ORIGINAL ARTICLE

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Influence of temperature on the growth of *Pseudomonas putida*

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Lots of reports on the generation of stress by increase in temperature in the bacterial cells especially in *Escherichia coli* has been observed so far. Current study further emphasized such effect on the cells of *Pseudomonas putida* (SUBP03). Conventional methods relating growth assessment of bacteria were employed. The optical density of bacterial cells at 600 nm (OD_{600}) in the minimal broth along with the culturable cells were assessed in the form of colony forming units (CFUs) in the minimal agar media at different temperatures (27 °C, 30 °C, 33 °C, 37 °C and 40 °C). Morphological observations were made to further clarify the bacterial physiology and the spot tests were performed to examine the cell viability. Cells of *P. putida* (SUBP03) were found to grow vigorously at 30 °C, while the growth was found to decline at lower temperature (27 °C) and along with the increase in temperature (at 33 °C, 37 °C and 40 °C). However, the morphological changes were insignificant. Furthermore, cells were noticed to completely lose culturability at 40 °C after 48 hours.

Key words: Critical growth temperature; High temperature stress; Optimum growth temperature; *Pseudomonas putida*; Viable but non culturable (VBNC) cells

Pseudomonas species are well–known rod–shaped, flagellated, gram–negative bacterium (1, 2). However, *Pseudomonas* often persisting in the environment like *Escherichia coli*, which may face many growth retarding stress factors such as nutrient depletion, temperature fluctuation, variation in pH and redox potential, limited water activity (aw), elevated level of reactive oxygen species (ROS), osmotic imbalance together with anomalous solute concentrations, etc. (3–23). Notably, bacteria were found to utilize unified strategies to deal with the environmental stress (3–5, 24–26). Nevertheless, several studies apparently suggested the expression of the global molecular chaperones, which maintain the cellular homeostasis, including *rpoE, rpoS* and *rpoH* genes in *E. coli*; *dnaK, dnaJ*, and *grpE* in *Pseudomonas* spp.; CspB and CspE in *Bacillus* spp. Cells; and GroEL and DnaK proteins in *Salmonella* spp. (3–5, 7, 8, 10, 12, 13, 19, 21–23, 27–40).

In acquiring stress, our earlier studies revealed four independent aspects, wherein (i) the influence of the temperature up–shift on the generation of oxidative stress (19, 22, 37); (ii) impulsive accretion of the reactive oxygen species (ROS) at the early stationary phase of bacterial growth (12), (iii) the origination of oxidative stress upon supplementation of the oxidative agent, H_2O_2 (21, 23) and (iv) the hindering effect of different aeration speed on the formation of colony forming units due to the suggestive endogenous oxidative stress (22).

In all instances, the physiological influence of the external and internal oxidative stress (12, 18, 22, 23) and heat stress (unpublished) in *E. coli* (SUBE01), *Pseudomonas aeruginosa* (SUBP01), *Pseudomonas fluorescens* (SUBP02), *Bacillus* spp. (SUBB01) and *Salmonella* spp. (SUBS01) has been inquired well and evidently brought the new information on the defense strategy against stress and the ascertainment of their critical growth temperature (unpublished) as observed through their sustainability in growth pattern. These previous findings led us to extend the research interest in other bacterial cells primarily to assess the optimum and critical growth temperature of our laboratory stock culture of *Pseudomonas putida* (SUBP03).

MATERIALS AND METHODS

Conventional experiments measuring the bacterial growth were conducted as described earlier by Nur et al. (23). Laboratory stock cultures of *Pseudomonas putida* (SUBP03) was used in this study. Minimal media (dextrose 1.0 g/L, dipotassium phosphate 7.0 g/L, monopotassium phosphate 2.0 g/L, sodium citrate 0.5 g/L, magnesium sulfate 0.1 g/L and ammonium sulfate 1.0 g/L) for both agar (MA) and broth (MB) were used for the assay of the bacterial culturability (19). After 24 hour incubation on minimal agar plates at 37 °C, one loopful of each of the bacterial culture was introduced into 5 ml minimal broth followed by 100 rpm (rotation per minute) 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 each was introduced into 2 different sets of 30 ml of minimal broth and incubated at 27 °C, 30 °C, 33 °C, 37 °C and 40 °C at shaking condition (100 rpm). At every 12 hours 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

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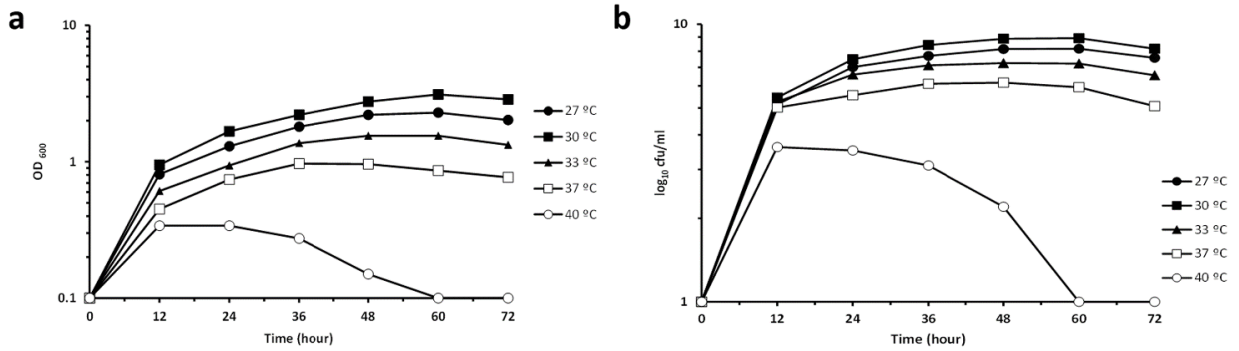

FIG. 1. Assessment of growth of *Pseudomonas putida* (SUBP03) at 27 °C, 30 °C, 33 °C, 37 °C and 40 °C in terms of OD_{600} (a) and CFU (b) in shaking condition up to 72 hours. Notably, when the *P. putida* (SUBP03) cells were grown at 27 °C, 33 °C and 37 °C, substantial reduction in cell turbidity (a) as well as in the generation of the colony forming units (CFUs) were observed (b) up to 72 hours of incubation periods in minimal media. Interestingly, a dramatically reduction in both cell turbidity and colony forming units (CFUs) were observed when the cells were challenged at 40 °C after 12 hours to 72 hours of incubation periods.

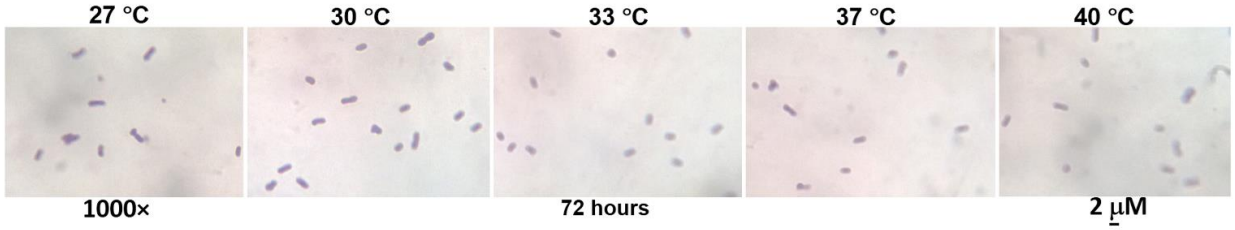

FIG. 2. Morphological changes of *Pseudomonas putida* (SUBP03) cells at 27 °C, 30 °C, 33 °C, 37 °C and 40 °C after 72 hours of incubation periods. Surprisingly, no morphological change was observed under light microscope.

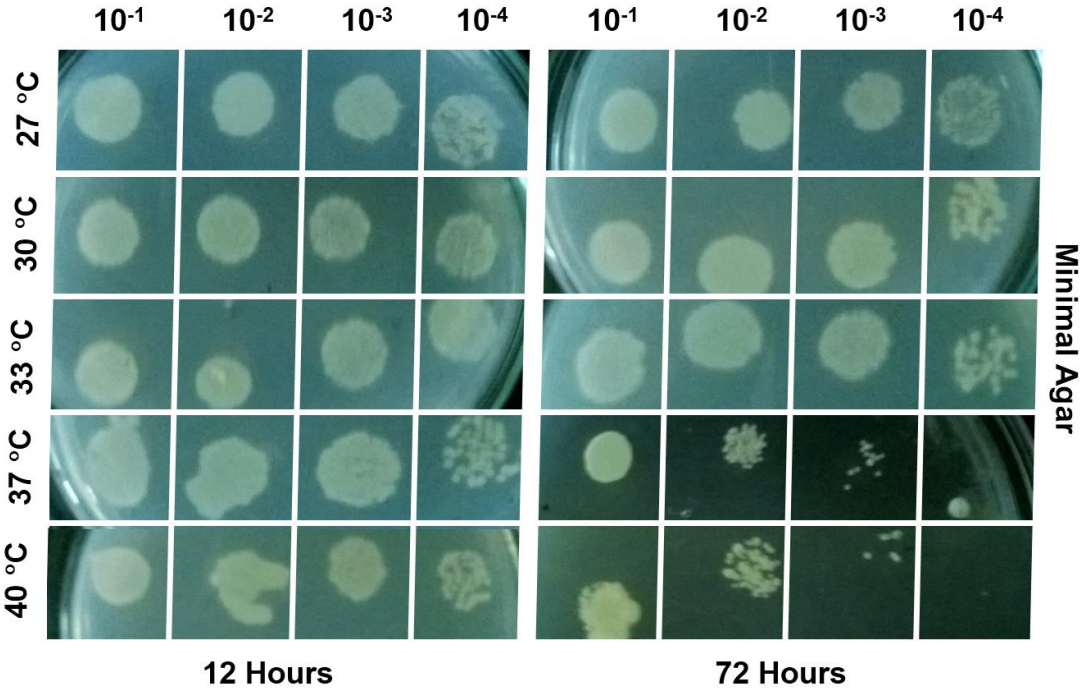

FIG. 3. Confirmative demonstration of culturability and survival potential of *Pseudomonas putida* (SUBP03) through spot tests in minimal agar media at 27 °C, 30 °C, 33 °C, 37 °C and 40 °C temperature after 12 hours and 72 hours of incubation periods. Notably, a relatively slower growth was observed when bacterial cells were grown at 37 °C and 40 °C on minimal agar after 72 hours of incubation periods.

(19, 21-23).

For the observation of cell morphology and arrangements, simple staining (Crystal Violet, Hucker's Solution) was applied as previously done (21-23). An aliquot of 10 µL from each bacterial culture suspension was removed by 12 hours intervals and the shape and organization of cells were observed under light microscope (Optima Biological Microscope G206, manufactured in Taiwan) at 1000× magnification (21). Finally spot tests were conducted to further confirm the bacterial viability under temperature stress. 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 (19, 21-23). From each dilution, an aliquot of 5 µl was dropped on to the minimal agar, dried off for 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 (19, 21-23).

RESULTS AND DISCUSSION

***Pseudomonas putida* (SUBP03) grows best at 30 °C.** The optimum growth temperature for *Pseudomonas putida* (SUBP03) was assessed through the measurement of optical density of bacterial cells at 600 nm (OD_{600}) in the minimal broth along with the detection of culturable cells in the form of colony forming units (CFUs) in the minimal agar media up to 72 hours. After 12 hours of incubation at 30 °C the cell number was found to be increase rapidly by approximately 4 logs (Figure 1), which was comparable to those cells grown at 27 °C, 33 °C, 37 °C and 40 °C. Surprisingly, under the light microscope, no morphological change was observed (Figure 2). Besides, a steady growth was noticed through spot at 27 °C, 30 °C and 33 °C (Figure 3). Hence, the optimum temperature of our test bacterial strain of *Pseudomonas putida* (SUBP03) was recorded to be 30 °C. Present finding is quite consistent with the earlier studies where *P. putida* has been noticed to exhibit highest growth at 30 °C too (3–5).

Critical growth temperature of *P. putida* (SUBP03) was recorded to be 40 °C. The inability to grow at 37 °C led our interest further to examine the critical growth temperature for the test bacterial strain of *Pseudomonas putida* (SUBP03). While a gradual decrease was observed in both CFU and the cell turbidity (Figure 1), when the cells were grown at 40 °C after 12 to 72 hours of incubation periods, respectively, a relatively shower growth was noticed through spot, when bacterial cells were grown at 40 °C (Figure 3), whereas a complete growth cessation was observed at 41 °C (data not shown). Previously, the maximum growth temperatures of *Pseudomonas putida* were recorded to be 35 °C by Balows et al. (3). In the current study, *Pseudomonas putida* (SUBP03) were found to lose the culturability completely at 41 °C. Hence the critical growth temperature of this *Pseudomonas putida* was recorded to be 40 °C, which is indeed corroborating with the data achieved as stated in the earlier investigation (4).

CONCLUSION

In a separate study, the oxidative stress events were investigated within *P. aeruginosa* (SUBP01) and *P. fluorescens* (SUBP02) against the oxidant 3 mM H_2O_2 in a concentration (unpublished). The findings of the current study revealed the heat shock state in case of *P. putida* (SUBP03), which may further increment the existing knowledge on the stress response in *Pseuomonas* spp. However, the limitation of this study underlies the lack of study in genetic level with the presentation of preliminary data. However, detection of the optimal and critical growth temperatures of *P. putida* (SUBP03) may draw interest within the closely related fields. Nevertheless, the expressional analyses of certain heat shock genes are worth to understand the detailed scenario as well as to complete the current investigation.

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