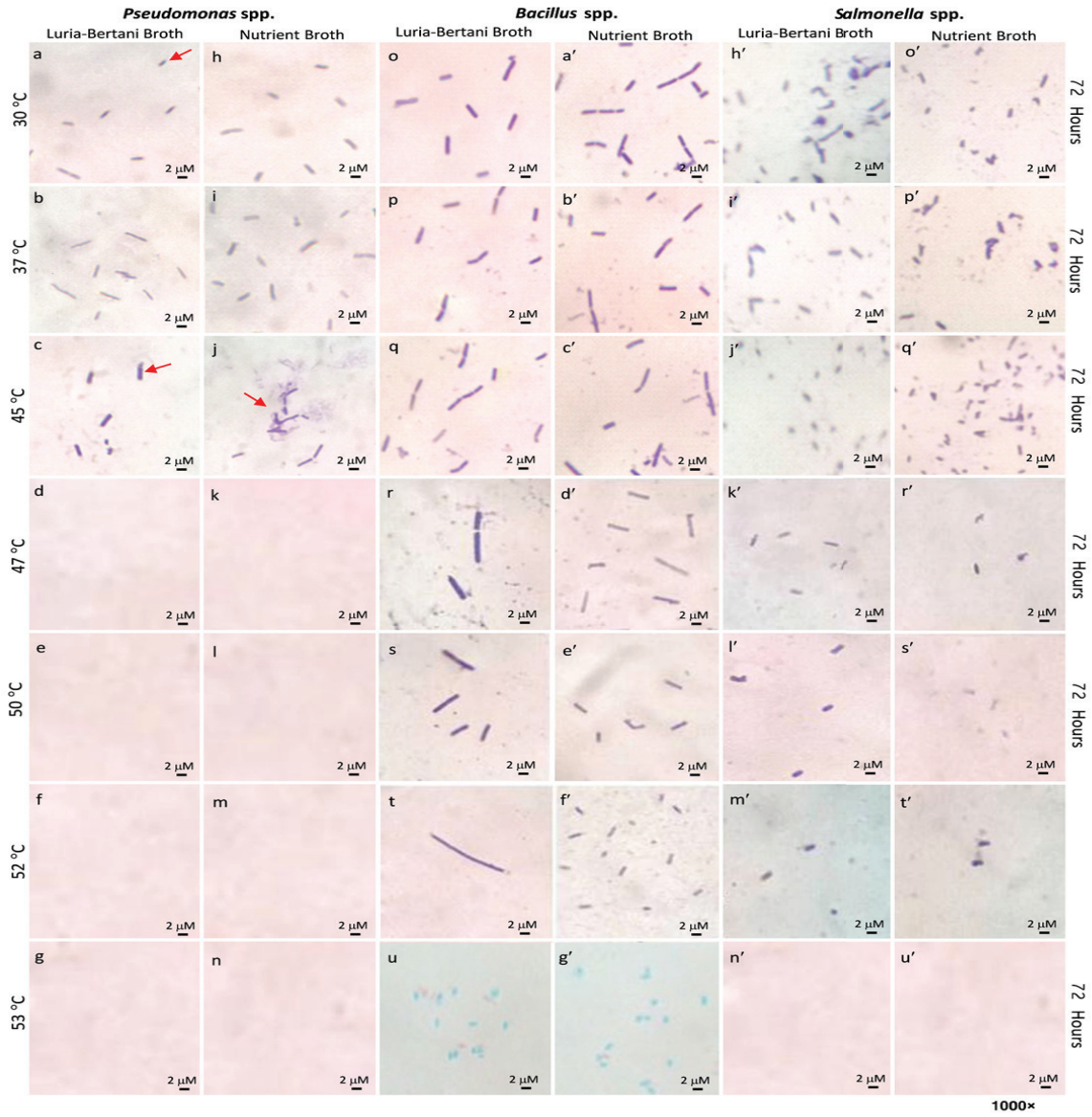

Figure 3. *Morphological observations of Pseudomonas (SUBP01), Bacillus (SUBB01) and Salmonella cells (SUBS01) at 30°C, 37°C, 45°C, 47°C, 50°C, 52°C and 53°C in NB and LB broth media.The morphologically defective cells of Pseudomonas spp. (a-n) was found at 30 °C and 45°C after 72 hours of incubation in the LB broth whereas Bacillus spp. (o-g¢) and Salmonella spp. (h¢-u¢) showed no changes in cell size whatsoever. Experiments were conducted in triplicate independently with the observation of at least 20 microscopic fields each time. The data were reproducible, and one representative data have been shown.*

temperature[36].Based on that fact, the current investigation imparted the detailed phenotypic changes among *Pseudomonas* spp. (SUBP01), *Bacillus* spp. (SUBB01) and *Salmonella* spp. (SUBS01) as a result of heat stress simulation, which might in turn would be of significance in terms of understanding the bacterial survival strategy[3,11].

Another imperative point is to ponder about the possible positve effect on the inhibited bacterial growth at 53°C upon the supplementation of Mg^{2+} and/ or ethylene diamine tetraacetic acid (EDTA) into the growing culture. Earlier research found that Mg^{2+} and EDTA were found to be capable of protecting the outer membrane from cell burst under the adverse conditions[7]. In the current study, when the growing cultures of *Pseudomonas* spp., *Bacillus* spp. and *Salmonella* spp. were treated with 20 mM $MgSO_4$, 5 mM EDTA and 20 mM $MgSO_4$ with 5 mM EDTA at their critical temperatures in LB broth and agar, all the bacterial cells were found to retrieve their growth after certain incubation periods. Such findings generated two facets: (1) Mg^{2+} and the EDTA were confirmed to facilitate the bacterial growth at high temeprature stress, and (2) establishment of the critical growth temperature of the test bacteria used in this study since the absence of Mg^{2+} and the EDTA caused the complete abolishment of the bacterial growth. Therefore, detecting the critical temperature after addition of Mg^{2+} and the EDTA would be of further interest.

The cellular impairment upon high temperature stress further led the interest to notice the impact of sudden induction of

temperature increment on bacterial cells. Thus, in order to achieve the complete phenotypic traits upon heat stress, rapid heat shock was applied along the test bacterial strains by two means: (1) growing the cells at 37°C for 2 hours followed by upshifting the temperature at 45°C; and (2) growing the cells at 30°C for 2 hours followed by upshifting at 47 °C.Earlier investigation revealed that the ÃH regulon, imparting protection against the cytoplasmic thermal stress, can be degraded by several proteases including FtsH, HslVU, and ClpAP during growth at adverse temperature[28]. However, sometimes the expression of *rpoH* gene remains at the transcriptional level via a feedback mechanism by the housekeeping Ã factor [36,45]. Based on this idea, in the current study, the laboratory stock cultures of *Pseudomonas* spp. (SUBP01), *Bacillus* spp. (SUBB01) and *Salmonella* spp. (SUBS01) were subjected to transient heat shock by temperature up-shifting from 37°C to 45°C (Figure. 4) and 30°C to 47°C (Figure. 5) in order to justify the bacterial heat shock response in the phenotypic state. Under these circumstances, *Pseudomonas* cells were found lose culturability, whereas *Salmonella* spp. and *Bacillus* cells remained uninfluenced. In cohort with the previous molecular studies, such results projected in the present study might be suggestive of the heat shock regulation by the housekeeping genes[3]. Besides, the experiments relating the transient heat shock effect also re-confirmed the data achieved as stated elsewhere[14].

Earlier investigations on the laboratory *E. coli* strain (SUBE01) also showed the typical stress response towards heat shock[1,3,8]. Further, the laboratory isolates of *E. coli* (SUBE01), *Bacillus* spp. (SUBB01) *Pseudomonas* spp. (SUBP01) and *Salmonella* spp. (SUBS01) under external and internal oxidative stresses revealed the accumulation of stressed cells which lost culturability with morphological defects while some of them remain uninfluenced[11]. Current investigation further described a detailed phenotypic comparison among an array of bacterial species in response to heat stress and nutrient depletion. Overall, the present study clearly showed that the growth of *Pseudomonas* spp. was suppressed at the lower temperatures (30°C) and drastically abolished at 45°C whereas *Salmonella* and *Bacillus* cells were significantly resistant which is in harmony with the previous suggestive reports on the genetic makeup of heat shock regulon in bacteria.

Despite the lack of molecular investigation, current study demonstrated an illustrative phenotypic comparison among *Pseudomonas* spp. (SUBP01), *Salmonella* spp. (SUBS01) and *Bacillus* spp. (SUBB01) against heat shock in different media. Previously it has been observed that with the increase in temperature, *E. coli* cells are found to lose its culturablity as has also been demonstrated in case of *Pseudomonas* cells in the current investigation. Apart from these two bacterial species, the

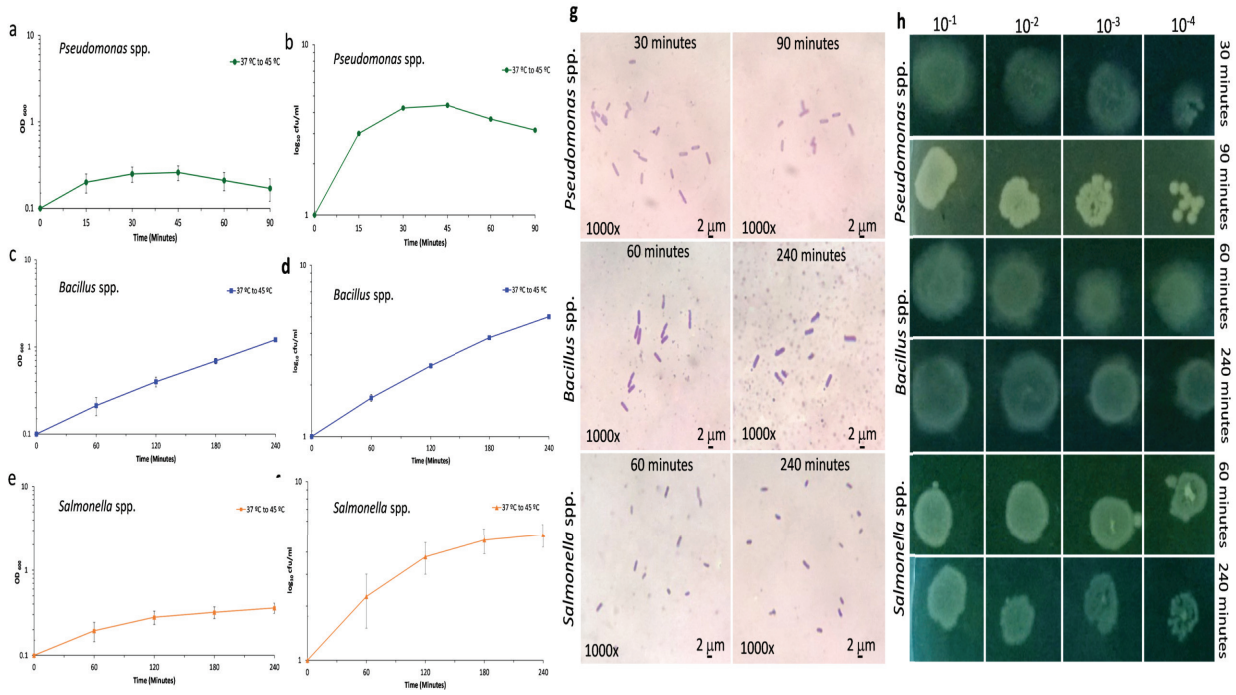

Figure 4. *Detection of transient heat shock response in bacterial species during the up-shift of the incubation temperature from 37°C to 45°C. The heat shock response was determined through the examination of cell turbidity (a,c,e), enumeration of CFU (b,d,f), morphological study (g) and the demonstration of loss of cell cultureability by means of spot test (h). Cells were grown at 37°C for 2 hours in the LB broth and then rapidly shifted at 45°C. Impairment of cellular homeostasis of Pseudomonas spp. (SUBP01) was observed during the transient heat shock by up-shifting the incubation temperature from 37°C to 45°C.*

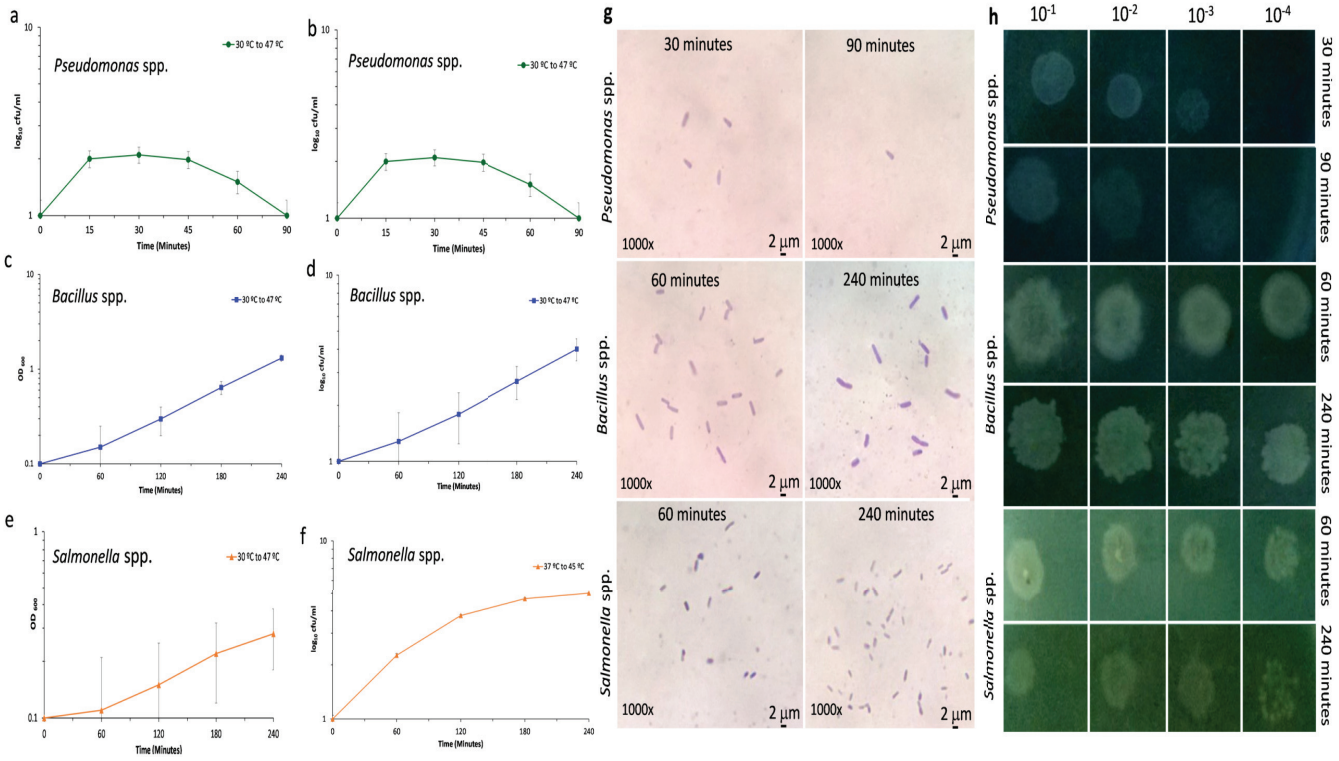

Figure 5. *Detection of transient heat shock response in bacterial species during the up-shift of the incubation temperature from 30°C to 47°C. The heat shock response was determined through the examination of cell turbidity (a,c,e), enumeration of CFUs (b,d,f), morphological study (g) and the demonstration of loss of cell culturablity by means of spot test (h). Cells were grown at 30°C for 2 hours in the LB broth and then rapidly shifted at 47°C. Impairment of cellular homeostasis of Pseudomonas spp. (SUBP01) was observed during the transient heat shock as observed earlier.*

survival traits of *Salmonella* spp. and *Bacillus* spp. reveal that they may respond towards the heat shock by eliciting different cellular stress response mechanisms as described in the earlier studies. An important aspect of the present investigation lies under the findings of the critical growth temperatures of the bacterial cells studied together with their growth behaviors towards transient heat shock.

Acknowledgements

Authors are thankful to the Department of Microbiology, Stamford University Bangladesh for the logistic supports.

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