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Research Article
Transformation of Heat Stressed Non-culturable *Bacillus cereus* Cells by Extracellular Extracts from *Pseudomonas aeruginosa*

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Abstract

Background and Objective: Study of bacterial stress response against heat shock has been well accomplished so far. Present study further investigated on the ability of specific heat stressed cells to be in the culturable form (i.e., cells those regained the colony forming ability on agar). **Materials and Methods:** Current study focused on the response of *Bacillus ceresus* cells when exposed to extremely highly temperature like 60°C (comparable with that grown at 37°C) using the traditional microbiological cultural methods including the enumeration of culturable cells as well as the spot tests. **Results:** While it was noticed that the cells become non-culturable at 60°C, interestingly a fraction of those cells were found to be culturable upon the supplementation of the extracellular fractions of *Pseudomonas aersuginosa*. **Conclusion:** The growth revival of *B. Cereus* from the viable but nonculturable (VBNC) state into the culturable cells is interesting to ponder the bacterial behaviour against stress signals. However, cells initially incubated 60°C were found to be devoid of forming the colonies on the agar.

Key words: Heat shock, *Bacillus cereus*, viable but nonculturable (VBNC), *Pseudomonas aeruginosa*, growth revival

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Study of the stress signal (variations in nutrient concentrations, temperature fluctuation, elevated levels of reactive oxygen species or ROS, etc.) transduction and the genetic makeup of the bacterial stress regulons and the investigation on the stress dealing chaperons and sigma factors have been conducted extensively so far[1-12]. As the first impact of the stress, the bacterial cells are likely to become non-culturable; they may become viable, but non-culturable (VBNC) and as a result they can't form the Colony Forming Units (CFUs) on the agar plates[13-16]. During heat stress, an array of heat shock genes in bacteria are activated which encode the corresponding Heat Shock Proteins (HSPs) in consortium of the activities of various sigma factors (s^S, s^E, s^H and s^E) as studied in bacteria and yeasts[16-19].

Bacterial VBNC state is quite interesting to study and such condition is sometimes considered to be one of the bacterial survival strategies under adverse conditions[4,9,16,20]. Interestingly some groups investigated on the resuscitation of the VBNC cells to the culturable forms especially in case of *E. coli*[21]. One of our recent studies showed that when the heat stressed culture of *E. coli* non-culturable cells were supplemented the extracellular fractions of *Bacillus* spp., the *E. coli* cells were capable to retrieve their growth, i.e., they became culturable from the VBNC state[4]. Another study showed the growth revival of *Pseudomonas aeruginosa* at a relatively high temperature when supplemented with the extracellular fractions of *Bacillus* species[5]. Along this line, present study further investigated the retrieval of the nun-culturable cells of *B. cereus* at 60°C to the culturable form with the supplementation of the extracellular fractions of *P. aersuginosa*.

MATERIALS AND METHODS

Study area: The study was carried out in the Laboratory of Microbiology, School of Life Sciences (SLS), Independent University, Bangladesh (IUB) from June, 01, 2019-December, 15, 2019.

Bacterial strains and culture media: Traditional microbiological methods of determining the culturable bacteria (i.e., by enumerating the CFUs) were applied. Luria-Bertani (LB) agar and broth were used both for the enumeration of CFUs and for conducting the spot tests[3,4]. After 15 h of aerobic incubation on LB plates at 37°C, one loopful of *B. cereus* culture was introduced into 3 mL LB followed by incubation at 37°C for 4 h at 100 rpm (preparation of pre-culture). After adjusting optical density of the pre-culture at 600 nm (OD_{600}) to 0.1, 30 µL of the *B. cereus* inoculum was introduced into each of the total of eight 100 mL conical flasks each containing 30 mL of LB broth.

Preparation of the extracellular extracts of Pseudomonas aeruginosa: One loopful culture of the laboratory stock cultures of *P. aeruginosa* was introduced into 3 mL LB followed by incubation at 37°C for 4 h at 100 rpm to make the pre-culture. After adjusting the OD_{600} to 0.1, 30 µL of the *P. aeruginosa* inoculum was introduced into 30 mL of LB broth which was incubated for 24 h at the condition stated above. After the OD_{600} reached to within 3-4, the culture was centrifuged at 5000 rpm for 5 min and the medium fraction (i.e., the extracellular extracts) was collected for the further study.

Examination of *B. cereus* growth retrieval: Retrieval of culturable cells was monitored using the method as we have conducted in our earlier studies[3,4]. Liquid cultures (8 flasks consisting of 30 mL LB media) of *B. cereus* cells were incubated for 2 h at 37°C and was shifted to 0, 55 and 60°C (2 flasks each; i.e., total 6 flasks) while two were kept incubated at 37°C. After incubation for 4 h, enumeration of culturable cells (the CFUs) was conducted. At this stage all the cells grown at 60°C were found to be non-culturable. In the next step, all the cultures already grown at 0, 37 and 55°C were shifted to 60°C (2 flasks each; i.e., total 6 flasks) and the extracellular extracts from *P. aeruginosa* were added to one set of these cultures (3 flasks) as well as to one of the 2 cultures already kept at 60°C. After an hour of incubation, enumeration of culturable cells was conducted for both the *Pseudomonas* extracts added cultures (3 flasks) and the non-added ones (3 flasks).

Assay of culturability: For the assay of Colony Forming Units (CFUs), each of the aliquots were serially diluted in 9 mL LB broth up to 10^{-8} and 100 mL was spread onto the LB agar from 10^{-4}, 10^{-6} and 10^{-8} dilutions. After drying plates were incubated aerobically at 37°C for 15 h. For conducting the spot tests, each of the *B. cereus* culture suspensions were diluted to obtain up to 10^{-4} fold dilution from which an aliquot of 5 µL was dropped onto the surface of the LB agar[4,5]. After drying off, the plates were incubated at 37°C for 12 h. All the experiments were performed three times independently and the results were carefully deduced to ponder the reproducibility.

RESULTS

The resuscitation of the nonculturable cells to the culturable cells is actually obscure since the specific non-culturable cell couldn't be detected to be transformed into its culturable form. However, grossly the fractions of the non-culturable cells were noticed to produce the CFUs only when the extracellular extracts of *P. aeruginosa* were added (Fig. 1, 2). As shown in Fig. 1, when the *B. cereus* culture was shifted from 55-60°C, all the cells lost culturability. However, when the extracellular extracts of *P. aeruginosa* (denoted by Sin Fig. 1) were added, the enumeration of CFUs was just 0.5-log less than that formed in 55°C. Similar scenario was noticed in case of shifting the culture from 37-60°C. Normally at 37°C, the CFU count was more than 10^5 which became nil when shifted to 60°C. However, when the extracts were added at 60°C, surprisingly the CFU level elevated more than 10^4, revealing the growth retrieval phenomenon.

Following the same way, when the culture was shifted from 0-60°C, all cells immediately became non-culturable which revived again upon the supplementation with the extracellular extracts of *P. aeruginosa*.

These results were further confirmed by employing the spot tests which clearly showed the appearance of culturable cells upon the supplementation with *Pseudomonas* extracellular extracts (Fig. 2). Diluted aliquots were employed from the same culture media to maintain the reproducibility of the method as well as the validity of the results. The appearances of the spots were in the accurate correlation with CFUs formation as shown in Fig. 1. At 60°C, only those cells has grown which got the extracellular extracts of *P. aeruginosa* as supplement. Thus, the spot test results further supported the process of being culturable from the non-culturable state at extremely high temperature where the cells initially got non-culturable.

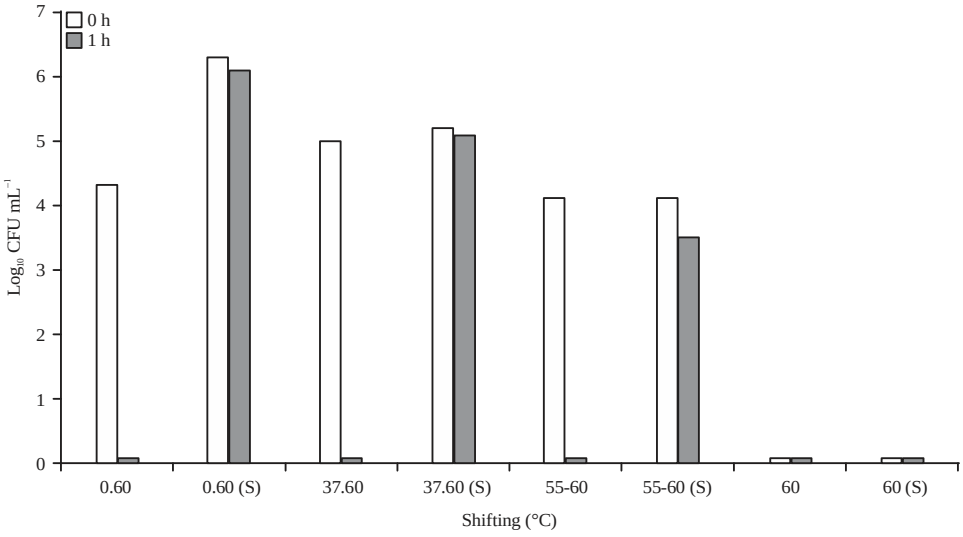

Fig. 1: Growth retrieval of *Bacillus cereus* when supplemented with the extracellular extracts of *Pseudomonas aeruginosa* after shifting the corresponding cultures from 0-60, 37-60 and 55-60°C

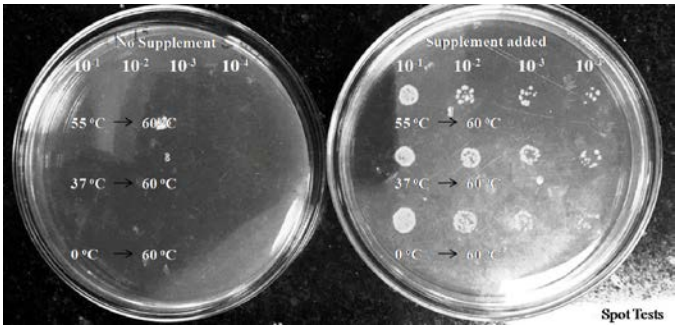

Fig. 2: Confirmation of the growth retrieval of *B. cereus* through spot tests

DISCUSSION

The findings of the current study principally adhered to 1 the formation of the non-culturable cells of *B. cereus* at 60°C and to 2 the subsequent revival of a fraction of these cells to the culturable form (capable of growth on agar) upon addition of the extracellular extracts of *P. aeruginosa*. Bacterial management of heat stress has been largely studied by several groups to chalk out the molecular mechanism underlying such resistance[8,16,19]. Moreover, among all other bacteria, the *Bacillus* cells are largely known to be resistant against heat stress[17]. Present study dealt with the VBNC cells, dead cells and finally the possible transformation of the VBNC cells into the culturable cells employing transient heat shock on *B. cereus* cells. The results clearly pointed growth retrieval of *B. cereus* when supplemented with the extracellular extracts of *P. aeruginosa* after shifting from 0, 37 and 55-60°C. The outcome of heat stress is enormously projected through the formation of both VBNC cells and the dead cells. Indeed a fraction of the VBNC cells and especially all the dead cells have been shown to undergo s^E-dependent lysis which further serve as nutrients for the remaining population in the culture[4-6]. Therefore, the resuscitation of the *Bacillus* cells in the current study can be explained by the transformation of the VBNC into the culturable cells since they are expected to utilize the extracellular materials from *P. aeruginosa* as nutrients and on the contrary the non-culturability of the initially incubated *Bacillus* cells at 60°C can be explained as the transformation of all the VBNC cells into dead cells which in turn could show no positive effect on the cellular culturability even there was s^E-dependent lysis of the dead cells. However, such complete absence of VBNC cells (which actually turned into dead cells) due to prolonged incubation at 60°C may be further confirmed by fluorescence staining methods as well as with molecular biological approaches[9,10].

SIGNIFICANT STATEMENT

This study discovered the growth retrieval of bacteria facing heat stress upon certain condition, in this case, with the addition of the extracellular extracts from different bacterial species which is expected to serve as nutrients or more specifically as stimuli triggering such transformation into culturable state from the non-culturable form. Such an interesting aspect can be useful to study the bacterial stress response in aquite fine tuned way regarding the necessary biosignals required for bacterial growth and survival in adverse conditions. In other words, this study would further aid to unravel the bacterial stress response completely if the additional molecular aspects are resolved. Thus a new facet can be projected in the field of research based on the bacterial stress response.

CONCLUSION

The induction of the non-culturable fraction of *B. cereus* cells to the culturable form with the addition of the extracellular extracts from *P. aeruginosa* showed a clear evidence of a possible phenomenon of the bacterial growth retrieval upon stressed condition. However, further study regarding the expression of the stress responsive genes would complement the current findings on the molecular basis.

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