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Drastic decline in optical density during stationary phase meant that *Bacillus subtilis* NRS-762 is not suitable as model organism in microbial survivability studies

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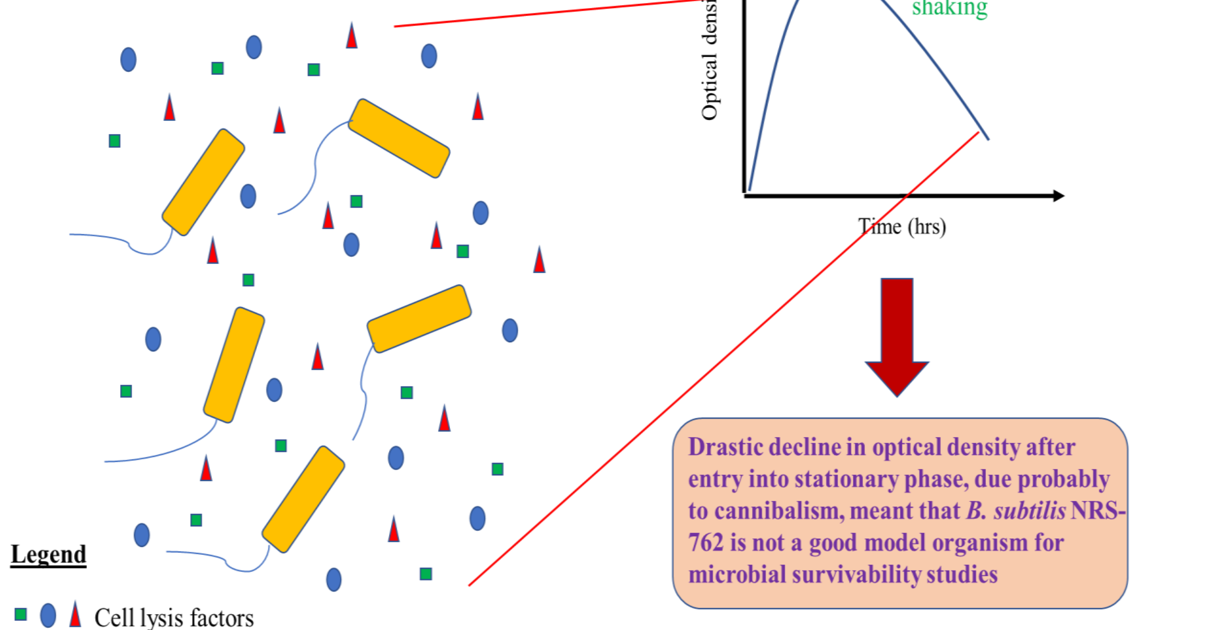
Graphical abstract



Short description: A soil bacterium common in many habitats and with significant metabolic capabilities for adapting to various environments as well as possessing many cellular differentiation pathways, *Bacillus subtilis* NRS-762 (ATCC 8473) is nevertheless not suitable as model organism in a variety of microbial survivability studies such as ones assessing the survival of microbes on the surfaces of drinking water pipelines. Specifically, drastic decline in optical density after entry into stationary phase in many commercial growth media such as LB Lennox and Tryptic Soy Broth pointed to cannibalism or prophage induced cell lysis as mechanisms. The relatively slow rate of decline in optical density revealed that cannibalism (where cell lysis factors are secreted by resistant subpopulations to lyse non-resistant ones for cellular nutritional content) may be dominant. With the ability to confound experimental readout on environmental factors' influence on cell survival, existence of drastic decline in optical density during stationary phase

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highlighted the non-suitability of *B. subtilis* NRS-762 as model organism for microbial survivability studies.

Highlights

1) A common soil bacterium, *Bacillus subtilis* NRS-762 (ATCC 8473) is not a good model organism for microbial survivability assays for various environmental related test scenarios.

2) Observations of drastic decline in optical density after *B. subtilis* NRS-762 culture enter stationary phase at different growth temperatures (25, 30 and 37 °C) in various growth media (LB Lennox and Tryptic Soy Broth) highlighted that its use as model organism could confound readout of survivability of microbes in different scenarios.

3) Cannibalism was likely the predominant mechanism accounting for the drastic decline in optical density, although prophage induced cell lysis was also likely operative in cultures grown at 37 °C, in what was a possible temperature activated entry into a prophage lytic programme.

4) Growth curve experiments revealed a reduced rate of optical density decline in cultures of *B. subtilis* NRS-762 grown at 30 °C in LB Lennox medium; thereby, pointing to a possible cell survival mechanism operative at 30 °C.

5) Ability of maintaining a stable optical density and cell population during stationary phase meant that *Escherichia coli* DH5α (ATCC 53868) may be a better model organism for assessing the survivability of microbes in different scenarios.

Abstract

Survival of microbes on various surfaces and habitats is a question of importance to basic science, as well as health care, water treatment and distribution, ecology, and search for life in other planetary bodies. To this end, various model organisms known to be resilient against a variety of environmental stressors are used for understanding the mechanisms underlying survival in extreme environments, or conditions mimicking those of the investigated habitats. Observations of drastic decline in optical density of *Bacillus subtilis* NRS-762 (ATCC 8473) in LB Lennox and Tryptic Soy Broth (TSB) at temperatures of 25, 30 and 37 °C, after the aerobic shake flask culture reached maximal cell density at stationary phase, pointed to possible cell lysis as mechanism for cell death. Specifically, optical density of the bacterium declined from 5.4 at 22.5 hours post-inoculation in LB Lennox medium to 2.5 after 38 hours of culture at 25 °C and 250 rpm rotational shaking. Similarly, optical density of *B. subtilis* NRS-762 also precipitously declined from 6.4 at 33 hours of culture to 1.8 at 51 hours post-inoculation at 37 °C in TSB. This is in stark contrast to aerobic growth of *Escherichia coli* DH5α (ATCC 53868) in LB Lennox medium at 37 °C and 230 rpm rotational shaking, where optical density remained stable during stationary phase. More importantly, observations of *B. subtilis* NRS-762 culture after autoclave decontamination revealed a lack of cellular debris; thereby, indicating massive cell lysis resulting in population collapse. Although *B. subtilis* is known to enter into various cellular differentiation programmes upon nutrient starvation, complete absence of cell debris that usually settle at the bottom of the shake

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flask after autoclave decontamination pointed to cannibalism or prophage induced cell lysis as key reasons underlying observed drastic decline in optical density of the culture. However, prophage induced cell lysis may be discounted as this would have led to rapid collapse of the entire cell population shortly after entry into stationary phase except during growth of *B. subtilis* NRS-762 at 37 ^{o}C where a temperature sensitive sensor might have activated prophage entry into the lytic programme. Hence, cannibalism, where a subpopulation of *B. subtilis* NRS-762 cells secrete cell lysis factors which other *B. subtilis* NRS-762 cells are not resistant to, likely resulted in massive cell lysis that released cellular contents that served as nutrients for the surviving population. Collectively, *B. subtilis* NRS-762 is not suitable as model organism for microbial survivability studies given its tendency to undergo differentiation into the cannibalism programme, which in killing a significant fraction of cells upon nutrient starvation, would confound experiments aimed at understanding the survivability of the bacterium under a variety of environmental conditions.

Keywords: cannibalism, prophage, cell lysis, *Bacillus subtilis*, model organism, optical density, drastic decline, viable cell population, cell differentiation, *Escherichia coli*,

Subject areas: microbiology, biochemistry, biotechnology, molecular biology, cell biology,

Importance of the work

Assessing the survival characteristics of microbes in different scenarios and under different surface and environmental conditions is of great importance to protecting human health and safety, as well as understanding the types of environment suited for microbial life. However, given the large diversity of microbes present on Earth, specific model organisms capable of recapitulating most of the key metabolic, signalling, cell differentiation, and resistance mechanisms available to microbes for coping with fluctuations in environmental conditions need to be used. One possible microorganism is *Bacillus subtilis*, given its versatile metabolism, capability of surviving in a variety of habitats on Earth, and ability to differentiate into resistant spores for coping with extreme conditions. However, observations of drastic decline in optical density of stationary phase cultures of *B. subtilis* NRS-762 (ATCC 8473) grown in LB Lennox and Tryptic Soy Broth at different temperatures (25, 30, 37 ^{o}C) meant that the bacterium is not suitable as model organism for microbial survivability studies. Specifically, relatively slow rate of optical density decline meant that cannibalism is likely the main factor accounting for the drastic decline in optical density, except during growth of *B. subtilis* NRS-762 at 37 ^{o}C in LB Lennox medium where a temperature sensitive prophage likely entered into lytic programme and prompted rapid cell lysis in a large fraction of cells. Thus, drastic decline in optical density during stationary phase at different growth temperatures meant that *B. subtilis* NRS-762 is not suitable for microbial survivability studies.

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Introduction

Microbes are present on almost every ecological niche on Earth, from the depths of the oceans to the dry desert, due in large part to their metabolic diversity and adaptability. Adaptability enables microorganisms such as bacteria to occupy extreme environments (e.g., acid mine drainage) and even low nutrient ones (e.g., drinking water).[1 2 3 4 5 6 7 8] Hence, microbes' ability to survive various environmental conditions and habitats is a key object of investigations by scientists, which provides a window into the complex signalling and metabolic network that enables a microbe to tune its metabolism, cell differentiation and cellular characteristics to adapt to changing conditions in its habitat. More importantly, ability to sense changing chemical characteristics in its immediate environment allow microbes to switch cellular differentiation programmes to undergo morphogenesis into another cell fate more suited to the new environmental conditions prevalent in the locale.[9 10]

Given the ability of microbes to survive in various environments, it is important from a basic science and applied research perspective to understand their survivability as well as the mechanisms underpinning their survival. For example, it is important to characterise what organisms are able to survive for prolonged periods on the stainless steel surfaces of surgical knife, and more importantly, their duration and mechanisms of survival. On the other hand, it is also of great importance to determine the types and relative abundance of microbes that form the biofilm found on the inner walls of drinking water distribution pipelines.[11 12 13] Doing so will allow us to understand why certain species of microbes have preferential abilities to survive on certain surfaces such as plastic pipelines for drinking water.[14] More importantly, there is a need to know if there are specific surface coatings that would retard the growth and colonization of various surfaces by most microbial species.[15 16 17 18]

Hence, the field of microbial survivability is pre-occupied with investigations of which microbes are able to survive in a particular environment and why? However, given the myriad microbes present on Earth, exhaustive studies of the survival characteristics of individual microbial species is impossible, due primarily to the expense and manpower involved. Hence, model organisms are chosen to provide a general overview of how most microbes would behave and survive on a particular surface or under specified environmental conditions such as high temperature and nutrient starvation. But, how should scientists choose an appropriate model organism?

To this end, examining how the rod-shaped bacterium, *Pseudomonas aeruginosa*, is chosen as a representative bacterium for understanding microbial survivability in medical settings, would be able to lend a lens into the specific criteria important to selecting a microbe for the purpose. Firstly, the microbe should be common in the environment of interest; for example, *P. aeruginosa*,

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is a good bacterium model for understanding the survivability of microbes under medical sterilization procedures. Secondly, the metabolism and signalling characteristics of the model organism must be in sync with those necessary for survival in the studied environment or surface. Specifically, if survival at high temperature is necessary, the genome of the model microorganism should be endowed with sufficient number and types of heat shock proteins and chaperones able to allow the microbe to function metabolically under high temperature. Third, the cell differentiation pathways and lineages and fates available to the model organism should be similar to those thought to be available in microbiota present in the habitat or surface. Doing so will provide understanding of how environmental stress affect the survivability of individual species in the habitat, especially how different cellular differentiation programmes affect the growth and survival of the species in the environment. Finally, a stable cell population should exist prior to the start of the survivability test to help discern the effects of the environmental stressors imposed compared to intrinsic cell death in the microbe. Thus, microbes suitable for microbial survivability tests are typically those capable of maintaining a stable cell population after the onset of stationary phase.

Observations of drastic decline in optical density of *Bacillus subtilis* NRS-762 (ATCC 8473) after entry into stationary phase in LB Lennox medium revealed that the culture experienced significant cell lysis and cell death, that culminated in the lack of cellular debris at the bottom of the shake flask after autoclave decontamination. Specifically, optical density of *B. subtilis* NRS-762 culture decreased from 5.4 after 22.5 hours of cultivation to 2.5 at 38 hours post-inoculation at 25 ^{o}C and 250 rpm rotational shaking. Similar drastic decline in optical density also manifested in cultures of *B. subtilis* NRS-762 grown in Tryptic Soy Broth, and LB Lennox supplemented with 2 g/L glucose. More importantly, the phenomenon was also independent of temperature, with the same effect present in cultures of *B. subtilis* NRS-762 grown in the above three media at 25, 30 and 37 ^{o}C.

Hence, observations of precipitous decline in optical density of *B. subtilis* NRS-762 cultures in various commercial growth media at different growth temperatures revealed that cannibalism might be at work trying to ensure the survival of the clonal population. With such drastic decline in optical density after *B. subtilis* NRS-762 culture reached stationary phase, use of the bacterium in cell survivability studies would likely confound obtained results given the inability to disentangle the effects of the environment in inducing cell death in the species versus that inherent in the bacterium. Another factor that could account for the drastic decline in optical density during stationary phase might be the induction of lytic programme in prophages resident in the *B. subtilis* NRS-762 genome. Given that substantial number of cells remained and a residual optical density existed, it is unlikely that prophage induced cell lysis was the key factor accounting for observed drastic decline in optical density except during growth of *B. subtilis* NRS-762 at 37 ^{o}C,

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Materials and methods

Materials

LB Lennox medium and Tryptic Soy Broth were purchased from Difco and Merck (Darmstadt, Germany), respectively, while yeast extract was purchased from Oxoid. Composition of LB Lennox was [g/L]: Tryptone, 10.0; NaCl, 5.0; Yeast extract, 5.0. Composition of Tryptic Soy Broth was [g/L]: Pancreatic digest of casein, 15.0; Papaic digest of soya bean, 5.0; NaCl, 5.0. Composition of M9 minimal medium supplemented with 1 g/L yeast extract was [g/L]: D-Glucose, 4.0; NH_4Cl, 1.0; NaH_2PO_4, 3.0; Na_2HPO_4, 6.78; NaCl, 0.5; yeast extract, 1.0. Composition of LB Lennox supplemented with 2 g/L glucose was [g/L]: Tryptone, 10.0; Yeast extract, 5.0; NaCl, 5.0; D-Glucose, 2.0.

Growth experiments

Bacillus subtilis NRS-762 (ATCC 8473) and *Escherichia coli* DH5α (ATCC 53868) were inoculated from glycerol stock cultures maintained at -70 ^{o}C. Specifically, seed cultures were prepared by adding the glycerol stock cultures into 100 mL of LB Lennox medium in a 250 mL glass shake flask stopped with a cotton plug. The seed culture was incubated at 30 ^{o}C and 230 rpm rotational shaking in a temperature controlled incubator (YIH-Der, LM 570RD, Taiwan) for 24 hours. Experiment cultures were inoculated with 1 mL of seed culture in 100 mL of respective growth medium in a 250 mL glass shake flask and incubated at the following conditions: 25 ^{o}C and 250 rpm on IKA's KS 260 Basic shaker, 30 and 37 ^{o}C and 230 rpm rotational shaking on temperature controlled air incubator (YIH-Der, LM-570RD). Three biological replicates were performed for each experiment.

At appropriate time points during the culture, 5 mL of culture broth would be withdrawn and its optical density measured at 600 nm using a UV-Visible spectrophotometer (Shimadzu Biospec-Mini) and a quartz cuvette (pathlength = 1 cm, and volume = 3.5 mL). If the optical density is more than 1, appropriate dilution with deionized water was used. The pH of the medium was also measured using an Orion 9156 BNWP pH probe fitted to a Mettler Toledo Delta 320 pH meter.

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Results and Discussion

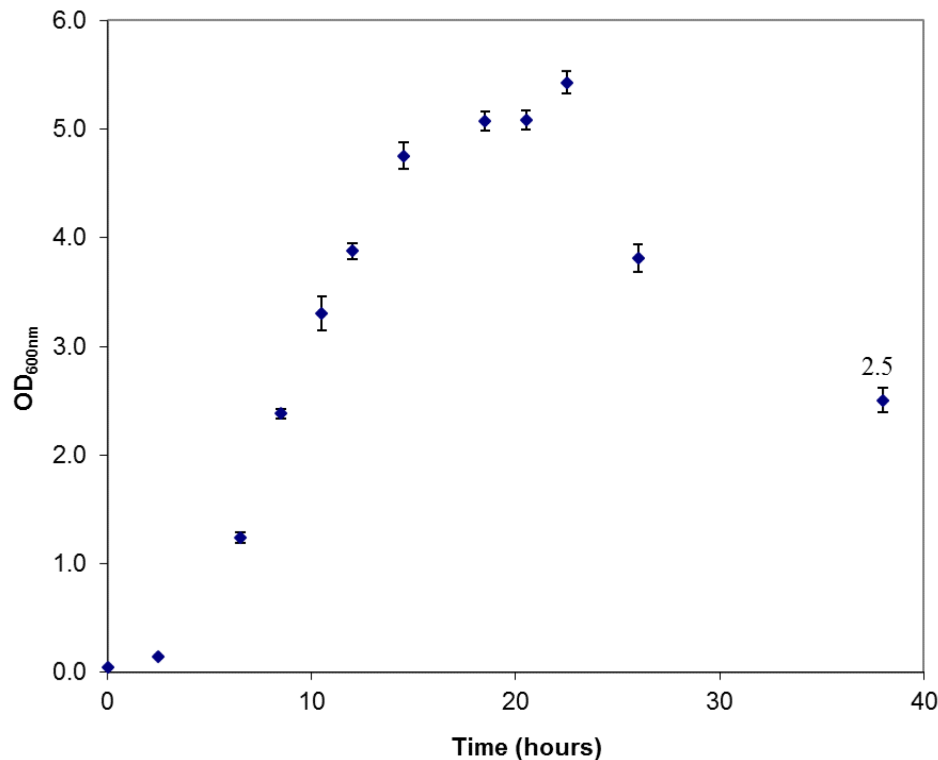

Figure 1: Growth of *Bacillus subtilis* NRS-762 in LB Lennox medium at 25 °C and 250 rpm rotational shaking. Optical density was measured at 600 nm.

Growth of *B. subtilis* NRS-762 in LB Lennox medium revealed that there was a drastic drop in optical density of the culture after the bacterium entered stationary phase (Figure 1). Specifically, optical density reached a maximal 5.4 after 22.5 hours of culture, but declined precipitously thereafter, with optical density at 2.5 after 38 hours of incubation. In addition, there was lack of cellular debris at the bottom of the shake flask after autoclave decontamination. Hence, *B. subtilis* NRS-762 demonstrated poor survivability after it has reached maximal cell density in its culture environment. One reason could be the induction of cannibalism, where specific subpopulations of *B. subtilis* NRS-762 cells secreted cell lysis factors that lysed neighbouring non-resistant cells, which helped release cellular contents that could serve as nutrients for the rest of the population.[19] Another reason could be the induction of prophage induced cell lysis where prophage resident in the genome of *B. subtilis* NRS-762 entered the lytic programme after the culture reached stationary phase and encountered nutrient starvation. However, such prophage induced cell lysis would likely result in rapid decline in optical density that would decimate the cell population within a few hours. Instead, significant residual optical density of 2.5 was observed

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16 hours after the induction of optical density decline in the culture. This indicated that cannibalism would be a more likely mechanism for the observed optical density decline.

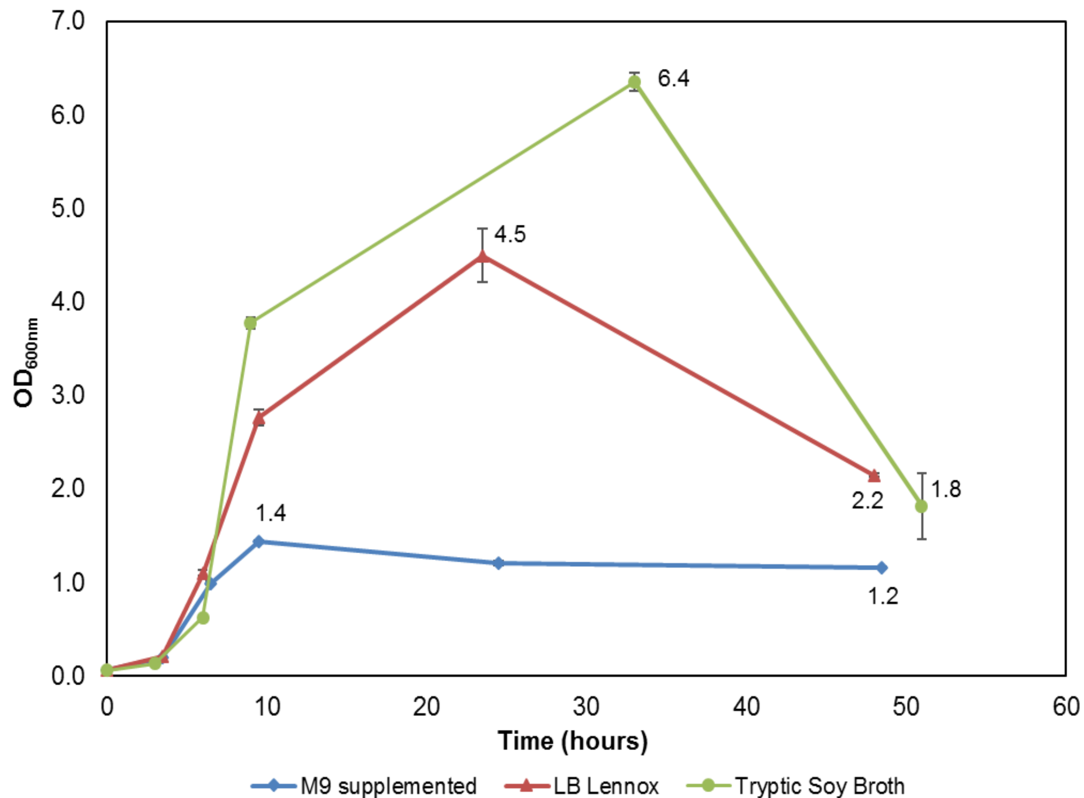

Figure 2: Growth of *B. subtilis* NRS-762 in different commercial media illustrating the same phenomenon of drastic decline in optical density after the culture reached maximal cell density at 25 ^{o}C and 250 rpm rotational shaking.

The same observation of drastic decline in optical density after *B. subtilis* NRS-762 culture reached stationary phase was also seen in Tryptic Soy Broth (TSB) (Figure 2). Specifically, optical density of *B. subtilis* NRS-762 in TSB reached 6.4 at 33 hours post-inoculation, but declined drastically to 1.8 at 51 hours post-inoculation. Similar drastic decline in optical density was also observed in *B. subtilis* NRS-762 grown in LB Lennox medium, where optical density declined from 4.5 at 23.5 hours post inoculation to 2.2 after 48 hours of cultivation. On the other hand, *B. subtilis* NRS-762 could not grow in M9 minimal medium as it lacked the ability to synthesize essential vitamins. After supplementing M9 minimal medium with 1 g/L yeast extract, *B. subtilis* NRS-762 attained an optical density of 1.4 at 9.5 hours post-inoculation, which declined to 1.2 at 48.5 hours post-inoculation. Hence, in both LB Lennox and TSB media, *B. subtilis* NRS-762 exhibited the same post exponential phase drastic decline in optical density, that suggested cell lysis either due to cannibalism or prophage induced cell lysis. However, given that significant

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residual optical density remained in the culture, cannibalism would be a more likely mechanism for the drastic decline in optical density.

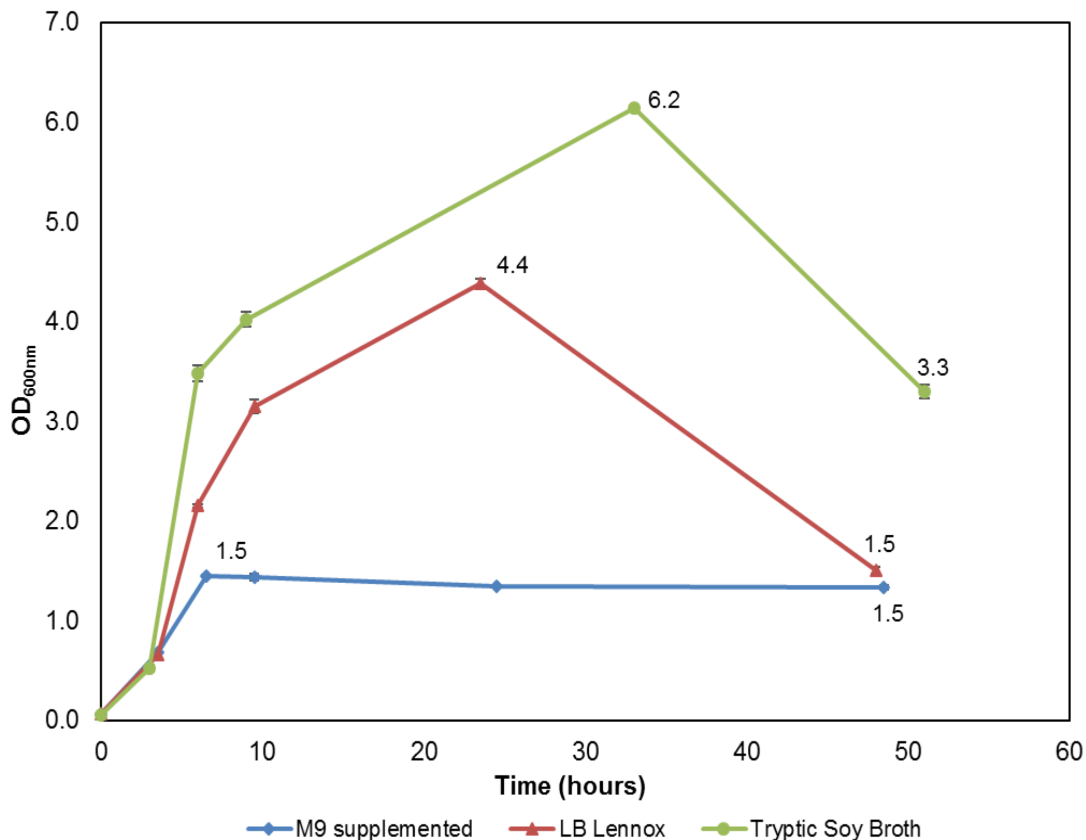

Figure 3: Growth of *B. subtilis* NRS-762 in various media at 30 ºC and 230 rpm in a temperature controlled incubator. While growth of *B. subtilis* NRS-762 in Tryptic Soy Broth and LB Lennox media exhibited drastic decline in optical density after the culture reached stationary phase, the same did not occur when *B. subtilis* NRS-762 was cultivated in M9 minimal medium with 1 g/L yeast extract.

Similarly, *B. subtilis* NRS-762 growth at 30 ºC in various media exhibited significant decline in optical density upon reaching stationary phase (Figure 3). But, curiously, the same did not happen in *B. subtilis* NRS-762 cultivated in M9 minimal medium supplemented with 1 g/L yeast extract. Specifically, upon reaching stationary phase at optical density of 1.5 after 6.5 hours of cultivation, the final optical density remained stable at 1.5 after 48.5 hours. On the other hand, optical density of *B. subtilis* NRS-762 grown in LB Lennox medium at 30 ºC and 230 rpm rotational shaking declined from 4.4 at 23.5 hours of incubation to 1.5 after 48 hours of cultivation. Similarly, drastic decline in optical density of *B. subtilis* NRS-762 culture was also observed in TSB, with optical density declining from 6.2 at 33 hours post-inoculation to 3.3 after 51 hours of

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incubation. Maintenance of significant residual cell population in cultures of *B. subtilis* NRS-762 grown in LB Lennox and TSB meant that cell lysis induced by prophages entering into lytic programmes could be discounted given that such lytic programmes would have resulted in a very small residual population and optical density. Thus, cannibalism was likely the main mechanism responsible for drastic decline in optical density of *B. subtilis* NRS-762 grown at 30 °C in LB Lennox and TSB media.

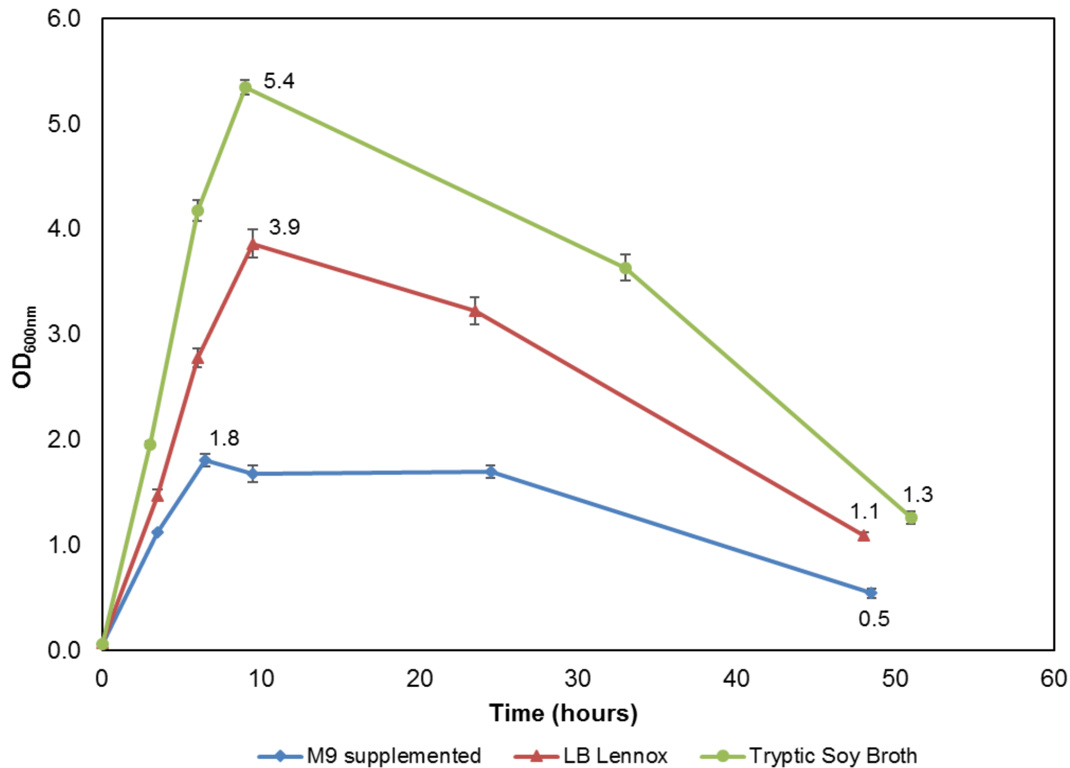

Figure 4: Similar drastic decline in optical density of *B. subtilis* NRS-762 grown in various media at 37 °C and 230 rpm rotational shaking in a temperature controlled incubator. Note that the residual optical density was small; thereby, indicating that a temperature sensitive switch might have activated prophage entry into the lytic programme during growth of *B. subtilis* NRS-762 at 37 °C in different growth media.

Growth of *B. subtilis* NRS-762 in various media at 37 °C and 230 rpm rotational shaking exhibited the same drastic decline in optical density after the culture reached stationary phase. Specifically, compared to growth at 30 °C and 25 °C, *B. subtilis* NRS-762 cultures entered stationary phase earlier after attaining their maximal optical densities. However, maximal optical densities recorded for *B. subtilis* NRS-762 growth in LB Lennox and TSB at 37 °C were lower than that for the same bacterium in the respective media at 30 °C; thereby, indicating that growth of *B. subtilis* NRS-762 was preferable at 30 °C compared to 37 °C. Residual optical density of 1.3 at 51 hours post-inoculation in TSB, 1.1 after 48 hours of culture in LB Lennox, and 0.5 at 48.5 hours post-inoculation in M9 minimal medium with 1 g/L yeast extract, indicated that prophage

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induced cell lysis probably accounted for the observed decline in optical density. Given that a small residual optical density was observed for *B. subtilis* NRS-762 cultured at 37 ºC in the respective growth media, the experimental data suggested that there was likely a temperature activated switch for prophage entry into the lytic pathway.

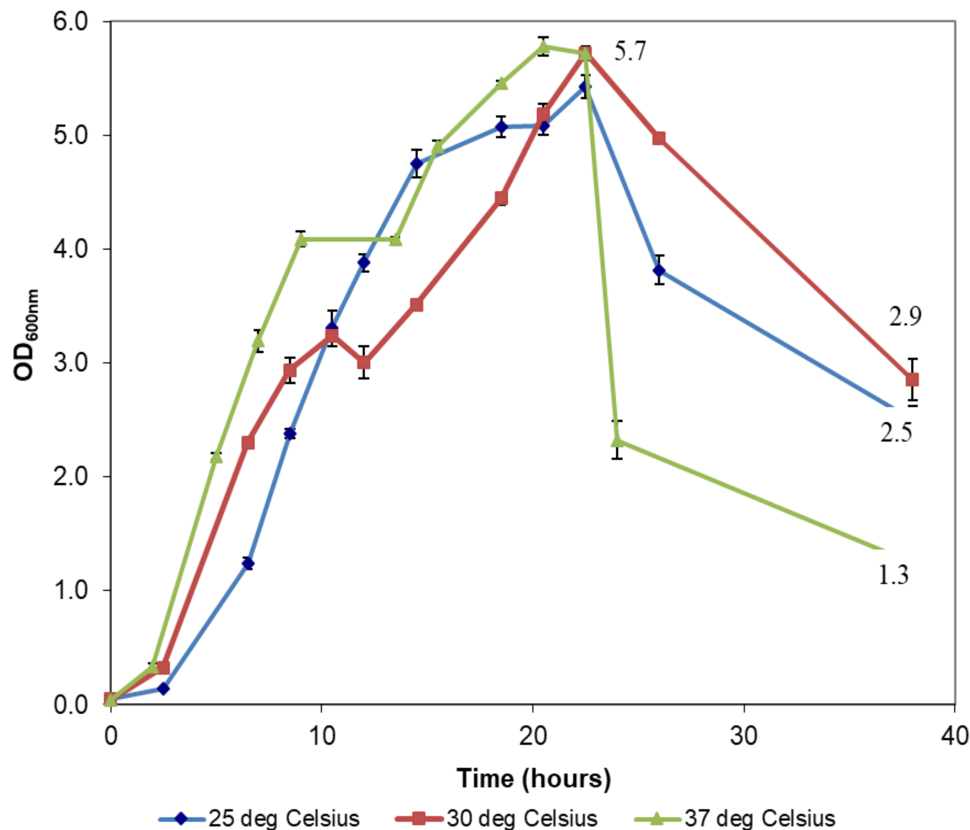

Figure 5a: Growth of *B. subtilis* NRS-762 at different temperatures (25, 30, 37 ºC) in LB Lennox medium. Irrespective of the growth temperatures, drastic decline in optical density occurred after entry into stationary phase, with the decline being most rapid during growth at 37 ºC, where induction of a temperature sensitive prophage induced cell lysis programme might have occurred.

To gain further insights into the decline of optical density after *B. subtilis* NRS-762 cultures reached stationary phase, full growth curves of high temporal resolution were obtained for the bacterium at temperatures of 25, 30 and 37 ºC. Growth of a microorganism at different temperatures offer a good gauge of its sensitivity to growth temperatures as well as offering insights into possible existence of temperature sensitive cell differentiation mechanisms; for example, temperature activated prophage induced cell lysis. Comparing *B. subtilis* NRS-762 growth in LB Lennox medium at different temperatures of 25, 30, and 37 ºC revealed that, while drastic decline in optical density after entry into stationary phase existed for all three temperatures, that which occurred at 37 ºC was significantly faster than those at 25 and 30 ºC, and suggested possible temperature activated entry into cell lysis programme by prophages resident in the

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genome of *B. subtilis* NRS-762. Specifically, optical density of *B. subtilis* NRS-762 declined rapidly from 5.7 at 22.5 hours post-inoculation to 2.3 after 24 hours of culture during growth at 37 ºC (Figure 5a). This is in stark contrast to the much slower decline observed in *B. subtilis* NRS-762 grown at 25 and 30 ºC. For example, optical density of *B. subtilis* NRS-762 grown at 25 ºC declined from 5.4 at 22.5 hours post-inoculation to 3.8 after 26 hours of culture, while that of *B. subtilis* NRS-762 grown at 30 ºC declined from 5.7 at 22.5 hours into the culture to 5.0 after 26 hours of cultivation. Relatively slow decline in optical density observed in *B. subtilis* NRS-762 culture grown at 30 ºC suggested possible presence of a cell survival mechanism that enabled the differentiation of cells into a cellular state different from cell lysis or cannibalism. While cannibalism likely existed in subpopulations of *B. subtilis* NRS-762 cells, co-existence of the cell survival programme in other subpopulations retarded the rate at which optical density declined. Nevertheless, observations of similar rates of optical density decline in *B. subtilis* NRS-762 cultures at 25, 30, and 37 ºC after 24 hours of culture, suggested that cannibalism and release of cell lysis factors might account for the observed drop in optical density in all three cultures. Thus, induction of prophage induced cell lysis at 37 ºC in *B. subtilis* NRS-762 was possibly followed by cannibalism.

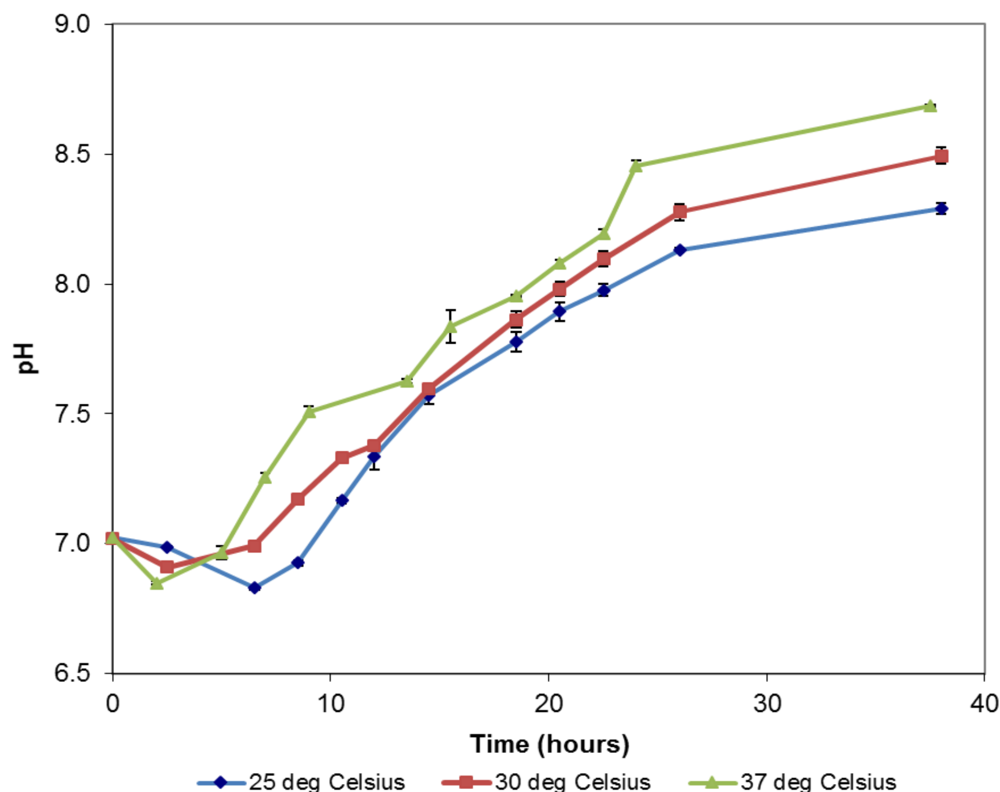

Figure 5b: pH variation in LB Lennox broth for *B. subtilis* NRS-762 grown at different temperatures of 25, 30 and 37 ºC.

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Comparison of the pH variation in LB Lennox broth for *B. subtilis* NRS-762 cultures grown at different temperatures revealed that while the same profile was observed for growth at different temperatures of 25, 30 and 37 °C, the overall pH profile became more alkaline the higher the growth temperature (Figure 5b). Additionally, after entry into stationary phase, the growth medium became more alkaline irrespective of the temperature of culture.

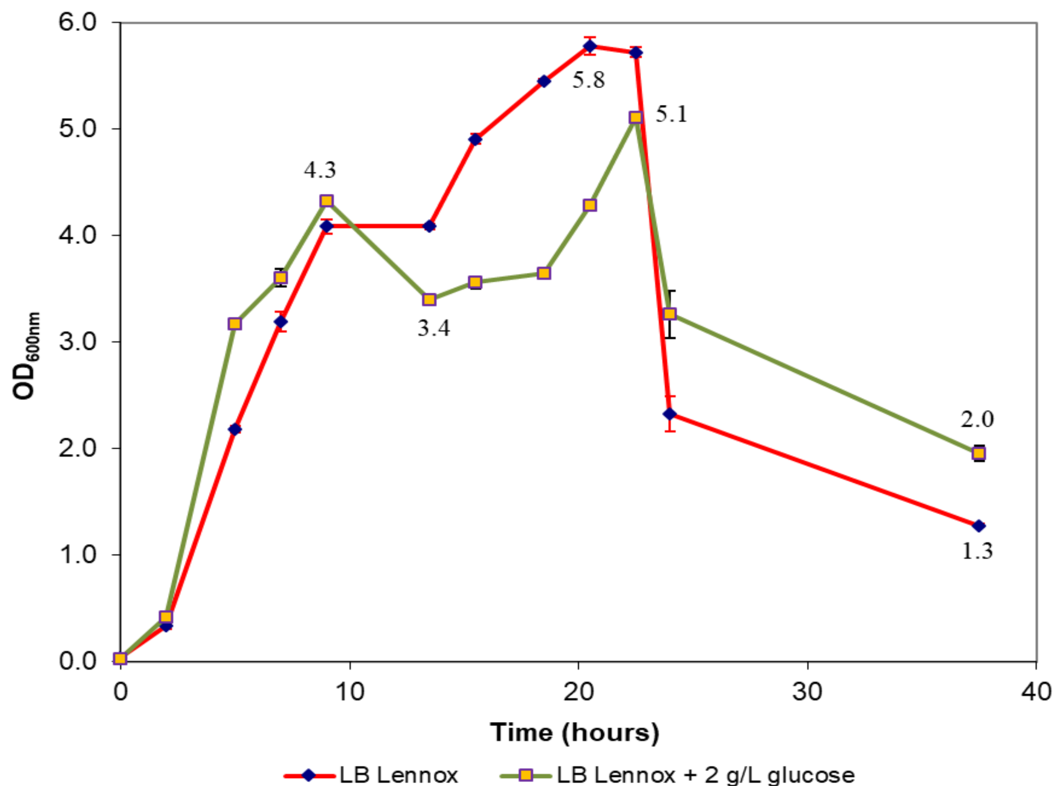

Figure 6a: Growth of *B. subtilis* NRS-762 in LB Lennox and LB Lennox supplemented with 2 g/L glucose at 37 °C and 230 rpm rotational shaking.

Growth of *B. subtilis* NRS-762 in LB Lennox medium supplemented with 2 g/L glucose revealed two distinct periods where there was decline in optical density (Figure 6a). Specifically, upon reaching maximal cell density in the first growth phase fuelled by glucose and other medium components, there was decline in optical density from 4.3 at 9 hours post-inoculation to 3.4 at 13.5 hours of cultivation. This is surprising compared to the growth performance of many other bacteria such as *Escherichia coli* DH5α, where cell population did not decline during the lag phase. The cell density subsequently rose from an optical density of 3.4 at 13.5 hours post-inoculation to 5.1 at 22.5 hours of cultivation, whereupon, there was drastic decline in optical density from 5.1 to 3.3 in 1.5 hours, which could be due to prophage induced cell lysis. Incidentally, similar drastic decline was also observed in *B. subtilis* NRS-762 grown in LB Lennox at the same time points. Specifically, optical density declined from 5.7 at 22.5 hours post-inoculation to 2.3 at 24 hours of cultivation, which could be due to prophage induced cell lysis.

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Finally, while optical density continued to decline between 24 and 37.5 hours post-inoculation, the rate of decrease in optical density and cell population was slower, which indicated that another mechanism of cell lysis and cell death was likely dominant. This was likely to be cannibalism, where cell lysis factors were secreted by resistant cells towards the entire population, where cell lysis and release of cellular contents as nutrients occurred in the non-resistant population. What accounts for the cessation of prophage induced cell lysis and induction of cannibalism would be a fascinating research question for cell biologists and microbiologists.

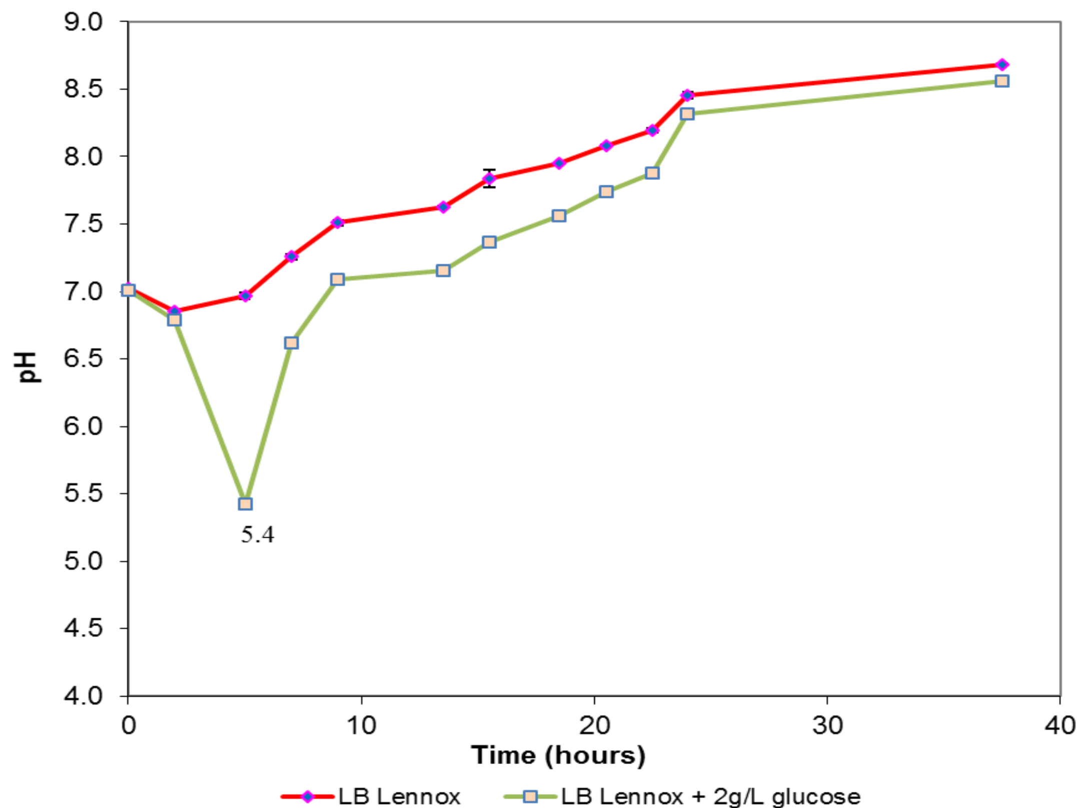

Figure 6b: Drastic decline and rebound in pH during growth of *B. subtilis* NRS-762 in LB Lennox medium supplemented with 2 g/L of glucose. After the decline, pH in the supplemented medium moved in lock-step with that of unsupplemented LB Lennox medium.

Whether secreted metabolites play a critical role in regulating *B. subtilis* NRS-762 decision to enter specific cellular differentiation pathways or inducing prophages to enter the lytic programme remains unresolved, but Figure 6b revealed that pH variation and overall secreted metabolites' acidity and alkalinity had only a small imprint on variation of optical density during the cultivation. Specifically, besides the drastic decrease in pH as *B. subtilis* NRS-762 cultivated in LB Lennox supplemented with 2 g/L glucose grew with glucose as the main carbon source, pH of both supplemented and non-supplemented LB Lennox medium moved in step with each other and, most importantly, had a low impact on variation of optical density.

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Comparison with another common model organism, *Escherichia coli*, revealed in more concrete detail why a microorganism unable to maintain a stable cell population during stationary phase was not suitable for studies seeking to understand the survivability of microorganisms under different environmental stressors. Figure 7 illustrated the growth profile of *E. coli* DH5α (ATCC 53868) and *B. subtilis* NRS-762 in LB Lennox medium at 37 ºC and 230 rpm rotational shaking in a temperature controlled incubator.

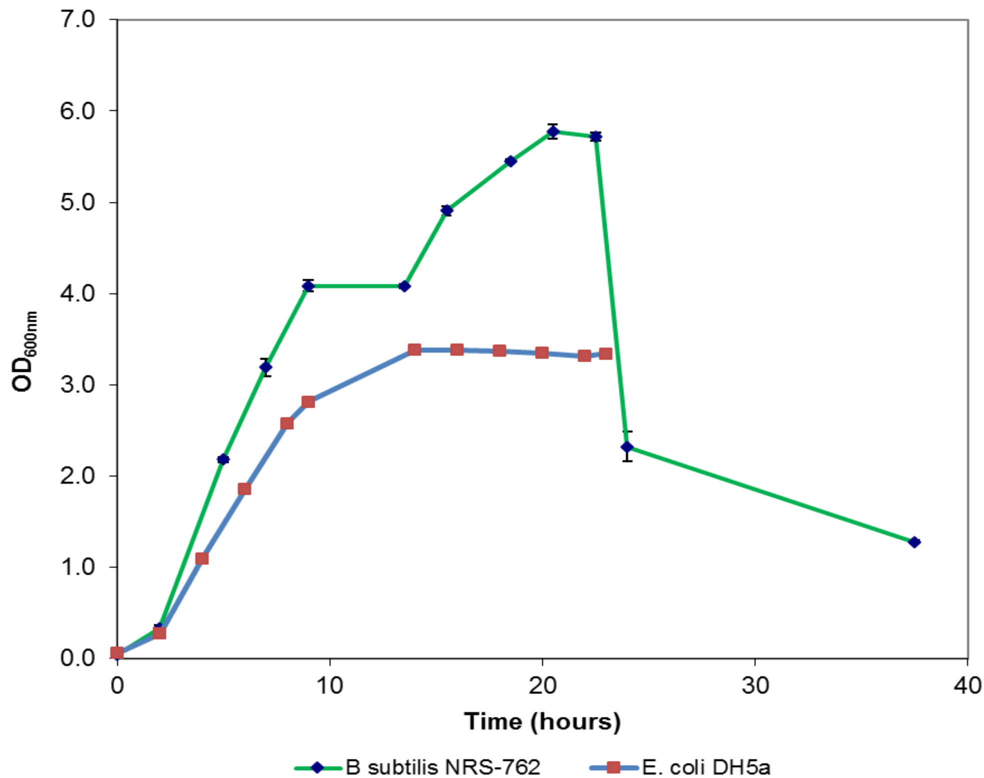

Figure 7: Growth curve of *E. coli* DH5α and *B. subtilis* NRS-762 in LB Lennox medium at 37 ºC and 230 rpm rotational shaking. Note the drastic decline in optical density and cell population of *B. subtilis* NRS-762 after it reached stationary phase, and the relatively stable optical density and cell population of *E. coli* DH5α after it reached maximal optical density. The growth profile also illustrated that *B. subtilis* NRS-762 was more efficient at biomass formation compared to *E. coli* DH5α due possibly to different mechanisms in which metabolic energy was allocated between cell maintenance and cell division.

Specifically, after reaching stationary phase, the optical density profile of *E. coli* DH5α remained stable, which highlighted that there was no significant cell lysis in the culture. The same was not true for *B. subtilis* NRS-762 cultured in LB Lennox at 37 ºC, where upon reaching stationary phase, there was a drastic decline in optical density, indicating significant cell lysis that could be accounted for by prophages entry into lytic programme and subsequent differentiation of the residual population into the cannibalism pathway. Hence, *E. coli* DH5α was a better model

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organism for understanding the survival of bacteria under various environmental stressors given that a stable cell population could be maintained long after entry into stationary phase. Additionally, Figure 8 revealed that the broth pH of *E. coli* DH5α culture remained fairly stable after entry into stationary phase, which was in good correspondence with a stable optical density. Given that broth pH is a proxy indicator of the types of metabolites secreted, it could be concluded that *E. coli* DH5α population at stationary phase did not secrete metabolites that could drastically alter the pH of the culture medium. This is a plus for a candidate model organism for microbial survivability test.

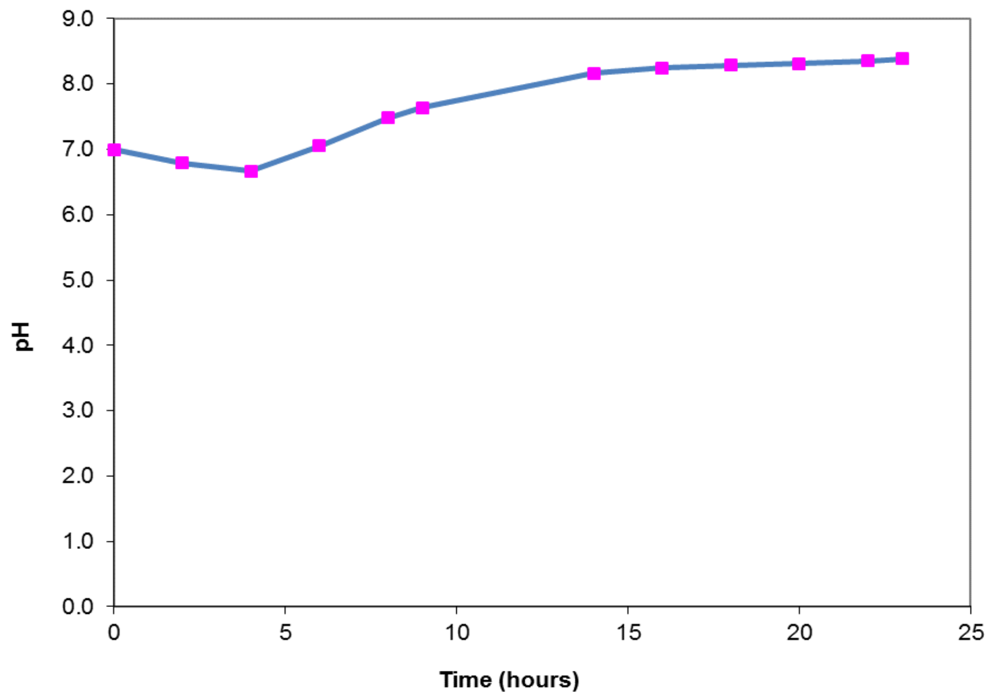

Figure 8: pH profile of *E. coli* DH5α grown in LB Lennox medium at 37 ^{o}C and 230 rpm rotational shaking, showing a stable pH profile after entry into stationary phase that corresponded with that of optical density, which highlighted that there was no significant alteration in broth's pH due to metabolites secreted during stationary phase.

Given that a stable cell population is needed as baseline in any microbial survivability test, drastic decline in optical density of *B. subtilis* NRS-762 at temperatures of 25, 30 and 37 ^{o}C during growth in commercial media would mean that the bacterium is not suitable as model organism for assessing the extent of fitness of the microbe in surviving various environmental stressors used in test scenarios. Hence, while *B. subtilis* NRS-762 possesses many characteristics useful as model organism in microbial survivability assays such as many cellular differentiation programmes for coping with nutritional starvation, its propensity to enter into either a cannibalistic differentiation programme or prophage induced cell lysis meant that it is not suitable as model organism for survivability studies where a stable cell population is a must as baseline. Induction of cannibalism

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can be viewed as a survival response for utilizing cellular contents of non-resistant cells as metabolic energy for maintenance metabolism, where the overall cell population no longer expands, but went into decline.

Conclusions

Survival of different microbes in differing environments and conditions is of interest given the importance of understanding how microbes behave in various environments, as well as efficacies of various methods used to kill them for purposes related to healthcare provision, drinking water treatment, probability of finding microbial life on other planets, and food processing. Given the large diversity of microbes present on Earth, model organism representative of microbial metabolism and propensity to survive under specific nutritional conditions need to be identified and used in microbial survivability assays for assessing the general survival characteristics of microbes under different conditions. But, what are the criteria for such a model organism? The primary selection criterion would be the ability of the microbe in maintaining a large stable cell population after entry into stationary phase. Secondly, given the presence of different subpopulations in a habitat, each with a different metabolic programme in sync with the cellular differentiation lineage or fate entered, a model organism capable of entering a variety of cellular differentiation programmes is desired. This is in large part due to the need to understand how variation in environmental conditions could help kill a persistent microbe.

Hence, taking the above into account, *B. subtilis* is a candidate model organism for microbial survivability studies given its metabolic diversity and ability to enter into different cellular differentiation programmes on occurrence of nutrient starvation. Such cellular differentiation programmes include sporulation, vegetative growth, filamentation, competence, stringent response, persister state, and cannibalism. However, experiment observations of growth of *B. subtilis* NRS-762 in LB Lennox and TSB, revealed drastic decline in optical density after entry into stationary phase at 25, 30 and 37 ^{o}C in aerobic cultures. Thus, the bacterium fails the criterion for qualifying as a model organism for microbial survivability studies: i.e., the ability to maintain a stable cell population during stationary phase. More importantly, lack of cellular debris after autoclave decontamination of cell culture broth likely came about from cannibalism of non-resistant *B. subtilis* NRS-762 cells exposed to cell lysis factors secreted by a cannibalistic subpopulation. Cell lysis induced by prophages resident in *B. subtilis* NRS-762 genome likely accounted for the drastic decline in optical density after entry into stationary phase during growth at 37 ^{o}C in LB Lennox and TSB medium, but was not prevalent at other cultivation temperatures given the likely temperature sensitive nature of the switch to lytic programme by the prophage. On the other hand, an unknown cell survival programme was likely activated during culture of *B. subtilis* NRS-762 at 30 ^{o}C in LB Lennox medium that reduced the rate of optical density decline. Comparison of cell survival during stationary phase revealed that *E. coli* DH5α was a better model organism for microbial survivability studies given the ability of the bacterium in maintaining a stable cell population during stationary phase. pH profile of *E. coli* DH5α also indicated that there

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was relatively small fluctuation in the pH of the culture broth which correlated with a stable cell population during stationary phase. Overall, *B. subtilis* NRS-762 achieved high cell density during growth in TSB and LB Lennox medium using a metabolism unable to synthesize essential vitamins (and thus, unable to grow in unsupplemented minimal medium). But, drastic decline in optical density after entry into stationary phase meant that it is not suitable as model organism for microbial survivability studies.

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Conflicts of interest

The author declares no conflicts of interest.

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