American Journal of Microbiological Research, 2014, Vol. 2, No. 1, 47-51
Available online at http://pubs.sciepub.com/ajmr/2/1/7

DOI:10.12691/ajmr-2-1-7

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

Md. Sakil Munna, Sadika Tamanna, Most. Rumana Afrin, Gulshan Ara Sharif, Chaity Mazumder, Konica Sarker Kana, Nusrat Jahan Urmi, Md. Aftab Uddin, Tasmina Rahman, Rashed Noor*

Department of Microbiology, Stamford University Bangladesh, Dhaka, Bangladesh
*Corresponding author: noor.rashed@yahoo.com

Received December 30, 2013; Revised January 10, 2014; Accepted February 09, 2014

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

Cite This Article: Md. Sakil Munna, Sadika Tamanna, Most. Rumana Afrin, Gulshan Ara Sharif, Chaity Mazumder, Konica Sarker Kana, Nusrat Jahan Urmi, Md. Aftab Uddin, Tasmina Rahman, and Rashed Noor, "Influence of Aeration Speed on Bacterial Colony Forming Unit (CFU) Formation Capacity." *American Journal of Microbiological Research*, vol. 2, no. 1 (2014): 47-51. doi: 10.12691/ajmr-2-1-7.

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 (H_2O_2) [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 H_2O_2, 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 H_2O_2 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 (OD_{600}) 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 OD_{600} 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.

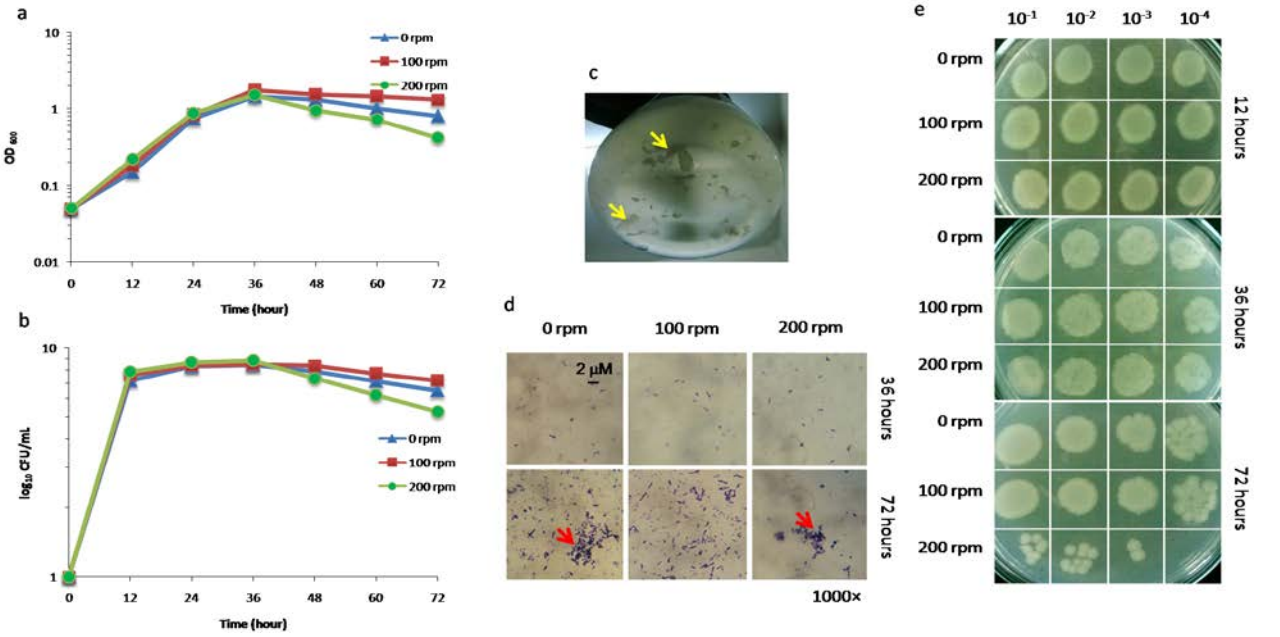

Figure 1. Assessment of stress response in *Escherichia coli* through the examination of cell turbidity **(a)**, enumeration of CFU **(b)**, observation for the accumulation of cell aggregates in culture media **(c)**, morphological study: arrowheads indicate impaired cells **(d)** and the demonstration of loss of cell culturability by means of spot test **(e)**. Cells were grown in nutrient broth at 37°C under 0, 100 and 200 rpm as described in Materials and Methods. At the times indicated, all examinations were carried out

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].

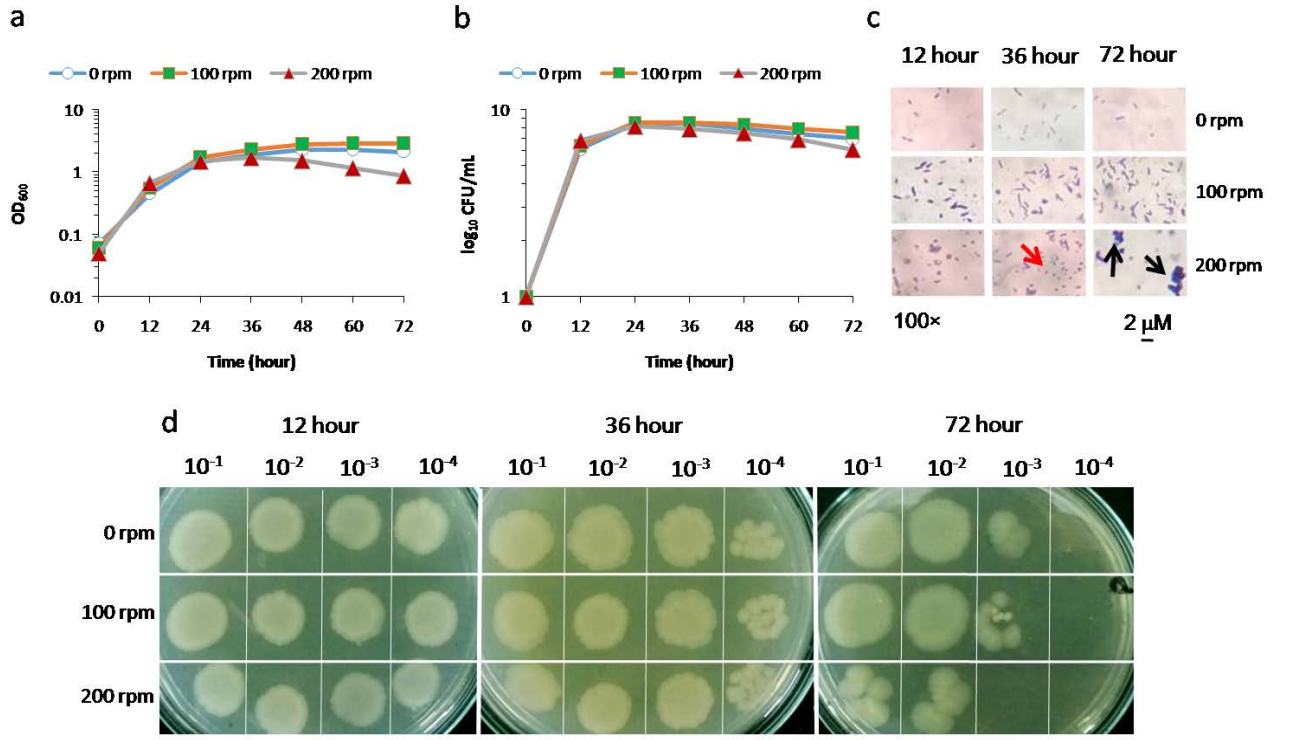

Figure 2. Assessment of stress response in *Pseudomonas* spp. through the examination of cell turbidity **(a)**, ability to form CFUs **(b)**, morphological observation: arrowheads indicate impaired cells **(c)** and the demonstration of loss of cell culturability through spot test **(d)**. Cells were grown in nutrient broth at 37°C under 0, 100 and 200 rpm as described in Materials and Methods. At the times indicated, examinations were carried out. No accumulation of cell aggregates in culture media was observed

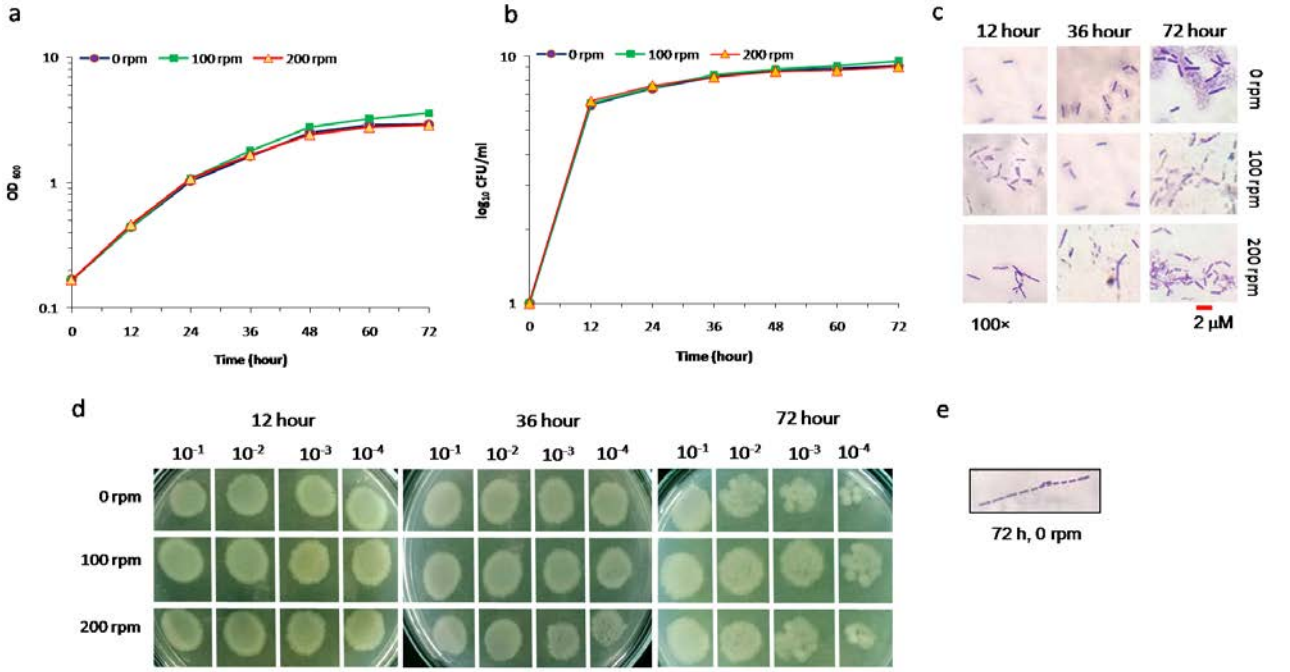

Figure 3. Assessment of stress response in *Bacillus* spp. through the examination of cell turbidity **(a)**, CFU formation **(b)**, morphological observation **(c)** and the demonstration of loss of cell culturability by means of spot test **(d)**. Cells were grown in nutrient broth at 37°C under 0, 100 and 200 rpm as described in Materials and Methods. At the times indicated, all examinations were carried out. The morphological change at 72 hour of incubation in static condition is shown **(e)**

3.2. Morphological Changes in Bacterial Cells in Relation to Cell Viability

All three bacterial species showed a relatively shower growth While at 36 hour of incubation, no significant changes in cell morphology and shape was 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,

Pseudomonas cells were also found to lose their culturability after 36 hours at 200 rpm (Figure 2 d). Thus, both in the cases of *E. coli* and *Pseudomonas* cells, morphologically defective cells were observed which were worth to explain the ultimate non-culturability of these strains (Figure 1 e, Figure 2 d) at 72 hours under 200 rpm [3,4,6,9].

One important thing is to ponder that unlike *E. coli*, *Pseudomonas* culture media did not show any accumulation of cell aggregates; however, interestingly the pigmentation in the *Pseudomonas* culture media was found to be eliminated concomitantly with the loss of their culturability. Distinct from *E. coli* and *Pseudomonas* cells, no significant changes in colony phenotype or cell morphology and arrangement were observed for the *Bacillus* spp. cells up to 72 hours upon the variation in the aeration speed. (Figure 3 c). The comparative resistance might be due to the unique cellular survival mechanism imparted by *Bacillus* spp. [39,40,41,42,43].

An interested morphological trait was observed in case of *Bacillus* cells in static condition. At 72 hour under 0 rpm, a significant fraction of cells were observed to be segmented (Figure 3 e). Such event was indicative of the existence of dividing cells resulting in the cellular culturability possible due to the presence of stressosome regulating the secondary messenger levels [36,44,45]. This notion is in cohort with the load of final bacterial load at 72 hour under 200 rpm; i.e., *Bacillus* cells were found to form around 3-log higher CFU/mL than both *E. coli* and *Pseudomonas* spp. (Figure 1, Figure 2 and Figure 3). Another important facet of growth of *Bacillus* spp. lied under the fact that these cells showed a comparatively higher growth than the other two all along at the static condition. Such a growth response irrespective of the aeration speed again could be explained through the stressosomal activities of *Bacillus* spp. [36,44].

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.

Acknowledgements

Authors are thankful to Kamal Kanta Das, Mrityunjoy Acharjee and Saurab Kishore Munshi for technical assistance, valuable suggestions and careful monitoring during the experiments. Authors also gratify Stamford University Bangladesh for the financial and logistic supports.

Conflict of Interest

None of the authors have conflict of interest.

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