Influence of Aeration Speed on Bacterial Colony Forming Unit (CFU) Formation Capacity

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Abstract
Bacterial homeostasis depends on an array of physical and chemical stimulants. Current investigation assessed the impact of one of such factors, the speed of aeration, on cell viability and culturability of Escherichia coli, Pseudomonas spp. and Bacillus spp. Each of the bacterial strain was incubated at 37°C with a shaking speed of 0, 100 or 200 rotation per minute (rpm) separately up to 72 hours, with a simultaneous monitoring of morphological changes and cell culturability. All bacterial species were found to optimally grow at 100 rpm whereas at 0 rpm growths of E. coli and Pseudomonas spp. were bit slower compared to that of Bacillus spp. The capacity to form colony forming units (CFUs) of E. coli and Pseudomonas spp. on Luria Burtani (LB) agar plates were observed to be inhibited after 36 hours of growth at 200 rpm; i.e., approximately 3-log reduced CFUs than those formed by Bacillus spp. Besides, morphologically impaired cells were observed for the former two bacteria cultivated at 200 rpm. Taken together, it is assumed that the high speed shaking might evolve the oxidative stress endogenously which possibly rendered the cells lose their culturability.

Keywords: Escherichia coli, Pseudomonas spp., Bacillus spp., aeration speed, colony forming units (CFUs), oxidative stress

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1. Introduction

Study of stress responses in bacteria is indeed an exhilarating vicinity of basic and applied biomedical and microbiological research. Variations in any of the growth influencing factors including nutrient availability, temperature, pH, aeration, redox potential, water activity, solute concentrations, and the culture volume along with the vessel size may affect bacterial growth rate, which is universally known as stress phenomenon [1-6] Upon entry of into the stationary phase, Escherichia coli cells have been shown to lose their ability to reproduce on standard nutrient plates, i.e., cells enter into a viable but non-culturable (VBNC) state, possibly due to the accumulation of reactive oxygen species (ROS) [3,4,8-15]. However, such oxidative stress can be combated by catalase and superoxide dismutase mediated response in E. coli [3,4,9,10,11,16,17,18]. Other bacteria including Pseudomonas spp. and Bacillus spp. have also been reported to elicit responses against such oxidative stress [19-26].

Effects of agitation on bacterial growth kinetics have been studied earlier; however, neither the association between the shaking speed with the advancement of endogenous oxidative stress nor the influence of agitation speed on cell culturability has been deliberated [27,28,29]. Our previous studies showed three independent aspects in evolving stress: (i) a close proportional relation with the temperature up-shift with the generation of oxidative stress [30,31]; (ii) spontaneous accumulation of ROS generating oxidative stress at the early stationary phase [3] and (iii) the commencement of such stress upon the addition of 3 mM hydrogen peroxide (H2O2) [3,32,33]. In all cases, E. coli strains were found to deteriorate their viability with a reduced rate of the formation of colony forming units (CFUs) [2,3,6,30,32]. Apart from the external induction by H2O2, temperature up-shift or nutrient starvation, one of the critical parameters decreasing cell viability could be the culture shaking speed [27,28,34,35]. An imitation of such stress responsive event with other bacterial strains or species might be of interest to the inclusive insight of the cellular responses. Along these lines, current investigation assessed the effect of aeration speed apart from the external of addition of H2O2 on E. coli, Pseudomonas, and Bacillus spp.

2. Materials and Methods

2.1. Bacterial Stain, Medium and Culture Condition
Bacterial strains used in this study were previously preserved on Nutrient Agar (NA) at 4°C [32]. After 2 hours of growth of freshly grown culture (pre culture) in 5 ml nutrient broth (NB), the OD at 600 nm (OD₆₀₀) of the culture broth was adjusted to 0.1. A volume of 30 μL was introduced into 3 different sets of 30 ml of nutrient broth and was incubated at 37°C with shaking at 0, 100 and 200 rotation per minute (rpm). At every 12 hours of intervals cell growth was monitored by measuring OD₆₀₀ and the colony forming units (CFU)/ml was estimated by counting the colonies on nutrient agar (NA) at every 24 hours [4,30,32].

2.2. Microscopy

Samples were collected at every 12 hours of intervals and the shape and arrangement of the bacterial cells were observed by using simple staining (Crystal Violet, Hucker’s Solution) under light microscope (Optima Biological Microscope G206, manufactured in Taiwan) at 1000 × magnification [32].

2.3. Spot Test

The bacterial suspension was serially diluted in 9 ml nutrient broth up to 10⁻⁴. From each of the 4 dilutions, 5 μL of the bacterial suspension was spotted onto NA plate. After inoculums dried off, plates were incubated at 37°C for 24 hours. Spotting was done after every 12 hours interval [30,32].

3. Results and Discussion

3.1. Growth Retardation of E. Coli and Pseudomonas spp. Under 200 rpm

All three bacterial species showed a relatively shower growth at 0 rpm compared to those at 100 rpm (Figure 1 a, Figure 1 b, Figure 2 a, Figure 2 b, and Figure 3 a, Figure 3 b). While shaken at the highest speed of aeration, i.e., at 200 rpm, the colony forming units (CFUs) were observed to be significantly reduced after 36 hour in case of E. coli and Pseudomonas cells. A minor reduction in CFU in Bacillus cells was also observed after 48 hour; however, the trend was insignificant compared to the former strains.

As depicted in Figure 1 c, E. coli culture medium was observed to be filled with cell aggregates, suggestive of the defective cell lysis phenomenon [3,4,6]. Such defective cells are suggested to consist of a major fraction of VBNC cells, which was further confirmed through the loss of culturability [1,2,3,4,6,9,11]. Besides, flat colonies characteristic of stressed cells [6] were observed for E. coli cells after 36 hours and the non-pigmented colonies were observed for Pseudomonas spp. upon prolonged under 200 rpm aeration. Thus the decline in CFUs as well as the colony characteristics referred to the stress commencement in Pseudomonas cells upon cultivation at 200 rpm. No such impaired phenotypes in Bacillus colonies were observed nor was the accumulation of cell aggregates detected in their culture media. Rather a steady growth was monitored all along the incubation period in case of Bacillus spp. at 200 rpm (Figure 3 a, Figure 3 b), possibly due to the upregulation of the general stress regulon by the unique stressosome complex as described previously [36].

Earlier studies showed Pseudomonas cells to exhibit a reduced growth rate under high oxygen tension [8,23]. However, P. aeruginosa is known to express the pqrCBAR genes which in turn may neutralize the oxidants [18,21,37,38]. In our experimental condition, the reduction trend in CFUs by Pseudomonas cells after 36 hour might be due to the impairment of such defense mechanism which is in cohort to the previous studies [8,23].
3.2. Morphological Changes in Bacterial Cells in Relation to Cell Viability

All three bacterial species showed a relatively shower growth. While at 36 hours of incubation, no significant changes in cell morphology and shape were observed in *E. coli* at all rpm; however, both the static condition and the highest agitation (i.e., 200 rpm) resulted in cell aggregates with a cessation in regular rod size (Figure 1 d). This is in consistent with the observed changes in colony morphology after 36 hours, with a paralleled impact on the reduction of culturability (Figure 1 e). Interestingly, under 200 rpm aeration, most of the *Pseudomonas* spp. cells were found to transform to spherical shape after 36 hours, and at 72 hours these cells were observed to be in aggregated condition (Figure 2 c). Like *E. coli* cells,


4. Conclusion

It’s been a long while the aeration speed for bacterial cultivation known to influence the growth rate [46,47,48]. In the current investigation, the preliminary experiments achieved a bit expected result that the excessive aeration caused significant impact on Escherichia coli and Pseudomonas spp. cells viability and culturability and that the Bacillus cells were bit unaffected by the aeration speed. Thus our earlier results relating to the cessation of bacterial culturability caused by upshifting temperature or by externally adding oxidant might recruit the elevated aeration speed as another stress evoking stimulant [3,30,32]. However, the suggestive relation between increased aeration and the commencement of oxidative stress needs to further concluded by the direct measurement of ROS. Besides, the genetic revelation of the phenotypes studied here would unveil the mechanism of cellular survival against intrinsic stress stimuli.

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Conflict of Interest

None of the authors have conflict of interest.

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