

The Effects of Different Carbon Sources on the Growth of *Rhodobacter sphaeroides*

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Abstract

Rhodobacter sphaeroides is a purple non-sulfur bacterium that belongs to the α -3 subdivision of *Proteobacteria*. *R. sphaeroides* is a model bacterial species because of its complex genome structure and expanded metabolic capabilities. The genome of *R. sphaeroides* consists of two circular chromosomes and five endogenous plasmids. It has the ability to grow under a wide variety of environmental conditions. It grows aerobically (~20% O₂), semi-aerobically (~2% O₂), and photosynthetically (under anaerobic condition plus light). It has been previously shown that many bacterial species utilize a number of alternate carbon sources for their optimal growth under a variety of growth conditions. We hypothesize that different or an additional carbon source in the minimal medium differentially affects the bacterial growth under dark-aerobic conditions. The bacterial growth kinetics and the number of cells in the bacterial culture were analyzed by measuring the optical density (OD at 600 nm) and the colony forming units (CFUs) at regular intervals of bacterial cultures. Results reveal that sodium succinate is the preferred sole carbon source for the optimal growth of *R. sphaeroides*. The results of growth kinetics and CFUs together concluded that from the tested carbon sources, sodium succinate is the best single carbon source in the minimal media for the optimal growth of *R. sphaeroides*. Interestingly, cell culture grown in SIS supplemented with sodium acetate exhibits a prolonged lag phase with the lowest ODs and CFUs that later switches to the growth-burst phase support previously discovered similar phenomenon of the growth-rate switch in the presence of acetate metabolism. Future work will utilize the aerobically grown *R. sphaeroides*' cells as a biocatalyst to deplete the oxygen levels from natural gas streams and industrial gas pipelines.

Keywords

Rhodobacter sphaeroides, Aerobic, Sistrom (SIS), Carbon Source, Optical Density (OD), Colony Forming Units (CFUs)

1. Introduction

Around the world and in the United States, the corrosion of natural gas pipelines has been causing millions of dollars to be wasted each year, and is an important issue of economic concern [1]. The prevention and reduction of the corrosion of gas pipelines is desirable for both financial and human safety. Several methods of preventing the corrosion of gas pipelines, such as coating the inside of the pipe and using a cathode are popularly used. These methods still pose threats and are not very effective.

In order to develop environmentally- and eco-friendly methods to deplete oxygen from the gas pipelines and natural gas streams, *Rhodobacter sphaeroides* and other related species can be utilized as biocatalysts [2]. *R. sphaeroides* is a gram-negative bacterium that belongs to the α -3 subdivision of *Proteobacteria*. The complex genome of *R. sphaeroides* consists of two chromosomes [3], and encodes for an expanded range of metabolic capabilities. *R. sphaeroides* grows in a variety of laboratory growth conditions, which include aerobic (~20% O₂), semi-aerobic (~2% O₂), anoxygenic photosynthesis under light, and anaerobic growth conditions [4]. These distinct traits make *R. sphaeroides* adaptable to grow in a diverse set of environments naturally and in the laboratory. Using *R. sphaeroides* as a biocatalyst to prevent the corrosion of gas pipelines has a variety of advantages. *R. sphaeroides* is easy and cost-effective to grow, ecologically-friendly, nonpathogenic, and is less cumbersome to manipulate compared to other microorganisms [5]. Employment of *R. sphaeroides* as a biocatalyst can be potentially utilized for the depletion of oxygen and in the bioremediation of contaminants and undesirable byproducts such as amines, glycol, and organic acids [6] [7]. Therefore, the characteristics that *R. sphaeroides* possesses makes it more suitable to develop for industrial applications and bioremediation processes.

In aerobic respiration, *R. sphaeroides* uses oxygen as the final electron acceptor in the electron transport chain [8]. Introducing *R. sphaeroides* into the gas pipeline system will reduce the levels of oxygen by consuming oxygen present in the gas mixture. *R. sphaeroides* will grow in conditions that mimic the growth parameters of the gas pipeline system. This includes growing the bacterium in dark-aerobic conditions and a nearly neutral pH. Therefore, the understanding of the growth characteristics and kinetics of *R. sphaeroides* under these conditions will provide important insights to benefit future research that lay down the foundation for future projects.

Previous experiments revealed that a variety of different bacterial species utilize alternative carbon sources to reach optimal growth in a range of environments [9] [10] [11]. This study is designed to develop a minimal media in which *R. sphaeroides* can grow optimally under dark aerobic growth conditions. The optimal bacterial growth will be determined by assaying optical density (OD) and colony-forming units (CFUs) of the bacterial cultures grown in media with either a single carbon source or combination of different carbon sources. We test

the two following hypotheses: 1) Growth minimal media containing different carbon sources will have different effects on *R. sphaeroides* growth, and 2) the optimal minimal media with additional carbon source will have different growth effects on *R. sphaeroides*.

2. Materials and Methods

2.1. Bacterial Strain and Growth Media

The wild-type bacterial strain, *R. sphaeroides* 2.4.1 (originally isolated by C.B. Van Niel) was used in this study. The bacterial cells were originally grown aerobically in SIS minimal medium [12], which contains sodium succinate as a carbon source. The bacterial cell cultures were stored in 15% glycerol at -80°C .

2.2. Growth Conditions and Carbon Source

Bacterial strain was obtained from the frozen stock and streaked out on a minimal SIS-Agar plate to isolate a single colony. The plate was incubated at 30°C for 96 hours to grow bacterial colony optimally. A single *R. sphaeroides* bacterial colony was isolated and inoculated into a 10 mL liquid SIS medium. The liquid culture was grown aerobically for 72 hours reaching a logarithmic phase of growth with an OD of ~ 0.700 at 600 nm of visible light. The optimal log phase of growth was determined to be in the range of 0.600 - 0.800 [13]. All samples were inoculated with *R. sphaeroides* and carbon source on the same day at the same time as previously described.

Using SIS minimal media recipe, a modified version of this recipe was formulated with alternate carbon sources or SIS supplemented with additional carbon source with a final 50 mM concentration. Carbon sources included fructose, sorbitol, sodium acetate, L-arabinose, sodium butyrate, sodium citrate, sodium pyruvate, maltose, or mannose.

2.3. Sample Preparation

For suitable experiment purposes a 50 mL centrifuge tube was used for each bacterial culture. In each centrifuge tube, 28.2 mL of SIS, 1.5 mL of carbon source, were added. 300 μL of *R. sphaeroides* pre-grown cells was inoculated into the 50 mL tube containing ~ 30 mL fresh liquid medium. Three replicate bacterial cultures for each carbon source were grown for a total of 15 samples. The cultures were incubated at 30°C for five days in a shaking incubator at 120 rpm. All samples were grown aerobically at an atmospheric concentration in $\sim 20\%$ oxygen in the dark.

All samples were inoculated with *R. sphaeroides* and carbon source simultaneously. At every 24-hour time interval (24, 48, 72, 96 and 120 hours), the tubes were removed, and the 3 mL of the culture samples were collected for a total of 120 hours for CFU analysis and OD measurements at 600 nm in **Table 1**.

Table 1. CFU collected from both experiments over 120 hours.

Obs.	Carbon	Treatment	24 h	48 h	72 h	96 h	120 h
1	SIS	A	6.00E+07	1.00E+08	2.16E+08	1.79E+08	9.60E+07
2	SIS	A	5.50E+07	7.20E+07	2.90E+08	1.61E+08	1.82E+08
3	SIS	A	7.50E+07	9.90E+07	2.48E+08	2.29E+08	1.66E+08
4	SIS + Sodium Acetate	B	4.00E+06	7.00E+06	2.40E+07	6.30E+07	1.26E+08
5	SIS + Sodium Acetate	B	5.00E+06	2.00E+06	2.30E+07	8.30E+07	1.24E+08
6	SIS + Sodium Acetate	B	2.00E+06	4.00E+06	1.70E+07	5.70E+07	1.13E+08
7	SIS + Sodium Pyruvate	C	8.70E+07	7.30E+07	1.36E+08	1.21E+08	1.05E+08
8	SIS + Sodium Pyruvate	C	8.40E+07	9.20E+07	1.14E+08	1.27E+08	1.30E+08
9	SIS + Sodium Pyruvate	C	6.00E+07	7.70E+07	1.28E+08	1.36E+08	1.50E+08
10	SIS	D	8.60E+07	8.50E+07	1.90E+08	1.73E+08	8.50E+07
11	SIS	D	1.03E+08	1.63E+08	2.02E+08	1.27E+08	1.39E+08
12	SIS	D	7.70E+07	6.30E+07	1.54E+08	1.79E+08	1.34E+08
13	Sodium Acetate	E	3.30E+07	3.70E+07	8.50E+07	1.73E+08	7.10E+07
14	Sodium Acetate	E	3.20E+07	8.10E+07	1.39E+08	1.27E+08	4.30E+07
15	Sodium Acetate	E	3.40E+07	7.70E+07	1.34E+08	1.79E+08	2.10E+07
16	Sodium Pyruvate	F	7.90E+07	8.60E+07	1.25E+08	1.23E+08	1.08E+08
17	Sodium Pyruvate	F	8.60E+07	6.60E+07	1.32E+08	1.14E+08	1.19E+08
18	Sodium Pyruvate	F	6.80E+07	1.08E+08	1.28E+08	1.11E+08	1.09E+08

Numbers represent the total viable cells for each treatment group. Treatment group refers to the type of carbon source contained in the media (A: SIS, B: SIS + Sodium Acetate, C: SIS + Sodium Pyruvate, D: SIS, E: Sodium Acetate, and F: Sodium Pyruvate).

2.4. Optical Density, Colony Forming Units, and Colony Morphology

Analyses of growth-kinetics were performed for different time intervals of bacterial growth. A growth curve was plotted using time and absorbance of each sample collected with OD measurements in **Table 2**. The OD was measured twice for each sample to ensure accurate reading at 600 nm.

The CFUs were counted to determine the number of viable cells in each sample. A serial dilution (in range of 10^{-2} to 10^{-10}) was prepared for each sample, and 100 μ L of each diluted sample was plated out containing plastic beads. The beads were removed after swirling and sitting for 15 minutes. The plates were incubated at 30°C for 9 - 120 hours to grow full-size bacterial colonies. All colonies were counted, and then extrapolated as CFUs/mL in the original culture. Colonies were also examined for their normal and altered colony morphology.

2.5. Statistical Analysis

Statistical analyses were performed in the R version 3.6.0 with the R stats package [14]. The optical density and colony forming unit data were analyzed using the repeated measures ANOVA with Tukey's honest significant difference test, which uses pairwise comparisons to determine differences between groups. Statistical significance was set at $p < 0.05$ on all cases.

Table 2. Absorbance collected from both experiments over 120 hours.

Obs.	Carbon	Treatment	0 hr	24 h	48 h	72 h	96 h	120 h
1	SIS	A	6.00E-03	4.84E-01	1.28E+00	1.74E+00	1.81E+00	1.74E+00
2	SIS	A	6.00E-03	4.58E-01	9.75E-01	1.59E+00	1.91E+00	1.75E+00
3	SIS	A	6.00E-03	6.71E-01	1.62E+00	1.95E+00	1.81E+00	1.69E+00
4	SIS + Sodium Acetate	B	6.00E-03	2.30E-02	6.80E-02	1.58E-01	5.39E-01	1.78E+00
5	SIS + Sodium Acetate	B	6.00E-03	2.70E-02	7.10E-02	1.69E-01	6.10E-01	2.01E+00
6	SIS + Sodium Acetate	B	6.00E-03	2.20E-02	6.25E-02	1.42E-01	4.64E-01	1.67E+00
7	SIS + Sodium Pyruvate	C	6.00E-03	8.39E-01	2.38E+00	3.24E+00	4.00E+00	4.08E+00
8	SIS + Sodium Pyruvate	C	6.00E-03	8.30E-01	2.38E+00	3.41E+00	3.69E+00	4.31E+00
9	SIS + Sodium Pyruvate	C	6.00E-03	8.48E-01	2.47E+00	3.26E+00	3.96E+00	4.27E+00
10	SIS	D	6.00E-03	8.64E-01	1.42E+00	1.86E+00	1.81E+00	1.70E+00
11	SIS	D	6.00E-03	7.88E-01	2.02E+00	1.79E+00	1.69E+00	1.60E+00
12	SIS	D	6.00E-03	5.63E-01	1.16E+00	1.73E+00	1.92E+00	1.74E+00
13	Sodium Acetate	E	6.00E-03	3.81E-01	9.25E-01	7.84E-01	7.28E-01	6.84E-01
14	Sodium Acetate	E	6.00E-03	3.97E-01	7.61E-01	6.38E-01	6.38E-01	6.26E-01
15	Sodium Acetate	E	6.00E-03	4.11E-01	7.16E-01	6.08E-01	6.06E-01	5.96E-01
16	Sodium Pyruvate	F	6.00E-03	4.62E-01	9.54E-01	9.48E-01	8.92E-01	8.26E-01
17	Sodium Pyruvate	F	6.00E-03	4.70E-01	9.46E-01	9.18E-01	8.62E-01	8.48E-01
18	Sodium Pyruvate	F	6.00E-03	4.81E-01	9.25E-01	8.92E-01	8.50E-01	8.08E-01

Numbers represent the absorbance of the cell culture at 600 nm wavelength for each treatment group. Treatment group refers to the type of carbon source contained in the media (A: SIS, B: SIS + Sodium Acetate, C: SIS + Sodium Pyruvate, D: SIS, E: Sodium Acetate, and F: Sodium Pyruvate).

3. Results and Discussion

As an initial screening (data not shown), results indicated that the rates of *R. sphaeroides*' growth were increased for the cell culture grown in SIS media supplemented with sodium pyruvate or sodium acetate, compared to the growth rates obtained for the cell culture grown in either SIS medium alone or SIS supplemented with fructose, sorbitol, L-arabinose, sodium butyrate, sodium citrate, maltose or mannose. This initial observation prompted further investigation of *R. sphaeroides*' growth using sodium succinate, sodium pyruvate or sodium acetate as a sole carbon source, and SIS minimal media that contain sodium succinate with additional carbon source, sodium pyruvate or sodium acetate.

3.1. Bacterial Growth in Minimal Media with Single Carbon Source

The results of ODs and CFUs for the bacterial cultures grown in the minimal media with a single carbon source (sodium succinate, sodium pyruvate or sodium acetate) are shown in **Figure 1(a)** and **Figure 2(a)**, respectively. Results for the ANOVA analyses for ODs and CFUs shown in **Figure 1(a)** and **Figure 2(a)** are given in **Figure 1(b)** and **Figure 2(b)**, respectively. Results exhibit that the ODs reflecting the cellular biomass over a continuous time-course of the

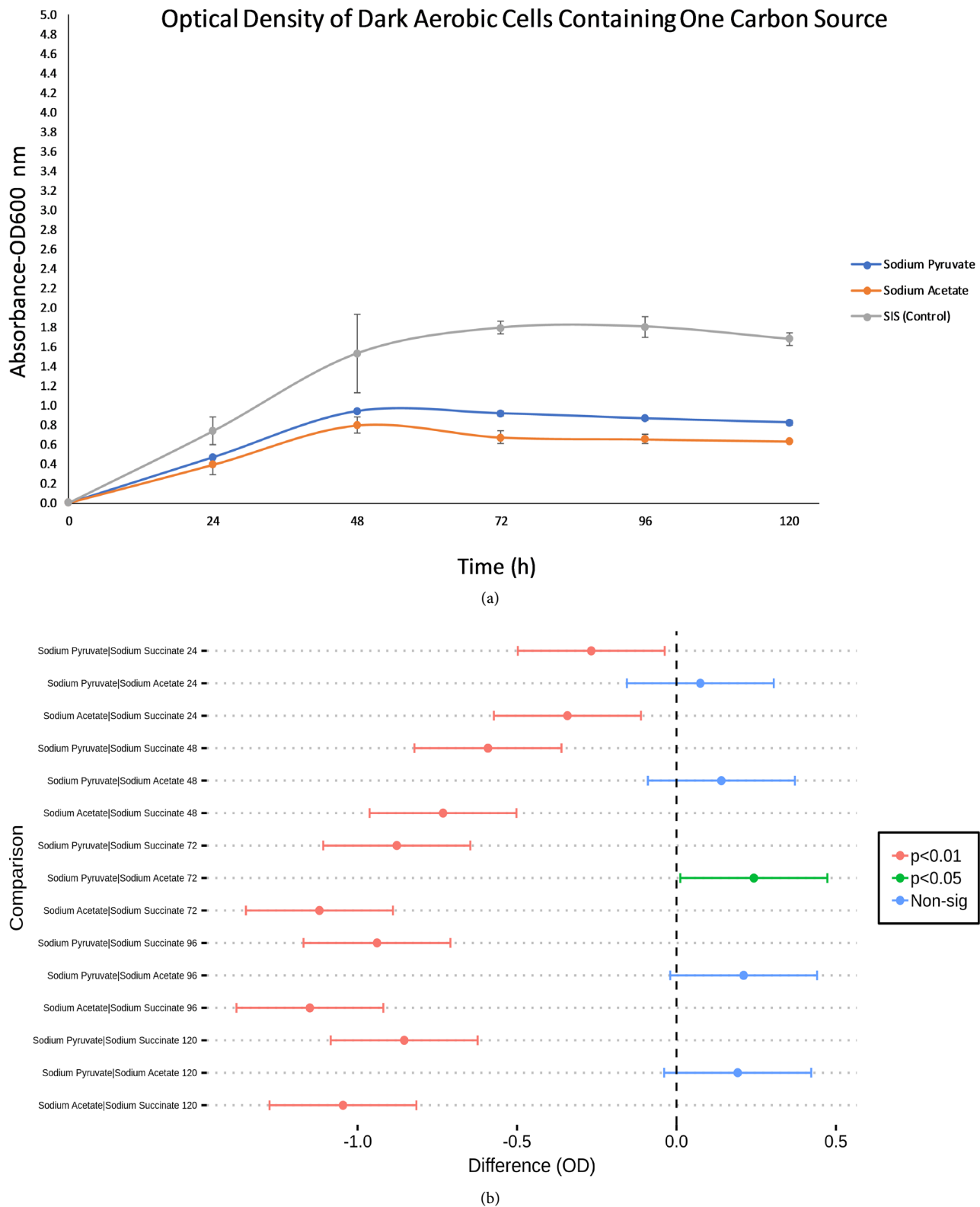


Figure 1. (a) A growth curve plot containing standard error bars was constructed using the OD readings of each sample of *R. sphaeroides* containing one carbon source. Two samples from each treatment were collected every 24 hours for 120 hours. The cells are represented in treatment groups D, E, and F in **Table 1**. Error bars represent mean \pm SE; (b) Tukey's pairwise comparison of the OD values for different carbon source groups at each time point.

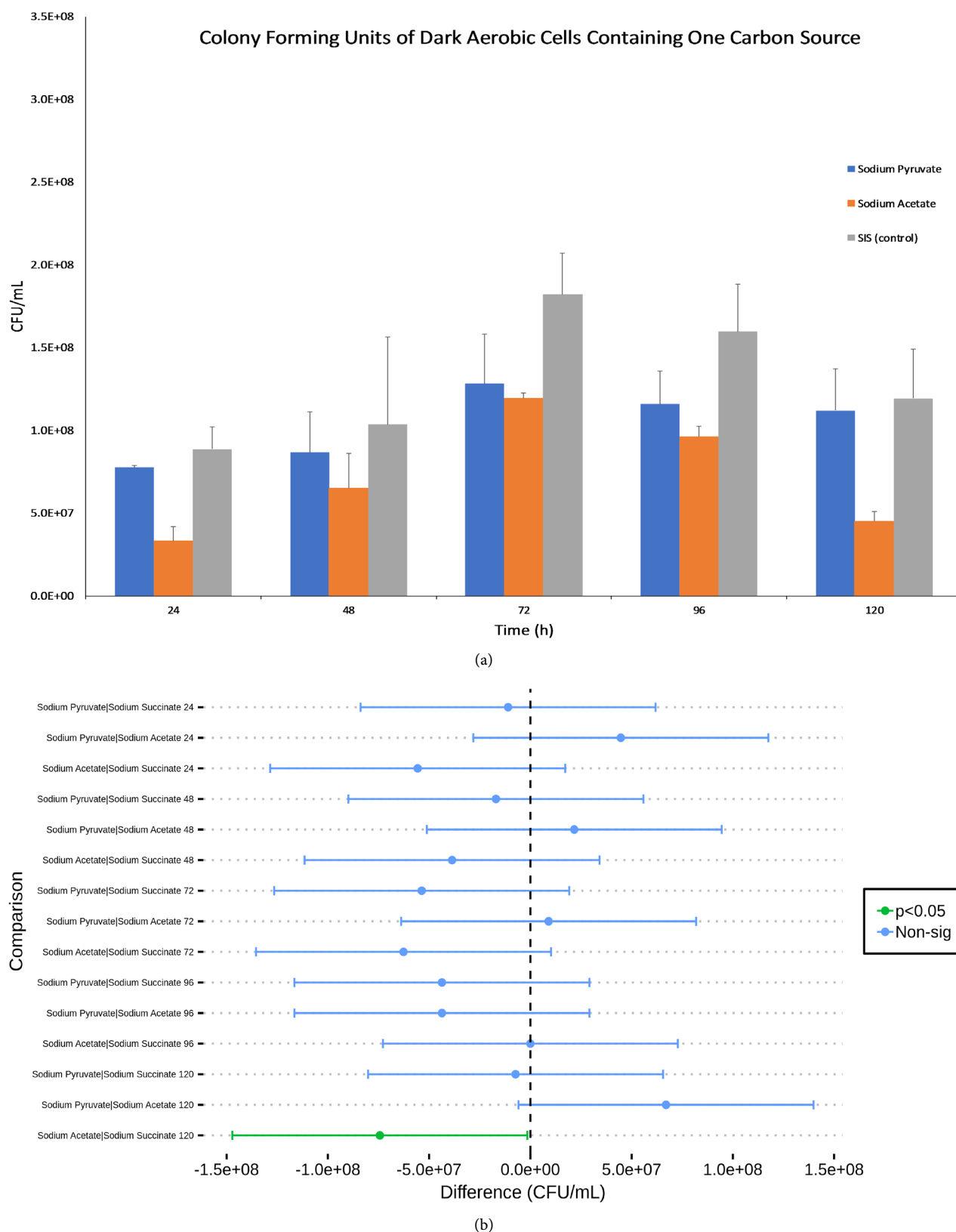


Figure 2. (a) A distribution of live viable cells of *R. sphaeroides* grown with one carbon source. The figure contains the mean of CFU collected every 24 hours for 120 hours. The cells represent treatment groups D, E, and F in **Table 2**. Error bars represent mean \pm SE; (b) Tukey's pairwise comparison of CFU values for different carbon source groups at each time point.

bacterial cultures grown in the media with sodium succinate are significantly higher ($p < 0.01$) than the ODs of the bacterial cell culture grown in either sodium pyruvate or sodium acetate. Although the ODs of the bacterial culture grown in sodium pyruvate are higher than the ODs of the bacterial culture grown in sodium acetate, the OD differences are not significantly different, except at the 72-hour culture ($p < 0.05$). This could be due to a small sample size of three bacterial cultures. The CFU counts of the bacterial cultures grown in media containing sodium succinate (SIS) were also higher than the CFU counts in the bacterial cultures grown in media containing sodium pyruvate or sodium acetate. However, CFUs differences are not significantly different in most of these comparisons, except between bacterial cultures grown in sodium succinate or sodium acetate at the 120 hour time point ($p < 0.05$). The ODs and CFUs of the bacterial cultures grown in media containing sodium acetate were the lowest of the three treatment groups. The results of growth kinetics and CFUs together concluded that from the tested carbon sources, sodium succinate is the best single carbon source in the minimal media for the optimal growth of *R. sphaeroides*.

3.2. Bacterial Growth in Minimal Media with Additional Carbon Source

Sodium succinate is the original formulation of the SIS minimal media [15]. In order to examine if additional carbon source (sodium pyruvate or sodium acetate) added to the SIS media produce any significant growth-rate effects on *R. sphaeroides*, SIS minimal media was further supplemented with sodium pyruvate or sodium acetate. The ODs and CFUs of the bacterial cultures grown in SIS as well as SIS media supplemented with sodium pyruvate or sodium acetate are shown in **Figure 3(a)** and **Figure 4(a)**, respectively. Results for the ANOVA analyses for ODs and CFUs shown in **Figure 3(a)** and **Figure 4(a)** are given in **Figure 3(b)** and **Figure 4(b)**, respectively.

Bacterial cultures grown in SIS supplemented with sodium pyruvate have higher ODs ($p < 0.01$) compared to the bacterial cultures grown in either SIS (sodium succinate) alone or SIS supplemented with sodium acetate throughout all time points. In addition, bacterial cultures grown in SIS (sodium succinate) have higher ODs ($p < 0.01$) compared to the bacterial cultures grown in SIS supplemented with sodium acetate throughout all time points, except at 120 hour time point, where the difference in ODs are not significantly different. It is noteworthy that bacterial cultures grown in SIS supplemented with sodium acetate have the lowest OD through a prolonged adaptive lag phase followed by a growth burst phase observed at 72-hour time point. This finding is consistent with the growth-switch phenomenon in acetate supplemented SIS media. Cellular growth is impeded by acetate fermentation where acetate is selected as the first choice of carbon source, and then later bacterial cells switch to sodium succinate for aerobic respiration. In contrast, the CFU counts are surprisingly not consistent with the observed ODs of the bacterial cultures grown under these growth conditions. Cell

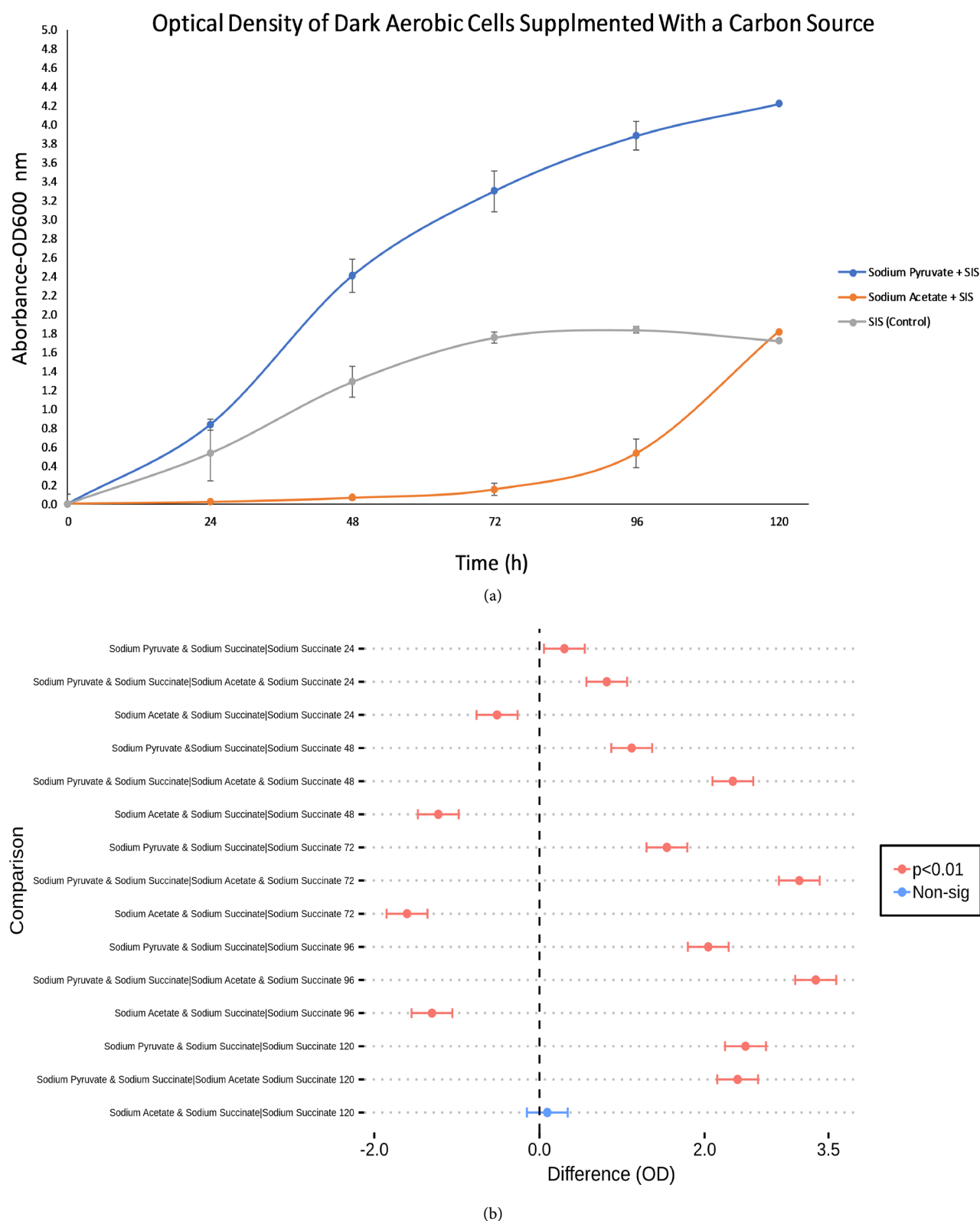


Figure 3. (a) Growth curve was constructed using the OD readings of each sample of *R. sphaeroides* containing two carbon sources. Two samples from each treatment were collected every 24 hours for 120 hours. The cells are represented in treatment groups A, B, and C in **Table 1**. Error bars represent mean \pm SE; (b) Tukey's pairwise comparison of the OD values for single and supplemented carbon source groups at each time point.

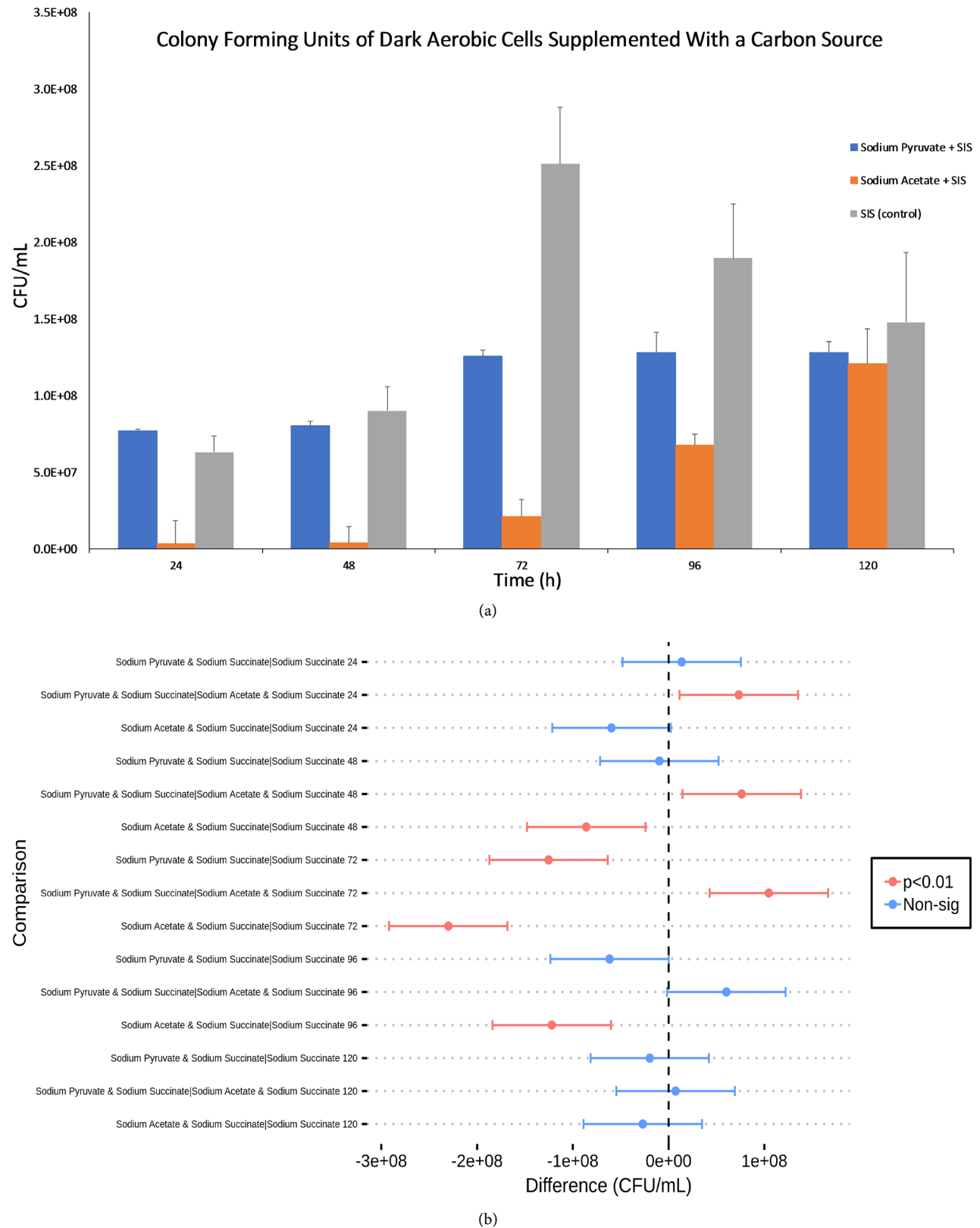


Figure 4. (a) A distribution of live viable cells of *R. sphaeroides* grown with cells supplemented with a carbon source. The figure contains the mean of CFU collected every 24 hours for 120 hours. The cells represent treatment groups A, B, and C in **Table 2**. Error bars represent mean \pm SE; (b) Tukey's pairwise comparison of CFU values of cells.

cultures grown in SIS media alone have higher CFUs counts compared to the CFU counts for the cell cultures grown in SIS supplemented with sodium pyruvate, however the CFUs differences are not significantly different, except at the 72 hour time point ($p < 0.01$). Also, CFUs of the bacterial cultures grown in SIS alone are significantly higher ($p < 0.01$) than the CFUs of the bacterial cultures grown in SIS supplemented with sodium acetate at three (48, 72, 96 hours; $p < 0.01$) of the total five time points. In addition, CFUs of the bacterial cultures grown in SIS supplemented with sodium pyruvate are significantly higher than the CFUs of the bacterial cultures grown in SIS supplemented with sodium acetate at three (24, 48, 72 hours, $p < 0.01$) of the total five time points. These above results concluded that on the basis of CFU counts, minimal medium with sodium succinate carbon source alone is the preferred carbon source for the optimal growth of *R. sphaeroides*. It is usually assumed that high ODs correlate with the high number of cells, but the unexpected results contradict this assumption. This may be due to the fact that the OD measures the cellular biomass that includes both live and dead cells, but CFUs only provides the counts of the live cells. Therefore, CFUs is a reliable indicator of the bacterial growth kinetics in the liquid culture.

Cell culture grown in SIS supplemented with sodium acetate exhibit a prolonged lag phase with the lowest ODs and CFUs, and then later switches to the growth burst phase at about 72-hour time point. This observation was corroborated with the previous finding, which has previously discovered the similar phenomenon of the growth-rate switch in presence of acetate metabolism [16]. The growth-rate switch is observed where bacteria utilize anaerobic acetate-fermentation as a means of ATP production regardless of other available metabolites and high oxygen tension [16]. Bacteria revert to the acetate metabolism, which allows the low energy production because of the finite space within their inner bacterial membrane [17]. The metabolites flood the membrane and the cost of holding them outweighs the advantage of producing energy because it impedes the electron transport function [18]. When supplemented with environmental resources, *R. sphaeroides* adapts well to its expanded metabolic capabilities, and becomes permeable to metabolites. This explains why the ODs shown in **Figure 3** do not correlate with the CFUs counts as shown in **Figure 4**. There are fewer viable cells that remained in the liquid culture as seen in the CFUs counts, however more dead cells are reflected in OD due to the explosion of the membranes or drastic loss of ATP synthesis. This two-carbon source system also clearly exemplifies surprising growth-switch effect on *R. sphaeroides*.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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