Journal of Experimental Microbiology and Immunology (JEMI) Vol. 1:1-8

The Role of Metabolic By-Product Secretion by *Escherichia coli* in Relation to Intracellular NADH Concentration and Re-Dox Potential

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Escherichia coli **utilizes by-product secretion in oxygen limiting conditions to recycle NADH and balance redox. NADH concentration and by-product secretions were qualitatively correlated with oxygen availability. NADH concentration was maintained in aerobic condition; however, increased as oxygen became limiting. GC analysis showed different by-product excretion patterns with varying oxygenation. Acetate was secreted equally under all conditions.**

Escherichia coli, like other facultative anaerobes, is capable of growth under both oxic and anoxic conditions (15). The availability of O_2 strongly influences the function of the catabolic pathways (1). In the presence of oxygen, energy can be obtained through aerobic respiration. Alternately, in the absence of oxygen or another suitable electron acceptor, fermentation is responsible for the production of ATP by substrate level phosphorylation (4). Sugars such as glucose can be used by *E.coli* cells as sole carbon and energy sources. Glucose is usually transported into the cell by the phosphotransferase system and catabolized to pyruvate (3). The availability of oxygen or another electron acceptor determines the further metabolism of pyruvate. With an electron acceptor, the NADH produced in glycolysis and the TCA cycle can be oxidized by the electron transport chain to produce energy by oxidative phosphorylation (3). In this way, energy is efficiently produced and the NADH/NAD^+ ratio is effectively balanced. When suitable electron acceptors are limited, fermentation begins (3). The TCA cycle and pyruvate dehydrogenases that produce large amounts of NADH become non-functional (3). However, the *E.coli* cell must still re-oxidize the NADH produced by glycolysis so the glycolytic reaction can continue (3).

Since the carbon substrate is only partially oxidized during fermentation, by-product secretion occurs. The secretion of metabolic by-products allows the generation of energy and balances the cellular redox. The types of fermentation by-products secreted are determined by the energy-to-redox ratios of the by-products and the energy and NADH recycling demands of the cell. To elucidate the relationship between by-product secretion and degree of oxygenation of a culture, by-product secretion patterns were investigated. These patterns were then related to the NADH concentrations and the redox potentials of an *E.coli* wild-type culture.

MATERIALS AND METHODS

E.coli K-12 wild-type strain B23 (supplied by Dr. Ramey, UBC), in M9 media supplemented with 0.2% glucose, was incubated overnight in a shaking water bath at 37°C. The overnight cultures (with turbidity greater than 1.0 OD_{460}) were diluted 1-in-50 in fresh media (M9 + 0.2% glucose) to a final volume of 1000ml. These diluted cultures were grown in a bioreactor (UBC Microbiology Workshop). Hot water pumped through tubing surrounding the bioreactor heated the culture to approximately 30°C. To create the aerobic test conditions, an air pump connected to an aquarium stone with tubing bubbled air through the culture. For the oxygen-stressed culture, oxygen was displaced from the bioreactor by bubbling compressed N_2 through the culture. 0.001g/L of reazurin was added to the oxygen-stressed culture as an anaerobic indicator. Reazurin has bright blue colour in oxygenated conditions, pink in oxygen limiting conditions and colourless in anaerobic conditions. Culture readings commenced only after the oxygen-stressed culture exhibited a bright pink colour.

When the culture concentration reached 0.1 OD_{460}, culture properties were followed for 2 hours. Redox and pH readings were collected using redox (ORP Redox Combination Electrode, Broadley James Corp.) and pH (Fisher) probes calibrated according to the manufacturer's protocol. The NADH concentration was determined using the FluroMeasure system (BioChem Technology Inc.). The data was acquired and compiled using FERMAC software (BioChem Technology Inc.).

Culture samples were collected every 10 minutes throughout the two-hour growth period and the concentration was determined by measuring the optical density with 460 nm light. Glucose concentrations for each sample were determined with the anthrone assay (10). Metabolic by-products production was determined by analysis of 0.5 µl of culture supernatant by gas chromatography with a DBwax capillary column (J&W Columns) and a flame ionization detector (Sigma 3B, Perkin Elmer). The injector and detector temperatures were 210 ^oC and 220 ^oC, respectively. The initial oven temperature was 50 ^oC and was ramped at 8 ^oC/minute to a final temperature of 150 ^oC. Helium was used as the carrier gas with a flow rate of 30 cm^3/min.

Under aerobic culture conditions, the concentration of NADH was relatively stable for the two-hour growth period, at approximately 200 Normalized Fluorescence Units (NFU) (Fig 1A). However, a small decrease was seen between 20 minutes and 60 minutes followed by an increase for the final 60 minutes. This decrease in NADH concentration occurred concurrently with a decline in growth rate. As seen in Fig 1B, the growth of the aerobic culture slowed slightly between 30 minutes and 60 minutes then increased again for the remainder of the 120-minute growth period. Because the NADH pool per viable cell mass remains relatively constant under a given metabolic state, cell concentration is proportional to NADH concentration (2). Therefore, the decline in NADH concentration is presumably due to the decrease in growth rate.

Additionally, in the final 30 minutes of the culture measurements, the NADH concentration fluctuated several times between approximately 195 and 190 NFU. Similar oscillations were also observed in the redox potential measurements. Redox reflects the reducing potential or the NADH concentration of the culture, so an increased NADH concentration would result in more reduced cell having redox potential. Therefore, it is logical that these trends correspond. In minutes 80 t0 90, an increase in redox was accompanied by a decrease in NADH; however, at 100 minutes an increase in redox was accompanied by an increase in NADH. The latter trends are contradictory. Therefore, these deviations may be better attributed to sensitivity of the instrumentation and not significant changes in the *E.coli* culture so can be ignored. The measurements were affected by slight movements of the equipment that may have occurred in the final forty minutes of the experiment.

RESULTS AND DISCUSSION

In the oxygen-stressed conditions, the NADH concentration was initially constant at approximately 206 NFU. Beginning at 40 minutes, the concentration increased steadily reaching a value of 232 NFU. Similarly, the redox potential measurements remained level for the first 20 minutes then dropped for the next 80 minutes. For the final 20 minutes, the redox decrease slowed and converged to approximately –150mV. As explained above, an increase in NADH concentration corresponds with a decrease in redox potential so the observed trends (Fig 1) were expected. As growth continued, the oxygen-stressed culture conditions became more oxygen limiting and metabolism may have shifted from aerobic to anaerobic. In the anaerobic metabolic state, the cell is more reduced and will have a more negative redox potential. Therefore, the drop in redox is a result of the metabolic shift.

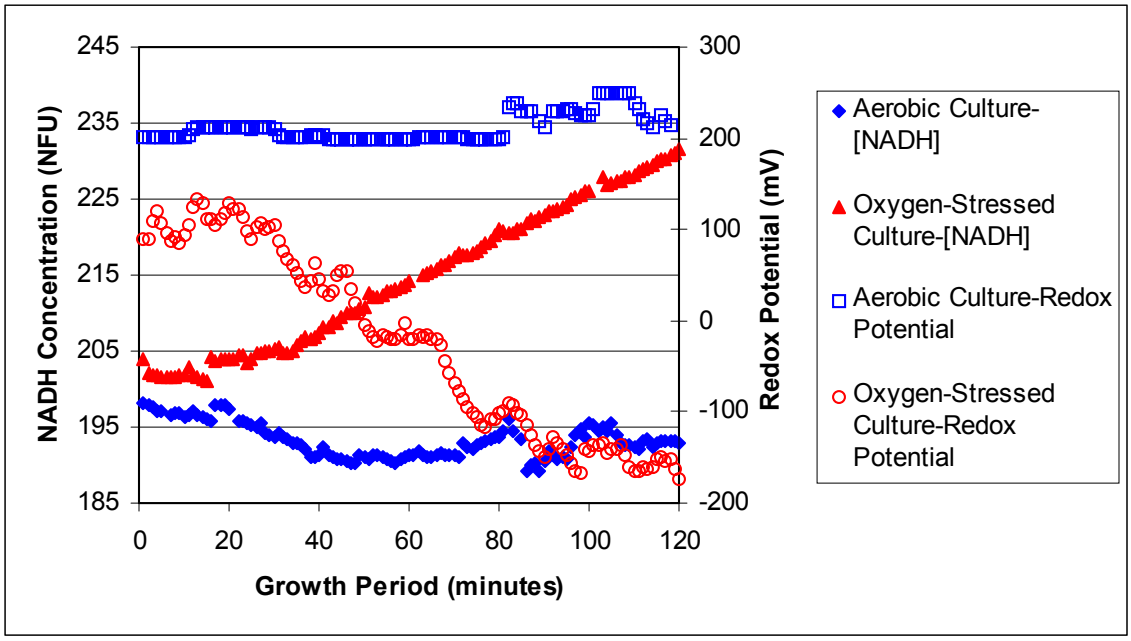
The growth rate of the oxygen-stressed culture (Fig 1B) gradually slowed for the first 50 minutes of the experiment and then increased for the final 70 minutes. The lag period may be a result of the shift from aerobic to anaerobic metabolism. For optimal function and to avoid energy waste, the synthesis of enzymes from various pathways in aerobic and anaerobic metabolism is tightly regulated at the transcriptional level (3). FNR, a global regulator, responds to redox potentials and represses the proteins such as cytochrome oxidase needed for aerobic respiration (8). Additionally, the two-component system ArcA/B, regulates aerobic respiration enzymes when exposed to anaerobic conditions (3). Another important regulator, FhlA, controls expression of formate hydrogen-lyase by sensing the formate, a key by-product of fermentation (3). The lag in growth is the period where synthesis of the components needed for anaerobic metabolism occurs. Once the synthesis is complete, fermentation can begin and growth rate will increase again.

Once fermentation begins, the recycling of NADH is not as efficient and is reflected by an increase in NADH concentration (Fig 1A). However, fermentation is capable of NADH recycling; therefore, NADH concentration will not infinitely increase but will be maintained at a higher concentration. Because the NADH concentration increased for the span of the experiment, the equilibrium concentration of NADH in fermenting cells may not have been reached. Alternatively, error in the results due to the fluorescent means of NADH concentration measurements may be masking the true trend. It is difficult to extract meaningful information from the complex NADH fluorescent measurement system (6). Humphrey et al concluded that monitoring a fermentation process using only NADH fluorescence was not sufficient (7). Inner filter effect and quenching processes decrease the sensitivity of measurement. In addition, the polychromaticity of the excitation and detection systems complicates the analysis of the measurement by obscuring the fluorescence yield due to intracellular NADH (14). Alternate means of NADH concentration measurements may be useful in future studies.

To relate NADH concentration and redox potential to metabolic by-product secretion patterns, the components of the culture media were monitored for the two-hour growth period. The glucose depletion trends are shown in Fig 2. Despite small increases in the oxygen-stressed culture, an overall decrease in glucose concentration was seen. The irregular glucose depletion may be a result of improper mixing. The slow bubbling of N_2 was the only means of stirring the culture and may have been insufficient. The aerobic culture trend was smoother because the air was bubbled more vigorously through the culture than the N_2 allowing for more complete mixing. Surprisingly, glucose depletion trends were similar under both conditions. Facultative anaerobes usually sustain a higher level of growth under aerobic conditions and therefore the glucose demands are higher (9). However, under these experimental conditions, the aerobic culture growth was only slightly higher than the growth of the oxygen-stressed culture (Fig 1B). It then follows that glucose depletion in the aerobic culture would resemble that of the oxygen-stressed culture in this experiment. Several experimental details may have contributed to the hindrance of the *E.coli* growth under the aerobic conditions. The experiment was conducted under non-aseptic conditions. Contaminants such as ethanol that was used to clean the bioreactor may have been present during the aerobic culture growth. In addition, the culture was heated using hot water pumped through tubing that encircled the bioreactor. This system of heating was not precise, and it was difficult to stabilize the temperature of the culture. The temperature may have been lower under the aerobic conditions as compared to the oxygen-stressed culture, thus retarding the growth rate.

Under fermentation conditions, as in the oxygen-stressed culture, many of the by-products produced are fatty acids that create acid culture conditions. Therefore, by-product accumulation can be ascertained by measuring the pH of

A.



B.

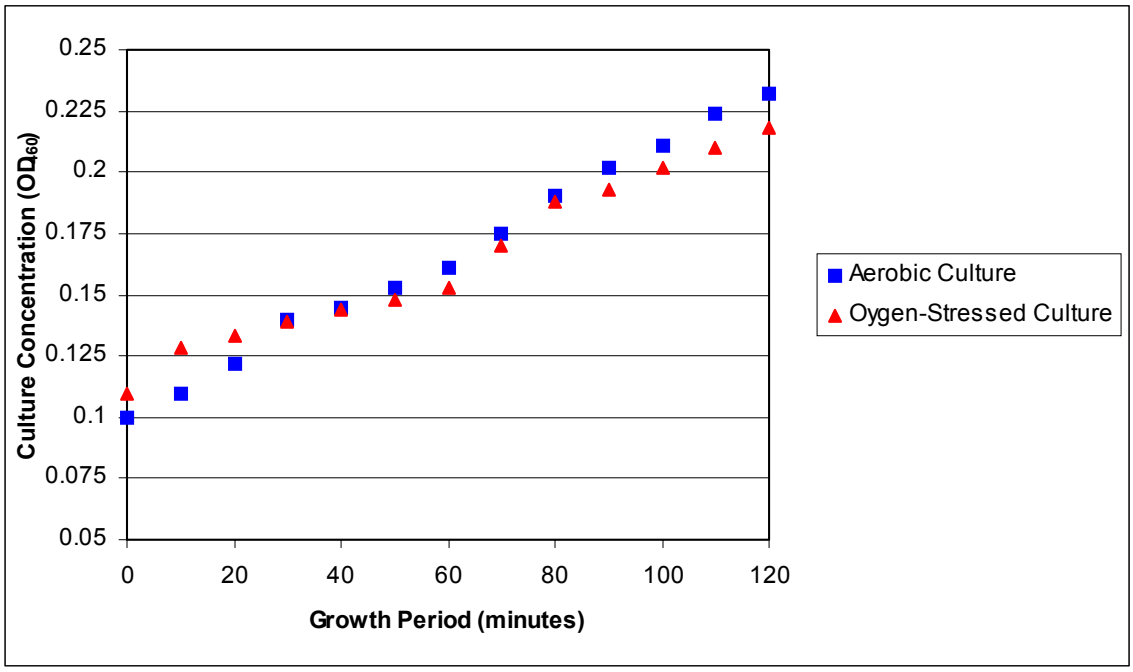


FIG 1. The relationship between (A) NADH concentration, (A) redox potential and (B) culture concentration of the aerobic and oxygen-stressed cultures. NADH concentration and redox potential measurements were acquired every five seconds for the two hour growth period using the FERMAC software. Measurements averaged over one minute time periods were plotted. Culture concentration was determined every ten minutes for the two hour growth period.

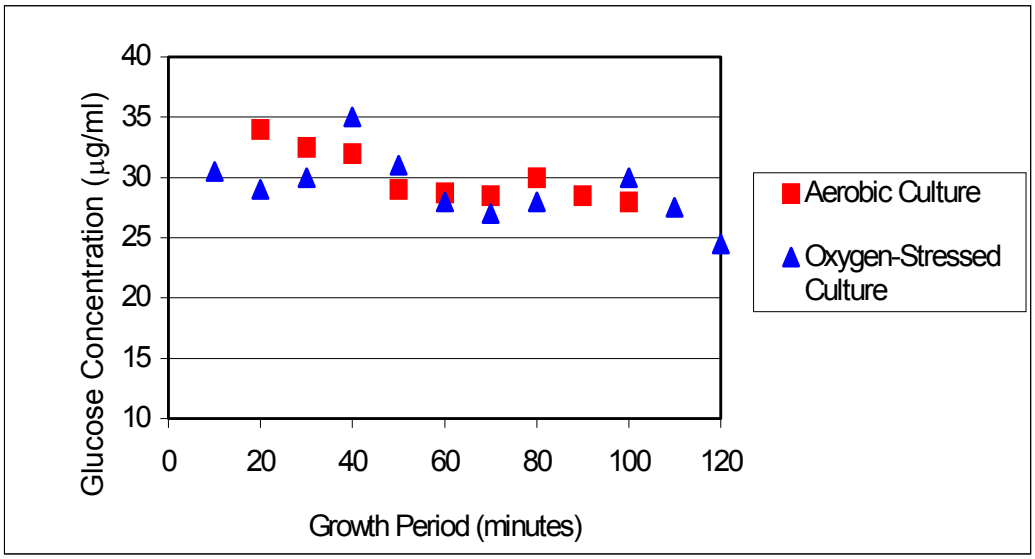


FIG 2. Glucose depletion from the M9 media supplemented with 0.2% glucose by the oxygen-stressed and the aerobic culture. Glucose concentration of the M9 media was determined at ten minute intervals for the two hour growth period.

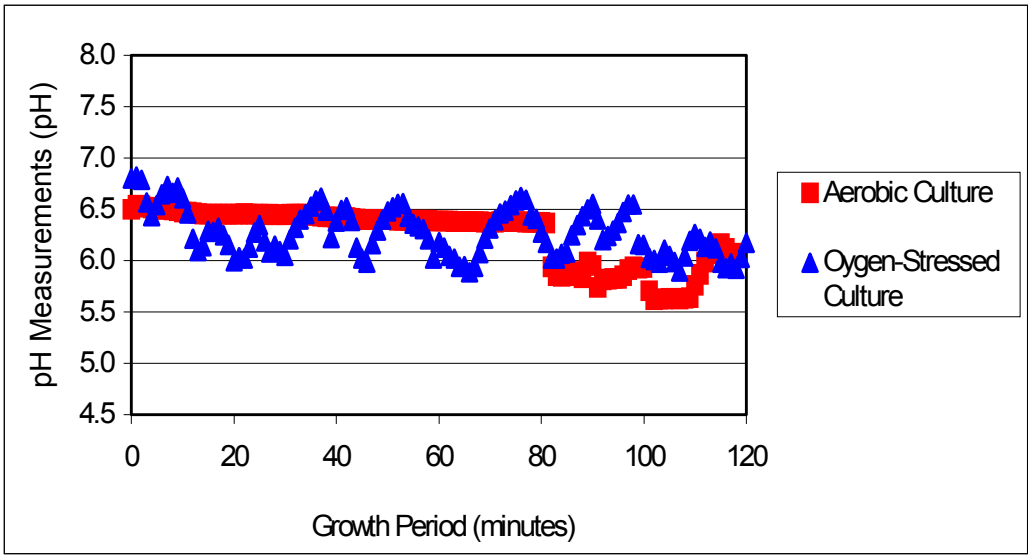


FIG 3. pH of the aerobic and oxygen-stressed cultures over the two hour growth period. pH measurements were acquired every 5 seconds using FERMAC software. The average pH for one minute periods were plotted.

the culture. As seen in Fig 3, both aerobic and oxygen-stressed cultures maintained a stable pH with small fluctuations between pH 6.8 and 5.5. The M9 minimal media contained significant amounts of phosphate that may have acted to buffer the culture; therefore, pH was a poor indicator of metabolic by-product accumulation. In future experiments, minimal media without buffering ability should be used to allow meaningful pH readings to be obtained.

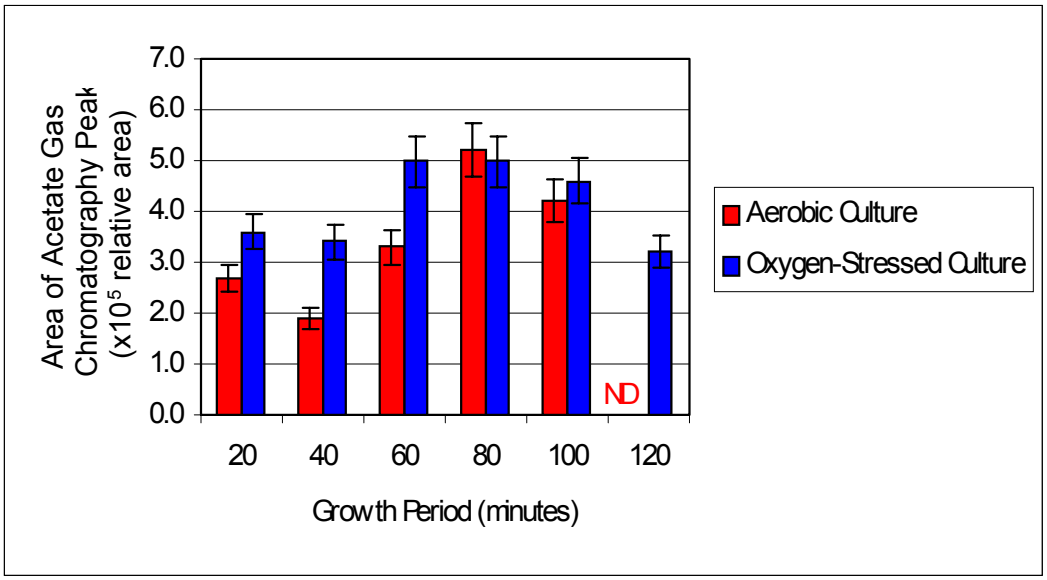


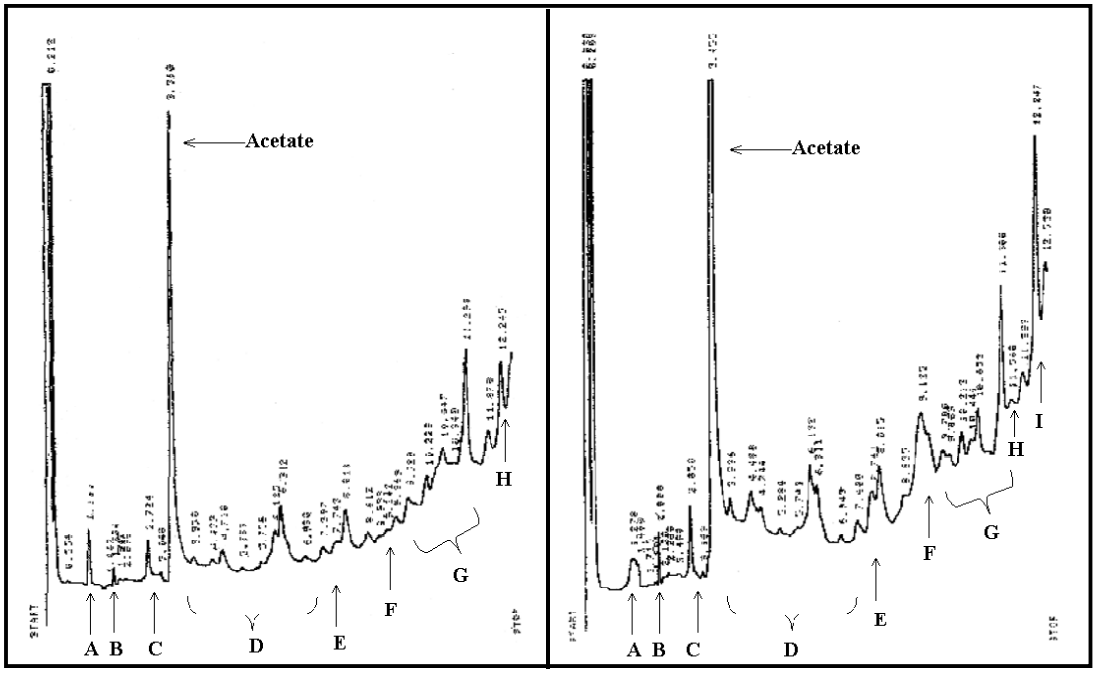
Fig 4. Relative area of the acetate peaks from the GC analysis of the aerobic and oxygen-stressed cultures. Culture media was removed at twenty-minute intervals for the two hour growth period. 0.5µl of sample was analyzed by GC and the acetate peak was identified by comparison to a standard. ND represents samples that were not analyzed by GC so the acetate peak area was not determined.

To further investigate by-product secretion patterns, culture samples were analyzed using gas chromatography (GC). An acetate peak was identified under both culture conditions. Fig 4 shows the relative amount of acetate produced over the 120-minute growth period. Under both culture conditions, the relative concentration of acetic acid increased through the growth period. In the oxygen-stressed culture, accumulation of acetate can be related to the onset of fermentation. The main by-products of fermentation are succinate, lactate, acetate, ethanol, formate, CO_2 and H_2 (8). As illustrated by the large GC peak (Fig 5), acetate was a major fermentation by-product. The concentration of acetate declined in the latter part of the growth period (Fig 4). This may indicate a shift in fermentation by-product secretion from acetate to other products (mentioned above). In the aerobic culture, acetate accumulation results from inefficient glucose usage. In aerated cultures containing glucose, *E.coli* excrete approximately 10-30% of the carbon flux from glucose as acetate (4). Acetate is produced when the uptake of glucose by the cells is greater than the demands for biosynthesis and energy production (4,5). This excretion of acetate has been contributed to limitations of the TCA cycle. In addition, Farmer et al showed acetate production was partially due to limitations of anaplerotic reactions such as the phosphoenolpyruvate carboxylase pathway and the glyoxylate bypass that lead to biosynthesis (4). Regardless, the utilization of glucose by *E.coli* is not optimal. Acetate may be dumped after energy has been derived by substrate level phosphorylation (4).

The GC patterns of culture supernatant analysis are shown in Fig 5. The media composition changed significantly from the 40-minute time point (Fig 5A and 5C) to the 100-minute time point (Fig 5B and 5D) under both culture conditions. In the aerobic culture (Fig 5A and 5B), peaks A, B, C, E, F and I increased with time. In addition, in areas D and G the patterns of the peaks changed substantially from 40 minutes to 100 minutes with several peaks increasing and new peaks appearing at the latter time point. The appearance and the increase of peaks indicate the accumulation of metabolic by-products. Compounds that volatize at higher temperatures such as those seen to the right of area D (Fig 5B) may represent fatty acid by-products. Furthermore, *E.coli* may metabolize substrates inefficiently when glucose is abundant in the culture causing the accumulation of " overflow metabolites" such as

A. Aerobic Culture: 40 minutes

B. Aerobic Culture: 100 minutes



C. Oxygen-Stressed Culture: 40 minutes

D. Oxygen-Stressed Culture: 100 minutes

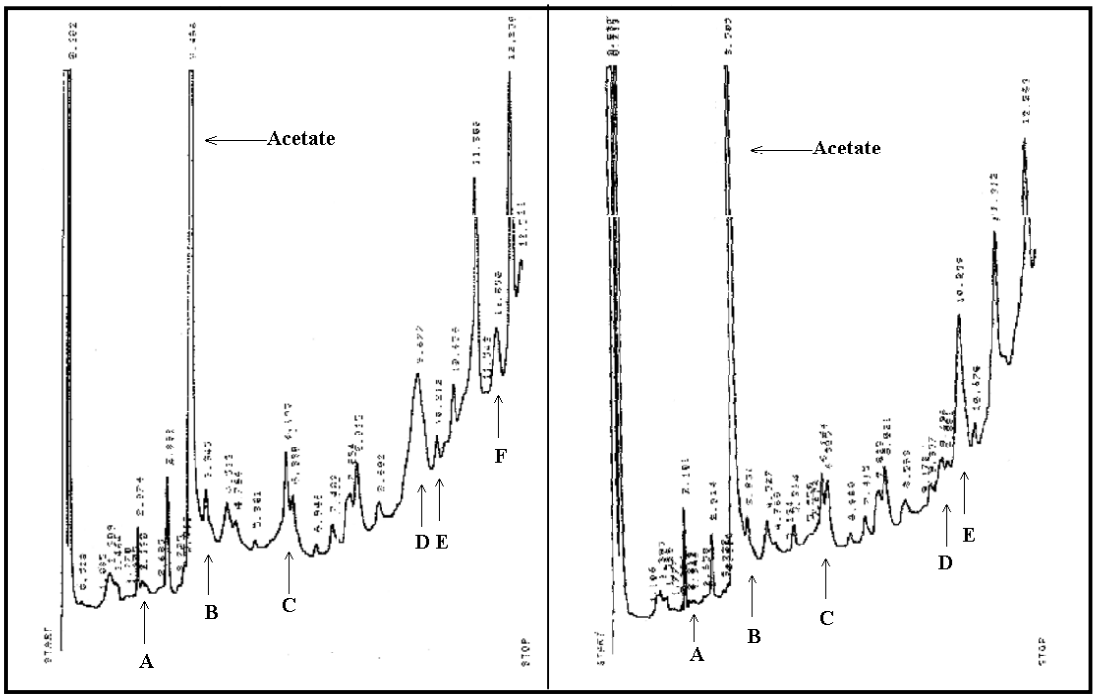


FIG 5. Gas chromatography analysis of the aerobic culture and the oxygen-stressed culture. 0.5µl samples from (A/C) the 40 minute and (B/D) the 100 minute time points were analyzed.

pyruvate, 2-ketoglutarate and D-lactate. These " overflow metabolites" may account for the appearance of peaks later in the growth period.

In the oxygen-stressed conditions (Fig 4C and 4D), peaks A, B and D decreased and peak F disappeared completely. Also, peaks C and E increased from 40 minutes to 100 minutes (Fig 5C and 5D). The decreased peaks may represent components of the media that are being depleted. However, the minimal media components would not be volatile in the GC conditions applied. Alternatively, these peaks may be by-products that were secreted in the early stages of the culture when oxygen is more abundant. As glucose and oxygen become limiting, these by-products may be further catabolized for energy; however, this phenomenon is usually associated with diauxic growth trends because enzymes for use of the new metabolites need to be induced. As diauxic growth trends were not evident, a more plausible explanation may be associated with the small GC sample size (0.5). If the media is not completely homogeneous, the GC samples may vary between analyses creating a variation in GC patterns. Finally, GC analysis has approximately a 10% error rate. GC patterns are not easily compared, as small changes are more likely due to error than differences of the samples. The increased peaks may indicate the accumulation of metabolic by-products such as formate, lactate, succinate or ethanol. Acetate secretion provides maximum energy from substrate level phosphorylation; however, it causes a redox surplus (16). Thus, acetate is produced when oxygen is still available so respiration can still function to eliminate NADH build-up. As the oxygen is exhausted, alternate fermentation products such as ethanol and succinate are produced to provide a suitable redox drop (16). In addition, formate is produced from the degradation of pyruvate to acetyl coenzyme A as a direct means of eliminating redox surplus (16). Ethanol is very volatile and the GC conditions of this experiment may not have isolated the ethanol peak from the earliest group of peaks. Lower initial oven temperature and a smaller ramp could verify the existence of ethanol as a by-product.

Since standards were not used in the gas chromatography analysis, the GC peaks could not be correlated to specific metabolic by-products. Therefore, quantitative comparisons between the aerobic and oxygen-stressed cultures could not be conducted. Using qualitative means, differences in GC patterns between the aerobic and oxygen-stressed cultures were identified. Peaks D and E of the oxygen-stressed culture were absent in the aerobic culture. In addition, peak F of the aerobic culture was missing in the oxygen-stressed culture. Furthermore, area G of the aerobic culture was significantly different than the equivalent area in the oxygen-stressed culture. Several large peaks such as peak E emerged in the oxygen-stressed culture. These differences in GC patterns are due to differences in by-products secretion. The aerobic and oxygen-stressed cultures employ different catabolic pathways to obtain energy and balance cellular redox. Consequently, different catabolic end products will accumulate in the two cultures and are reflected in GC patterns.

In future studies, by-product secretion analysis can be expanded. Quantitative analysis may be conducted by mixing internal standards into the sample or by running standards between sample runs. The peaks of the sample can be normalized against the standard peak so changes in baseline and peak area can be normalized. Furthermore, some metabolic by-products such as CO_2, H_2 and lactate may not be detected by the methods used in this experiment. Enzymatic bioanalysis kits are available from Boehringer Mannheim for the detection of lactate as well as formate and succinate (http://www.r-biopharm.com). High-performance liquid chromatography (HPLC) can also be used to determine carboxylic acids (12). Gas chromatography with a thermal conductivity detector may be used to analyze H_2 production (13). In addition, CO2 analysis is usually conducted by commercially available probes based on infrared detection methods or by mass spectrometry (12).

Taken together, a definite relationship between NADH concentration, oxygenation degree and by-product secretion of a wild type E.coli culture could not be ascertained. However, changes in NADH concentration and by-product secretion were qualitatively correlated to varied culture oxygenation. With validation of the NADH measurement method and accurate identification and quantification of excreted by-products, more defined relationships can be characterized in future studies.

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