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Original Article

Possible Growth Retrieval Simulation on the Heat-Stressed *Pseudomonas aeruginosa* (SUBP01) Cells

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Earlier observation of cellular damage of *Pseudomonas aeruginosa* (SUBP01) by the extrinsic oxidative agent (H_2O_2) and the assumptive accumulation of the reactive oxygen species (ROS) through aeration further led to the growth retrieval assay of the bacterium. Current study investigated the influence of the inorganic growth stabilizers (20 m*M* $MgSO_4$ plus 5 m*M* ethylenediaminetetraacetic acid, EDTA) and the organic extracellular fractions of the heat-resistant *Bacillus* species (SUBB01) and *P. fluorescens* (SUBP02) on the growth pattern of *P. aeruginosa* at 45° and 46°C. Bacterial growth (cfu) was measured on the minimal agar plates up to 72 hours, microscopic experiments to monitor the subsequent morphological changes, and finally by the spot tests to examine the cell viability under the given heat stressed conditions. As expected, growth revival was observed at both 45° and 46°C after 48 to 72 hours, upon supplementation with inorganic growth stabilizing agents and the extracellular fractions (secretions) of *P. fluorescens* compared to that of the control; *i.e.*, untreated with inorganic or organic supplements. Interestingly, compared to that of *P. fluorescens* extracellular extracts, no positive impact on growth retrieval was noticed by the application of the extracellular fractions of *Bacillus* species.

Keywords: *Bacillus* spp. (SUBB01), *Pseudomonas aeruginosa* (SUBP01), *P. fluorescens* (SUBP02), Heat-stress, Growth retrieval

Introduction

Pseudomonas species are common Gram-negative, rod-shaped bacteria prevailing the environment, where they may undergo unfavourable environmental circumstances including heat shock, oxidative stress, nutrient depletion, divergence within the optimal pH, restricted water activity (a_w), osmotic imbalance, etc.[1-5]. In response to such stress signals, an array of molecular chaperones, principally CspB and CspE in *Bacillus* spp., GroEL and DnaK proteins in *Salmonella* spp., RpoE, RpoS and RpoH proteins in *Escherichia coli* and *Pseudomonas* spp., has been reported to be expressed[6-19]. While such chaperone mediated defense mechanisms may vary along different bacterial cells. Interestingly, a remarkably strong defense strategy has been noticed in *Bacillus* spp. and *Pseudomonas fluorescens*[12-13,20-21]. The heat shock stimulon of *Bacillus* spp. is the most complicated ever described in bacteria, encoding around 200 heat shock genes, regulated by at least 6 different mechanisms depending on species[20-24]. Notably, *P. fluorescens* revealed the frequent occurrence of extracytoplasmic function (ECF) sigma (ó) factors, which serve as important regulatory defensive agents in response to the stress signals including the elevated temperature, irradiation, alteration in pH and the redox state, reactive oxygen species (ROS), toxic compounds, and nutrient depletions[1-5,17,25-27].

In our earlier studies, the cellular impact in response to the external and internal oxidative stresses[16-17,25-27] and heat shock (unpublished) in *E. coli*, *Pseudomonas* spp., *Bacillus* spp. and *Salmonella* spp. has been inquired well and evidently brought the new information on the defense strategy within *Bacillus* spp. and *Salmonella* spp. as observed through their sustainability in producing the colony forming units (CFUs). Under high aeration speed at 200 rotations per minute (rpm), with a concomitant elevation of internal oxidative stress, *E. coli* and *Pseudomonas* cells were found to loss viability, while *Bacillus* spp. remained uninfluenced[26]. Notably, a heterogeneous population was observed within the *Pseudomonas* spp. whereby a few unstressed cells evolved, which were later identified as *P. fluorescence* cells leaving the *P. aeruginosa* fractions as stressed cells[26]. In another study, supplementation of the hydrogen peroxide (H_2O_2, an external oxidant) was found to retard the growth of *E. coli* and *Pseudomonas* spp., whereas *Bacillus* spp. were found to be unaffected[27]. Such findings were quite interesting especially regarding *Pseudomonas* spp. since lots of stress related investigations have been chalked out within *E. coli* cells.

Based on such rationale on the identification of the heat resistant *Bacillus* and *P. fluorescence* cells, current study further endeavoured to scrutinize whether the supplementation of the

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extracellular growth enhancing factors of *Bacillus* spp. (SUBB01) or *P. fluorescence* (SUBP02) in the medium fraction could retrieve the growth of heat stressed *P. aeruginosa* cells (SUBP01), grown at high temperatures up to 46°C. Moreover, earlier studies reported that Mg^{2+} (in the form of $MgSO_4$) and ethylenediamine tetraacetic acid (EDTA) were capable to protect the outer membrane from the incidence of cell burst within *E. coli* upon heat shock as well as to protect the subsequent damage imposed on proteins and DNA[19,28-29]. Thus, besides the impact of organic supplements, the effect of the inorganic growth stabilizing agents ($MgSO_4$ and EDTA) was also investigated to bring about the comparative effects on bacterial cellular growth retrieval.

Methods and Materials

Bacterial stain, medium, and culture conditions

Laboratory stock cultures of *Bacillus* spp. (SUBB01), *Pseudomonas aeruginosa* (SUBP01) and *P. fluorescens* (SUBP02) were used in this study. Experiments demonstrating the bacterial growth in terms of cell turbidity (optical density at 600 nm, OD_{600}) and colony forming units (cfu) were conducted as described earlier[27]. Minimal agar and broth (Sigma-Aldrich, USA) were used for the assessment of culturability[7]. After 24 hour incubation on nutrient agar plates at 37°C, one loopful of the bacterial culture was introduced into 5 ml nutrient broth followed by incubation at 37°C for 4-6 hours at 100 rpm (pre-culture). After adjusting optical density of the pre-culture at 600 nm (OD_{600}) to 0.1, 30 µl each was introduced into 3 different sets of minimal agar broth (30 ml) and incubated at 45° and 46°C. At every 12 hours interval, the cell growth was monitored by measuring OD_{600} and by counting the colony forming units (cfu) up to 72 hours[7,25-27]. Revelation of culturable cells was further confirmed by the supportive spot tests[7,25-27]. As described previously, each the culture suspension was serially diluted in 9 ml nutrient broth to obtain up to 10^{-4} fold dilution[27]. 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.

Demonstration of morphological changes

Simple staining (crystal violet, Hucker's solution) was conducted to assess the viability and the cellular morphology as described previously[25-27]. An aliquot of 10 µl from the bacterial culture suspension was removed at 24 hours of growth, and the cellular morphology, shape and organization were observed under the light microscope (Optima Biological Microscope G206, Taiwan) at 1,000x magnification[25-27].

Preparation of organic and inorganic supplements

To prepare the extracellular fractions of bacteria (*Bacillus* sp. and *P. fluorescens*), cells were grown separately into 6 different sets of Durham's bottle containing 5 ml minimal broth, which were kept in shaking water bath at 100 rpm for 24 hours at 37°C. Subsequently, actively growing bacterial cells were centrifuged at 4,000 rpm for 15 minutes, and the resulting pellets were collected. Afterward pellets were centrifuged at 4,000 rpm for 15 minutes for 3 times with 10% glycerol and 50 m*M* $CaCl_2$ respectively[30]. The resulting supernatants were collected and used as organic supplement to observe the possible retrieval of *P. aeruginosa* cell viability both at 45° and 46°C. Subsequently, a mixture of 20 m*M* $MgSO_4$ and 5 m*M* EDTA were used as inorganic supplement to conduct the similar experiment[19].

Results and Discussions

Revolution of growth and viability of Pseudomonas aeruginosa

As stated above, our previous studies investigated the strong defense strategies of *Bacillus* sp. SUBB01[27] and *Pseudomonas fluorescens* (unpublished) in response to the oxidative stress artificially created by the supplementation of H_2O_2 into the growing culture. Notably, at the early stationary phase, *E. coli* undergoes lysis mechanism appears to remove VBNC (viable but nonculturable) cells accumulated at the transition period from exponential to stationary phases[16,31]. Moreover, heterogeneity in *E. coli* population was reported before it entered stasis[16,32], where *E. coli* were assumed to uptake the probable stress resistance factors and possibly balance the viable and culturable cell population (Figure 1). Based on this suggestive idea, present study designed an ingenious simulation model of growth retrieval, and employed a possible positive defensive strategy on the heat stressed *P. aeruginosa* growth at 45° and 46°C temperature by means of supplementation of the (1) extracellular (secreted into the culture media) fractions of *P. fluorescens* and of (2) *Bacillus*

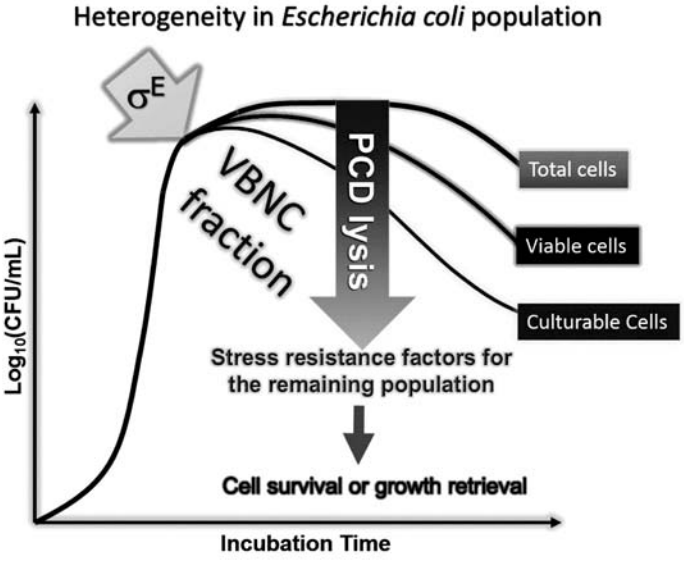

Figure 1. *Possible Growth revival strategies of stressed Escherichia coli cells*[7,16-17,31]. *The heat stressed cells of E. coli were assumed to be fractionated into the viable cells, viable but non-culturable (VBNC) cells and dead cells in the early stationary phase. The surviving viable and culturable cell fractions are thought to uptake the debris generated from the lysis dead cells and the VBNC cells triggered by* s^E *regulon gene transcription and translation.*

species as organic supplement which was further compared to that of (3) the inorganic supplement (Mg^{2+} with EDTA) into the culture media of heat stressed *P. aeruginosa* (Figure 2).

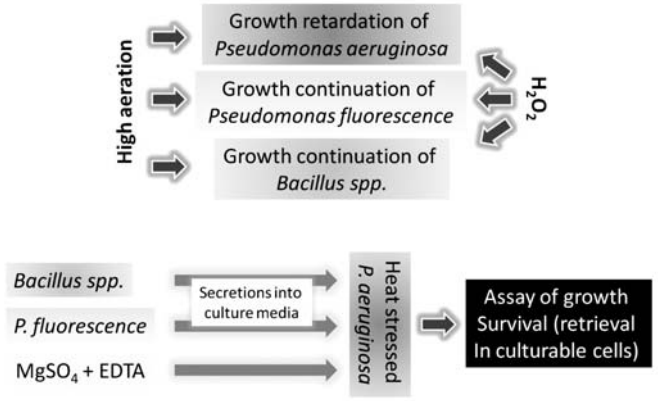

Figure 2. *Rationale and objectives of the current study. Previously data has shown that high aeration at 200 rpm and the addition of H_2O_2 caused the loss of culturability of Pseudomonas aeruginosa while Bacillus spp. and P. fluorescence were found to be comparatively resistant against such stresses*[26-27]*. Based on these findings, current study endeavored to examine whether the supplementation of the extracellular factors of Bacillus spp. (SUBB01) or P. fluorescence (SUBP02) in the medium fraction could retrieve the growth of heat stressed P. aeruginosa cells (SUBP01), grown at 45 °C and 46°C. Effect of the inorganic growth stabilizing agents ($MgSO_4$ and EDTA) was also investigated.*

In this study, when *P. aeruginosa* cells were grown with the extracellular fractions of *P. fluorescens* at 45° and 46°C, a growth revival was observed in cell turbidity (Figure 3 a, b, c and d) as well as in the generation of cfus compared to that of the control; *i.e.*, untreated and the cells treated with 20 m*M* $MgSO_4$ with 5 m*M* EDTA. Besides, no significant changes were observed when cells were grown with the extracellular fractions of *Bacillus* species (Figure 3). In consistent to the results obtained from the growth related experiments, no morphological change was observed under light microscope (1,000x), when cells were subjected to grown with the extracellular fractions of *Bacillus* species compared to those treated with 20 m*M* $MgSO_4$ with 5 m*M* EDTA, untreated (control). Notably, an increase in the number was observed when cells were grown with the extracellular fractions of *P. fluorescens* at 45° and 46°C (Figure 4).

Confirmative demonstration of growth retrieval of P. aeruginosa cells

As stated earlier, after 48 hours of incubation, *P. aeruginosa* cells were surprisingly found to retrieve their cell turbidity at 600 nm along with the generation of the culturable cells at 45° and 46°C upon supplementation with the extracellular fractions of *P. fluorescens* and inorganic supplement compared to the control and those grown with the extracellular fractions of *Bacillus* species led us to further cross-check of the growth retrieval trait of the cells through the spot tests as stated earlier[25-27]. Consistent

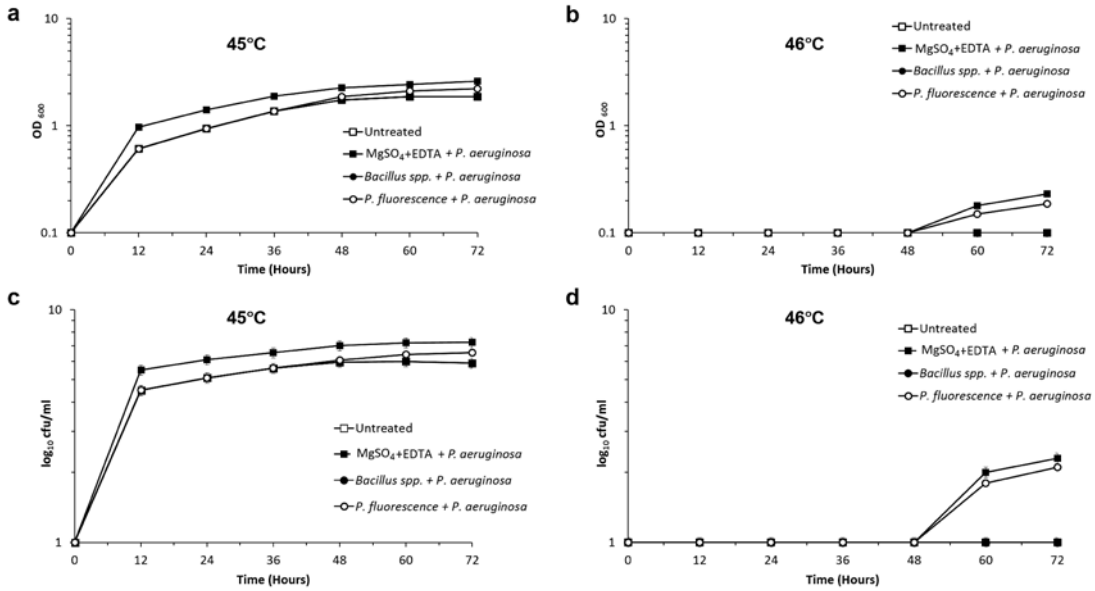

Figure 3. *Growth revival of Pseudomonas aeruginosa (SUBP01) in terms of cell turbidity (a, b) and colony forming units (c, d) at 45°C (a, c) and 46°C (b, d), upon supplementation with $MgSO_4$, and EDTA as inorganic supplement and the extracellular fractions of Bacillus species (SUBB01) and Pseudomonas ûuorescens (SUBP02) as organic supplement. The experiment carried out through the examination of cell turbidity by measuring OD_{600}. P. aeruginosa (SUBP01) were grown in minimal media at 45° and 46°C and after 10 hours of growth 20 mM $MgSO_4$ with 5 mM EDTA, the extracellular fractions of Bacillus species (SUBB01) and P. ûuorescens (SUBP02) were added seperatly. A growth revival was observed at both 45°C (a, c) and 46°C (b, d), grown with 20 mM $MgSO_4$ with 5 mM EDTA and extracellular fractions of P. ûuorescens (SUBP02) compared to that of the control; i.e., untreated, and the cells treated with extracellular fractions of Bacillus species (SUBB01).*

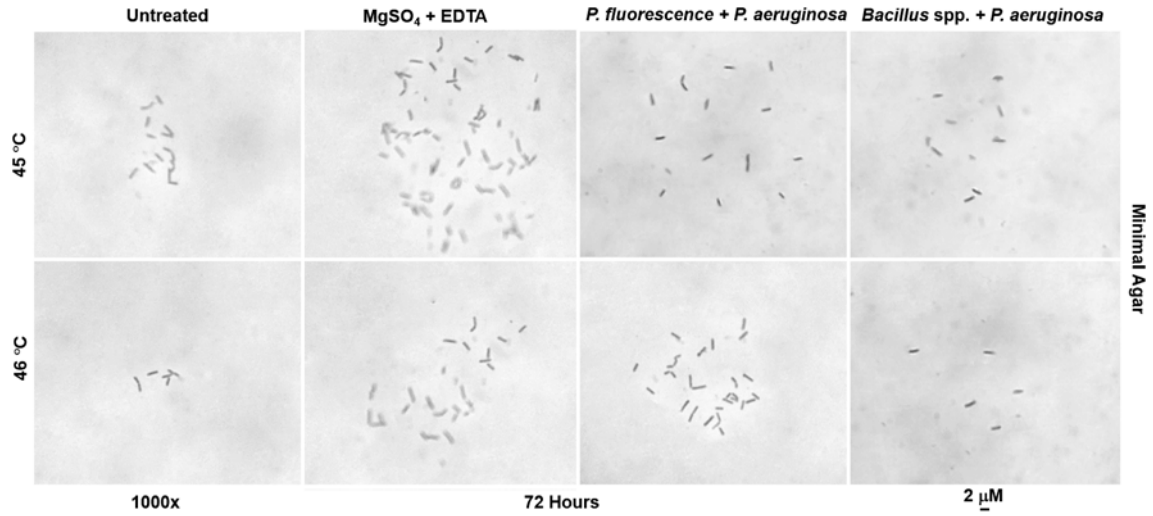

Figure 4. *Morphological impairment of Pseudomonas aeruginosa (SUBP01) at 45° and 46°C. However, morphologically recovered cells were observed after 72 hours of incubation periods when cells were grown with the extracellular fractions P. ûuorescens (SUBP02). Notably, no cells were observed at both 45° and 46°C, upon supplementation with the extracellular fractions of Bacillus species (SUBB01).*

to the growth experiments as shown in figure 1 and figure 2, a growth revival of *P. aeruginosa* cells was observed at 45° and 56°C upon supplementation with the extracellular fractions of *P. fluorescens* with minimal variation with the cells treated with 20 m*M* $MgSO_4$ with 5 m*M* EDTA in minimal agar (Figure 5). No significant changes were observed when cells were grown with the extracellular fractions of *Bacillus* species.

Earlier studies revealed that *E. coli* cells exhibited s^E dependent cell lysis at early stationary phase, which might be one type of programmed cell death (PCD)[15,17,32]. Several studies showed that various physiological and morphological changes during the early stationary phase appears to be similar in *Escherichia coli* and *Pseudomonas* spp.[12-13,33]. Considering the idea of nutrient support by cell lysis and the organism might make some kind of equilibrium in cell population, the current study also proposed a model based on the present findings (Figure 6). The model evidently figures out the possible positive effect on the heat stressed *P. aeruginosa* (SUBP01) growth at high temperature upon the supplementation of the extracellular fractions of *P. fluorescens* (SUBP02) as organic growth supplement.

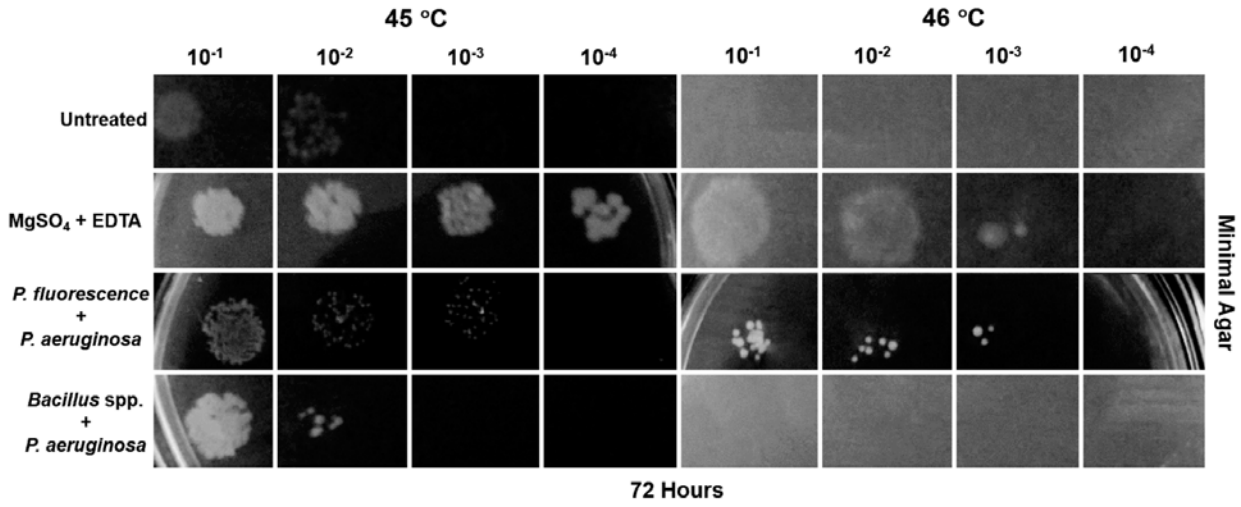

Figure 5. *Revolution of cell viability and culturability of Pseudomonas aeruginosa (SUBP01) in minimal agar (MA). In the MA agar, a steady growth was found at both 45° and 46°C after 72 hours of incubation periods upon supplementation with the extracellular fractions P. ûuorescens (SUBP02) compared to that of the control; i.e., untreated and the cells treated with extracellular fractions of Bacillus species (SUBB01).*

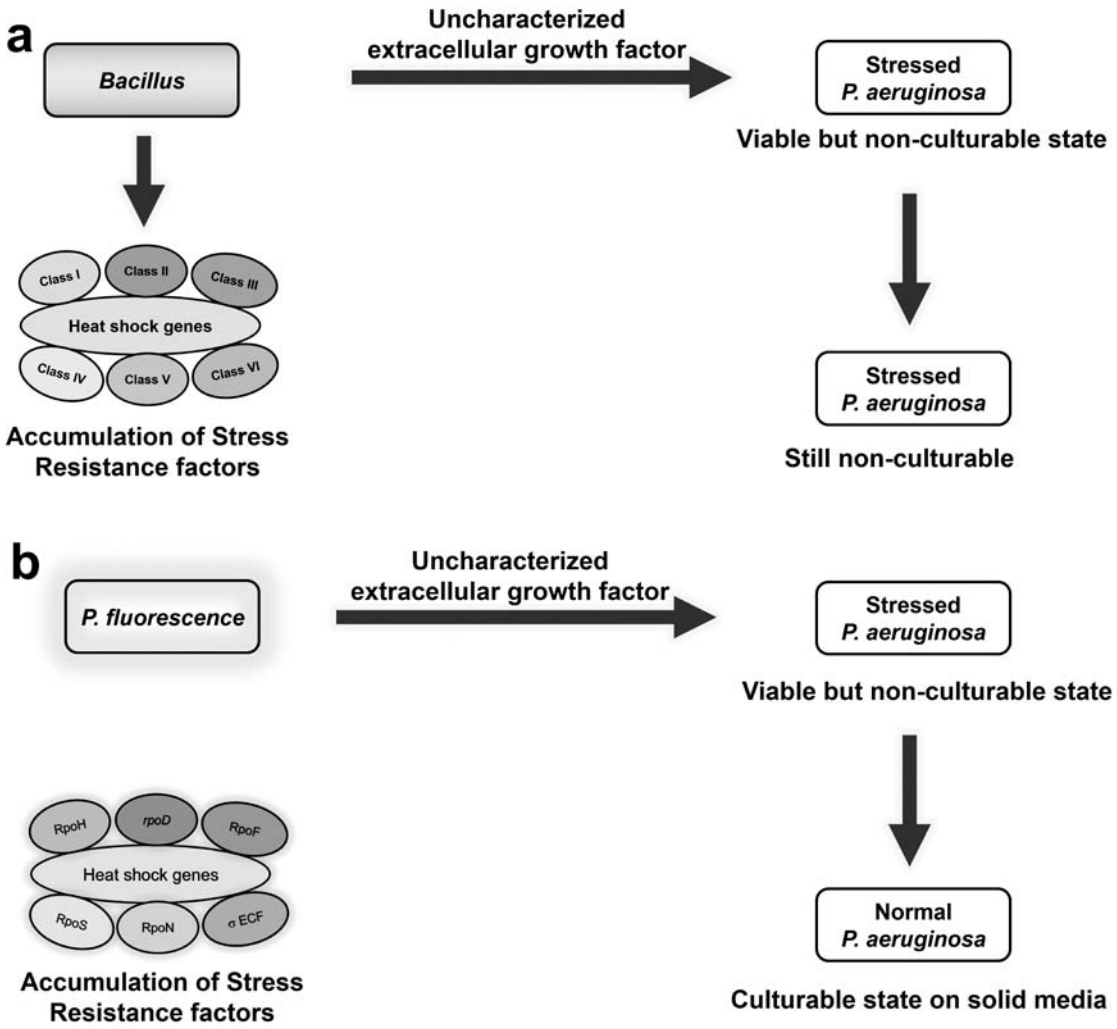

Figure 6. *The model of the growth retrieval of Pseudomonas aeruginosa (SUBP01). Notably, no significant changes were observed, when cells were grown with the extracellular fractions of Bacillus species (SUBB01) compared to those treated with the extracellular fractions of P. ûuorescens (SUBP02).*

Conclusion

Our previous study has demonstrated that *E. coli* and *P. aeruginosa* lose its culturability upon oxidative stress, where *Bacillus* spp. and *P. fluorescens* revealed an eliciting different cellular stress response mechanism. The current investigation further uravalled the revolution of growth and viability of *P. aeruginosa* at high temperature and co-metabolic relationship between *P. aeruginosa*, *P. fluorescens* and *Bacillus* species. Despite the lack of genetic investigation, current study has sufficiently exhibited the positive impact of bacterial extracellular extracts on growth resuscitation, which so far has been evident for the first time in case of *Pseudomonas* spp. Further studies at the genetic level would unveil the molecular mechanism underlying the reasons of growth retrieval of *P. aeruginosa* by *P. fluorescence* but not by *Bacillus* spp.

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