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Heat shock impact on the growth of *Bacillus* spp. (SUBB01) and its surveillance in minimal medium under shaking condition

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Heat shock proteins (HSPs) are a family of proteins that are produced by all living organisms in response to exposure to stressful condition. The present research aims to study the physiology of *Bacillus* spp. (SUBB01) under aeration in diverse culture media and temperature at 47, 48, 49, 50, 52, 53 and 54°C. Bacterial growth was measured through enumeration of the viable and culturable growing cells that are capable of producing the colony-forming units (CFUs) on Luria–Bertani (LB) and nutrient agar (NA) plates for 24 to 48 h. The work also focused on the repercussion of *Bacillus* spp. against excessive temperatures (37, 45 and 50°C) in minimal media under shaking condition. Isolated microbes were demonstrated under a light microscope to observe their cellular morphology, shape and organization. There was high-temperature shock up to 50°C in both LB, NA, and agar media, with the presence of a demanding defense mechanism against heat shock in these bacterial cells. However, further molecular studies on the genetic makeup of such stress responses as well as the growth retrieval mechanisms of *Bacillus* spp. through the exogenous organic factors would be very important.

Key words: Heat stress, *Bacillus* spp. (SUBB01), viable cells, minimal media, bacterial growth.

INTRODUCTION

Spore-forming pathogenic bacteria known as *Bacillus* spp. are repeatedly found in the environment. *Bacillus* spp. are hostile; they like *Escherichia coli have* with a number of growth defending stress factors like heat shock stress plus nutrient depletion, temperature fluctuation, variation in pH and redox potential (oxidation and reduction), limited water activity (a_w), elevated level of reactive oxygen species (ROS), osmotic disequilibrium

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along with unusual solute concentrations (Givskov et al., 1994; Kabir et al., 2004; Nystrom, 2005; Den Besten et al., 2009; Den Besten et al., 2013). Many of the heat shock proteins (HSPs) have been described as having heat shock, nutrient depletion, osmotic shock, toxic chemicals, and so on. Such HSPs have been reported to be induced in response to several stress factors. During heat shock stress the regulation of HSPs is specifically controlled by a single transcription factor; in eukaryotes, this regulation is accomplished by heat shock factor (HSF) (Klein et al., 2003). To withstand high-temperature stress, it is known as CspB and CspE (cold shock protein B and cold shock protein E) in *Bacillus* cells, and elevated levels of GroEL and DnaK proteins are reported in *E. coli* and *Salmonella* (Mayr et al., 1996).

Similar studies have shown that variation of temperature has an effect on the bacterial growth rates (Murata et al., 2009a, b; Noor et al., 2013). The impulsion of ROS provides hydrogen peroxide (H_2O_2), not just at the beginning of the early stage, but also in the growing culture (Kabir et al., 2004; Murata et al., 2009a; Munna et al., 2013; Nur et al., 2014) and the effect of several aeration speed on the cellular capability to produce the colony-forming units (CFUs) on agar plates (Munna et al., 2014). Above all interpretation, the physiological response of *E. coli* (SUBE01), *Pseudomonas* spp. (SUBP01), *Salmonella* spp. (SUBS01) and *Bacillus* spp. (SUBB01) was determined against oxidative stress through their sustainability in retaining the viable and culturable cells (Nitta et al., 2000; Noor et al., 2009a; Murata et al., 2011, 2012; Noor et al., 2013; Munna et al., 2013, 2014; Nur et al., 2014). Also the accumulation on the defense strategy of these bacteria peculiarly belonging to *Bacillus* spp. (SUBB01) under the static condition was detected through their phenotypic behavior (Nur et al., 2014). The recent investigation was conducted to further anatomize the heat-shock response in *Bacillus* spp. (SUBB01) under shaking condition (100 rpm) in minimal media.

MATERIALS AND METHODS

Bacterial strain, medium and culture condition

In this prominent experiment, the laboratory stock cultures of *Bacillus* spp. (SUBB01) were used for possible findings. Minimal agar (MA) and Minimal broth (MB) as well as nutrient agar, Luria-Bertani agar, nutrient broth and Luria-Bertani broth were used for the exploration of culture ability (Noor et al., 2013). After incubation for 24 h at 37°C in MA plates, one loopful of each bacterial culture was added into 5 ml of MB medium imitated by 100 rpm for 4-6 h (pre-culture) at 37 °C. Each bacterial isolate was introduced into 30 mL of MB and incubated at 37, 45 and 50°C, under shaking condition (100 rpm), after adjusting the optical density of the pre-culture at 600 nm (OD_{600}) to 0.1, 30 µl. At every 12 h interval, bacterial cell growth was observed by measuring at OD_{600} every 12 h, and the formation of CFUs was estimated by counting the bacterial viable and non-viable colonies up to 60 h at every 24-h intervals (Noor et al., 2009b, 2013; Munna et al., 2013, 2014). All the experiments were executed more than three times. Statistical analysis regarding bacterial growth was performed by determining P-value through t-test. The standard deviations were also measured.

Spot test

Each bacterial culture suspension was serially diluted in 9 mL nutrient broth to obtain up to 10^{-4} fold dilution described earlier, (Noor et al., 2013; Munna et al., 2013, 2014). An aliquot of 5 µl was dropped onto the nutrient agar and Luria-Bertani agar from each dilution. Then it was dried off for 15 min, and finally the agar plates were incubated at 37°C for 24 h. Spotting tests were done at every 12 h intervals of bacterial growth (Noor et al., 2013; Munna et al., 2013, 2014).

Demonstration of culturable *Bacillus* spp. (SUBB01) under heat stress

Laboratory stock culture of *Bacillus* spp. (SUBB01) and *E. coli* (SUBE01) were used in this experiment. Demonstrating the bacterial growth considering as cell turbidity (optical density at 600 nm) and CFUs were conducted as described in previous similar studies (Nur et al., 2014). To assay of culture ability of *Bacillus* spp. (SUBB01) and *E. coli* (SUBE01) by growing under the heat stress condition; nutrient agar (NA), Luria–Bertani (LB) agar, nutrient broth (NB) and Luria–Bertani broth were used as per standard policy. One loopful of each bacterial culture was added into 5 ml of MB medium mixed by 100 rpm for 4-6 h (pre-culture) at 37°C. Each bacterial isolates were introduced into 30 ml of MB and incubated at 37, 45 and 50°C at shaking condition (100 rpm), after adjusting the optical density of the pre-culture at 600 nm (OD_{600}) to 0.1, 30 µl. At every 12 h intervals, bacterial cell growth was observed by measuring OD_{600} at every 12 h, and the formation of CFUs was estimated by counting with the bacterial viable and non-viable colonies up to 60 h at every 24 h. All the experiments were conducted by three times. Statistical analysis regarding bacterial growth was performed by determining P-value through t-test. Standard deviations for all data have been indicated by error bars.
Assessing bacterial cell viability was further confirmed by the spot tests intervals (Noor et al., 2013; Munna et al., 2013, 2014; Nur et al., 2014). As delineated previously, each culture suspensions were serially diluted in 9 mL nutrient broth to obtain up to 10^{-4} fold dilution. From each dilution, an aliquot of 5 µL was dropped on to NA and LB agar, dried off for 15 min, and finally the plates were incubated at 37°C for 24 h. Spotting on the agar was accomplished at 24 h of growth.

Microscopic analysis of morphological changes in bacterial strains

Simple staining (Crystal Violet, Hucker's Solution) was used to observe the viability and cellular morphology of bacterial cells as previously described. Spore staining (malachite green oxalate, safranin O) was conducted to differentiate the bacterial spores from vegetative cells following standard procedures. An aliquot of 10 µl from the bacterial culture suspension was removed at 24 h of growth, and the cellular morphology, shape and organization were observed under the light microscope (Optima Biological Microscope G206, manufactured in Taiwan) at 1000× magnification (Munna et al., 2013).

RESULTS AND DISCUSSION

Previous study demonstrates the effective defense

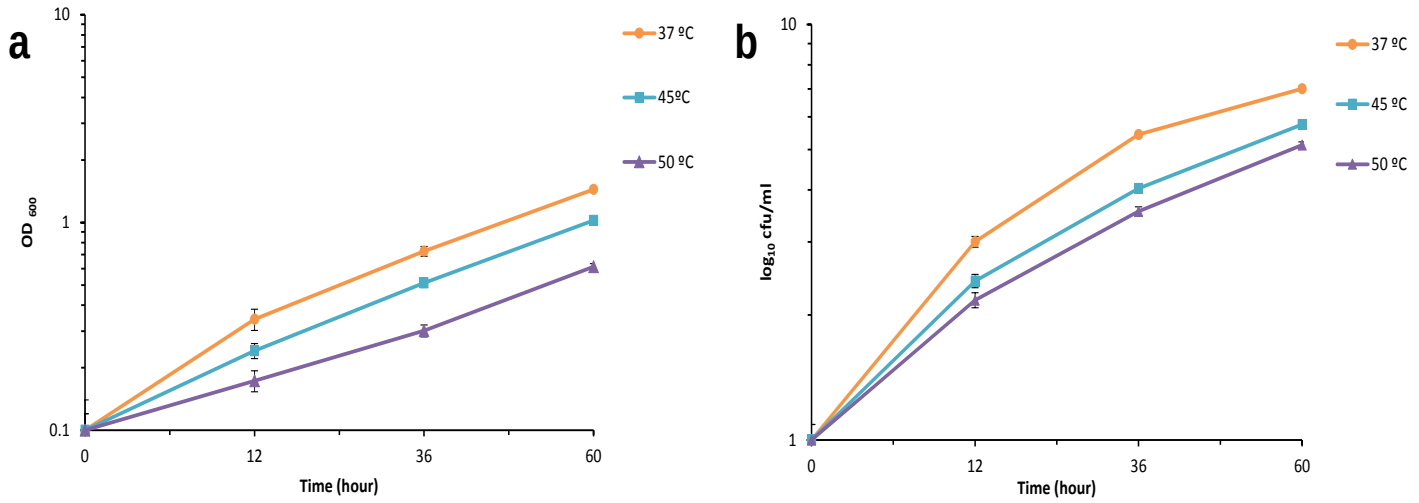

Figure 1. Categorization of growth of *Bacillus* spp. (SUBB01) at 37, 45 and 50°C. (a) Cell turbidity was monitored by measuring cells optical density and (b) Colony-forming units CFU/ml were determined at 100 rpm (rotation per minute) in minimal media.

strategies of *Bacillus* spp. (SUBB01) in response to the oxidative stress unnaturally produced by the supplementation of 6 mM H_2O_2 in the growing culture at an aeration speed of 100 rpm. Interestingly, the culturable cells of *Bacillus* spp. (SUBB01) decreased in their amount when these bacterial cells were compared with a nascent concentration (21 mM) of H_2O_2 (Nur et al., 2014). On the other hand, apart from this stressed condition, current investigation also engaged with a state of heat stress in the *Bacillus* cells since the increase in temperature is known to induce the accumulation of ROS inside the cells. This is turned into losing of cellular viability and culturability of bacterial cell largely (Yamada et al., 2009). In addition, when the *Bacillus* spp. (SUBB01) cells were grown at 37, 45 and 50°C, around 4-log reduction in cell turbidity (Figure 1a) as well as in the generation of the CFUs they were monitored (Figure 1b) up to 24 h of incubation periods in both nutrient and Luria–Bertani (LB) agar and broth. Surprisingly, bacterial growing cells produced CFUs up to 10^2 CFU/mL at 53°C. Interestingly, when the bacterial cells were compared with high temperature at 54°C, a complete excretion in both cell turbidity and CFUs was determined. This indicates the critical temperature for *Bacillus* spp. (SUBB01) is 53°C (Figure 1). Coherent with the results acquired in the growth-related experiments, no morphological changes were observed under the light microscope when cells were susceptible to growth temperatures of 48, 50, 52 and 53°C in both LB and nutrient broth for up to 24 h of incubation (Figure 2). Sporulation was also observed at 54°C. Previous studies showed that the stress of the signaling complex of *Bacillus* spp. is activated in response to several environmental stresses including the housekeeping heat shock stress (Munna et al., 2015).

Demonstration of growth viability and culturability of *Bacillus* spp. (SUBB01) under heat stress

Our previous study demonstrated that *E. coli* strain showed typical response against heat stress and cold stress conditions in minimal media (Noor et al., 2013; Shahriar et al., 2019). The consequence of our recent investigation indeed portrayed the phenotypic behavior of *Bacillus* spp. (SUBB01) in response to heat stress at 37, 45 and 50°C under shaking condition (100 rpm) in minimal media. No significant alterations were observed in cell turbidity (Figure 1a) as well as in CFU, Figure 1b) in minimal media. A steady growth at 37, 45 and 50°C was shown by *Bacillus* spp. (SUBB01) under shaking condition (100 rpm) up to 60 h of incubation periods (Figure 1). All the data found in this study were estimated as significant ($P < 0.1$). Aliquots were removed from respective bacterial cultures. There is no significant morphological or arrangement change observed with heat shock at 48, 50, 52, 53 and 54°C at 100 rpm (a–j). After every 12 to 60 h the *Bacillus* spp. (SUBB01) had steady growth at 37, 45 and 50°C.

The findings of the present investigation are quite consistent with earlier study on the critical growth temperature of *Bacillus* strain depending on the discernible impression of the descriptive nature of research condition. The results distinctly exposed the strong defense scheme of *Bacillus* spp. (SUBB01) in response to the heat shock at 37, 45, 47, 48, 49, 50, 52, 53 and 54°C in minimal media respectively. A quick drop in both cell turbidity and CFUs along with spores was observed after 12 to 24 h of incubation periods when cells were grown at 54°C in both Luria-Bertani and nutrient broth and agar.

In addition, the cells growth and viability spot tests were

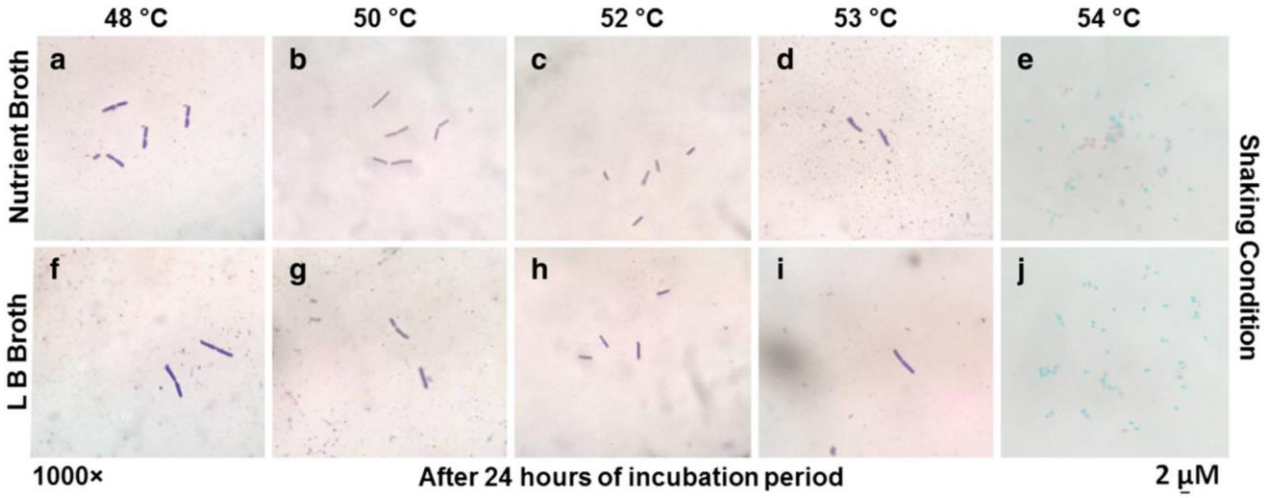

Figure 2. Microscopic analysis (morphology, size and shape) of *Bacillus* spp. (SUBB01) grown cells.

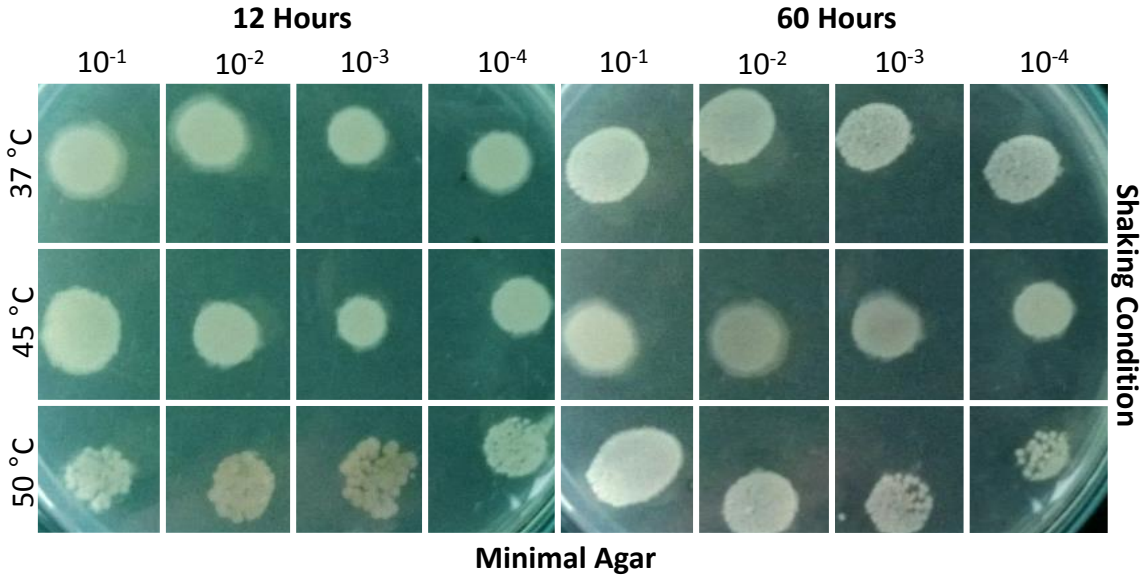

Figure 3. The confirmative manifestation of culturability and survival potential of *Bacillus* spp. (SUBB01) at 37, 45 and 50°C at 100 rpm in minimal agar media.

performed. Systematically, up to 10^{-4} dilution of MB culture, *Bacillus* spp. (SUBB01) on MA at 37, 45 and 50°C showed a steady growth (Figure 3). *Bacillus* spp. have six 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 σ^B and σ^F), Class III HSPs, HtpG of Class IV, HtrA and HtrB of Class V, and finally the VI HSps to survive heat shock (Price et al., 2001; Phillips and Strauch, 2002; Huillet et al., 2012).

However, the present experiment depicts changing the temperatures of growth surveillance that impact *Bacillus* spp. (SUBB01) strain remained uninfluenced. This corroborates with previous reports (Price et al., 2001; Phillips and Strauch, 2002; Periago et al., 2002; Huillet et al., 2012). It is well-known that upon shifting from optimum to high temperatures, *Bacillus* spp. and other bacteria have been found to synthesize increased amounts of the HSPs.

The study also clearly illustrated the phenotypical changes in the bacterial cell caused by the sudden heat stress at the optimum velocity of aeration, which is contemporary unlike any other bacteria such as *E. coli*. These findings are relatively new in the field of heat

shock response on the growth of *Bacillus* cells with their surveillance under shaking condition. Such preliminary findings could be worth increasing the existing knowledge on bacterial cell biology and signal transduction. Spore forming bacteria, *Bacillus* spp. (SUBB01) usually have heat resistance which can be considered to have two components: temperature alteration distinguishing the species and the stabilization conferred by the heat shock state.

The heat shock response in bacteria is a protective mechanism to cope with heat-induced damage to proteins by synthesizing a specific set of proteins known as HSPs (Lindquist, 1986). The alternative sigma factor σ32 mediates the heat shock response. Under stress conditions, an elevated environmental temperature causes a transient increase in *σ*32 transcription and transient stabilization of *σ*32 protein levels, which is usually unstable. The *σ*32 directs transcription of RNA polymerase (RNAP) from the heat shock promoters and, thus, results in the induction of HSPs. Most HSPs behave as molecular chaperones that function to bind to and stabilize non-native polypeptides that are generated during protein synthesis or by heat denaturation of existing proteins, modulate protein folding pathways to prevent miss-folding or aggregation of proteins, and promote protein refolding and proper assembly (Georgopoulos and Welch, 1993). HSPs play a crucial role in this stress response.

Conclusion

Heat stress resistance of bacterial spores such as in *Bacillus* spp. (SUBB01) were compared with the temperature adaptation of each strain as weighed by the optimum and maximum growth temperatures (37 to 54°C) and the heat stress resistance of its vegetative cells. The extensive variation of temperature like sudden heat shock that affected *Bacillus* spp. (SUBB01) was estimated to be 53°C. The findings of the present investigation are quite consistent with earlier study on the critical growth temperature of *Bacillus* strain. The results distinctly exposed the strong defense scheme of *Bacillus* spp. (SUBB01) in response to the heat shock at different temperatures in minimal media. A quick drop in both cell turbidity and CFUs along with spores was observed after 12 to 24 h of incubation periods when cells were grown at 54°C in both Luria-Bertani and nutrient broth and agar. However, further molecular studies on the genetic makeup of such stress responses as well as the growth retrieval mechanisms of *Bacillus* spp. (SUBB01) through the exogenous organic factors would be important.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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