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Controlling the Growth Rate of Escherichia coli by Limiting the Supply of Carbon in an Amylose-Amylase Nutrient System

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Bacteria in their natural habitat are often faced with adequate yet poor environmental conditions that limit their growth. Under such conditions, *Escherichia coli* (*E. coli*) cells have been shown to adapt and respond to their environment by altering the transcription of many genes involved in carbon and energy metabolism as well as the uptake of available carbon sources. Furthermore, previous studies have demonstrated that in environments with limiting nutrient, the rate of growth becomes proportional to the extracellular concentration of the limiting nutrient. In this study, such principles were exploited to devise a method by which the growth rate of *E. coli* could be specifically controlled through a controlled maltose-liberating amylose-amylase system. The effect of different amylase concentrations on the growth rate of *E. coli* in minimal media containing amylose were evaluated by turbidity-based growth curves. Our study suggested that there were no significant differences between the growth rates of the cells grown in the presence of different amylase concentrations. However, the cell production levels appeared to increase with increasing concentrations of the amylase. The similar growth rates could arise if the levels of maltose produced by the amylase are sufficient to saturate transport. The different production levels corresponded with different lag phases, and might indicate that the cells are sufficiently damaged or stressed at levels of maltose that are insufficient to sustain growth increases.

When faced with conditions where all necessary components for energy transduction and formation of cell constituents are supplied, bacteria are able to grow and adapt to the ambient milieu (6). Indeed, by studying the constant growth of bacteria during an exponential balanced growth, valuable insights into the many cellular processes that affect bacterial physiology have been obtained (6). However, unlike the conditions provided in a laboratory setting, bacteria in their natural habitat are often faced with adequate yet poor environmental conditions that limit their growth (4). Under favorable conditions, such as a laboratory rich medium, the specific growth rate of bacteria such as *Escherichia coli* becomes independent of the concentrations of nutrient and is ultimately limited by cellular processes and not by the medium constituents (4, 11). This is partly due to the fact that in the presence of excess substrates, saturation of the many transport proteins and intracellular enzymes that are involved in the uptake of nutrients and their metabolism within the cells results in a growth behavior that is analogous to an enzymatic reaction following saturation kinetics (11). However, in environments with limiting nutrient concentrations, the rate of growth becomes directly proportional to the extracellular concentration of the limiting nutrient (10). This is because when all necessary cellular practices, such as the energy to maintain ionic balances, membrane potential, *et cetera*, are taken into account, the carbon and energy sources left in the end are not enough to support rapid growth, and therefore, growth rate declines (2). However, bacteria have devised mechanisms to cope with such nutritional changes in their environments (4). For instance, with respect to sugar limitations, transcription of many genes involved in carbon and energy metabolism, as well as the transport and uptake of carbon sources are differentially expressed (4).

In *E. coli,* and other Gram negative enteric bacteria, the maltose system is a multi-component high affinity transport system that is involved in the uptake and catabolism of α(1-4)-linked glucose polymers (maltodextrins) of up to 7 to 8 glucose units (1). The substrate recognition of the system primarily involves a periplasmic maltose binding protein (MBP) that has high affinity for maltose and maltodextrins (K_D of 1μM) (1). The efficient uptake of maltose and maltodextrins, especially at

low concentrations, requires the presence of LamB which is the specific diffusion pore for maltodextrins and other carbohydrates in the outer membrane (1). Other components of the system include the membrane spanning proteins MalF and MalG, and the energy coupling protein MalK (1). In addition, MalK has been shown to be involved in regulating the expression of the maltose system by interacting with MalT, the transcriptional activator of all *mal* promoters (1).

In order to understand how changes in growth rate affect the ability of *E. coli* to survive stresses such as UV irradiation, it is important to devise methods in which the growth behavior of *E. coli* is controlled without affecting other cellular processes. In this study, we tried to devise a method in which the growth of *E. coli* could be specifically controlled through limiting the availability of nutrients. In the designed amylose/amylase system, the concentration of amylase added would maintain a growth-limiting supply of maltose in the growth media resulting in different growth rates during exponential phase. We hypothesized that by using different concentrations of amylase that limit maltose concentrations to non-saturating levels, we could control the growth rate of *E. coli*.

MATERIALS & METHODS

Bacterial strain. *E. coli* B23 was obtained from the MICB 421 culture collection in the Department of Microbiology and Immunology at the University of British Columbia.

Media. Bacteria were grown in M9 minimal media (7 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 0.2g/L $MgSO_4.7H_2O$, pH 7.1), supplemented with either 0.05% glucose, or 0.1% amylose (soluble starch, Merck, 74881) and 0.025%, 0.035%, 0.05%, or 0.1% amylase enzyme. 1% amylase enzyme stock solution was prepared by dissolving 0.10 g amylase powder (Sigma, A2771) in 10 ml sterile dH_2O and filter-sterilized.

Preliminary tests for growth of *E. coli* in different media. M9 media was prepared and supplemented with no carbon source, or 0.05% glucose, or 0.1% amylose with theoretical amylase concentrations of 0.0093% and 0.0139%. A second experiment was performed using the following concentrations of amylase: 0.001%, 0.010%, 0.025%, 0.050%, 0.10%, 0.20%, and 0.50%. The tubes were inoculated with 10 µl of overnight culture grown in M9 media supplemented with 0.05% glucose and were aerated at 37°C in a tube rotator. The growth was monitored over 3 days to determine which amylase concentration would be best suited for limiting growth.

Growth curve. Overnight culture of *E. coli* B23 was prepared using 60 ml of M9 minimal media, supplemented with 0.05% glucose, and inoculated with 100 µl of an overnight culture of *E. coli* B23. The culture was allowed to grow overnight in 37°C water bath at 100 rpm. The following day, the overnight culture was used to inoculate 80 ml of fresh M9 minimal media supplemented with 0.05% glucose, or 0.1% amylose and 0.025%, 0.035%, 0.05%, or 0.1% amylase to give an approximate starting OD_{460} reading of 0.1. Also, a control culture was prepared using M9 salt media lacking any carbon sources. The inoculated cultures were then put into a shaking water bath at 150 rpm and allowed to grow at 37°C; sampling was conducted every thirty minutes using the Spectronic 20D+ spectrophotometer until stationary phase was reached.

RESULTS

Controlling growth based on theoretical concentration values of amylase. The calculated theoretical concentrations required to maintain growth at limiting enzyme concentrations (0.0093% and 0.0139% amylase) resulted in no visible growth after three days of incubation (data no shown).

***E. coli* B23 growth in media with different amylase concentrations revealed different bacterial productivity**. The results shown in Figure 1 and Table 1 were used to determine the appropriate combinations of enzyme-substrate concentrations that would later be used to construct growth curves. These results demonstrate that the productivity, determined by the apparent final turbidity of the cultures, increased as the concentration of amylase in the media increased from 0.001% to 0.5%. However, over a 3 day period of continued incubation, no further change was observed in the apparent turbidity, and hence, productivity of the cultures. Absence of growth in the media containing no amylase confirmed the inability of our strain to use amylose as a carbon source in the absence of exogenous amylase. In addition, it demonstrated that there was nothing else available in the medium to permit significant growth.

Turbidity-based growth curves. To analyze the growth behavior of *E. coli* B23, two trials were carried out to obtain representative growth curves. Figure 2 represents the results of the turbidity–based growth curve for the second trial. The results of trial one were nearly identical to those of the second trial; however, no control was used in the first trial.

The growth of cultures containing different combinations of amylose-amylase concentrations demonstrated a short lag phase before entry into exponential growth. The lag phase appears to be shortest for the culture containing 0.1% amylase (45 minutes) and longest for the culture containing 0.025% amylase (60 minutes) (FIG. 2). However, the glucose-containing culture seemed to enter exponential growth without any significant lag phase.

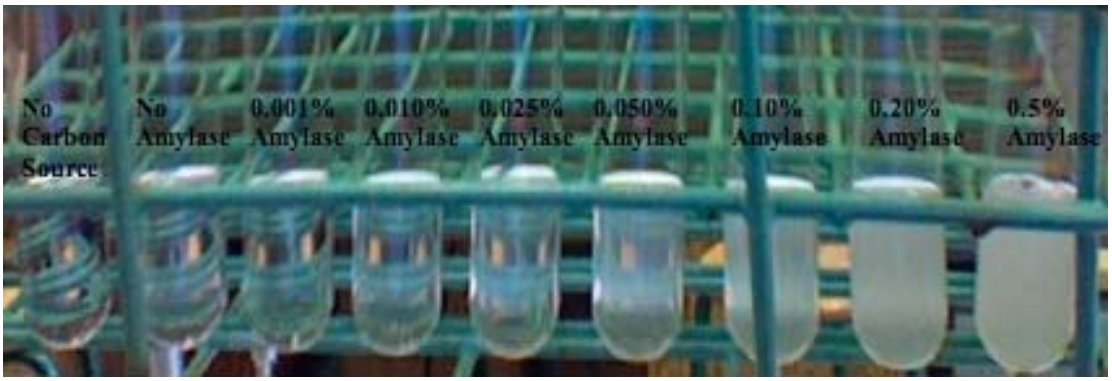

FIG. 1: Visual comparison of bacterial productivity based on the turbidity of cultures grown in M9 minimal media containing either no carbon source, or 0.1% amylose as the carbon source with increasing concentrations of amylase.

Turbidity-based growth rates and culture productivity. Analysis of the exponential growths showed similar rates for the majority of the cultures (FIG. 2). Indeed, as shown in Figure 3A, numerical analysis of the rates showed overlapping growth rates for all of the growth cultures. However, there is a slight but insignificant decrease in the growth rate of cultures containing concentrations of amylase lower than 0.05% (FIG. 3A). In addition, no growth was observed in the control culture, suggesting minimal glucose carry over from the overnight inoculum (FIG. 2).

Differences in productivity in the various growth cultures are evident by the differences in their final turbidity readings shown in Figure 3B. It demonstrates a clear and reproducible pattern of decrease in production levels that corresponds to the decrease in the amylase concentration. The highest turbidity-based productivity, obtained from the glucose sample, was 0.807. The lowest turbidity-based productivity, however, was 0.250, which was observed for the 0.025% sample. Lastly, cells of glucose and 0.1% amylase cultures appeared to enter stationary phase after 3.5 hours of growth. The remaining amylase cultures entered stationary phase earlier, within 3 hours of growth.

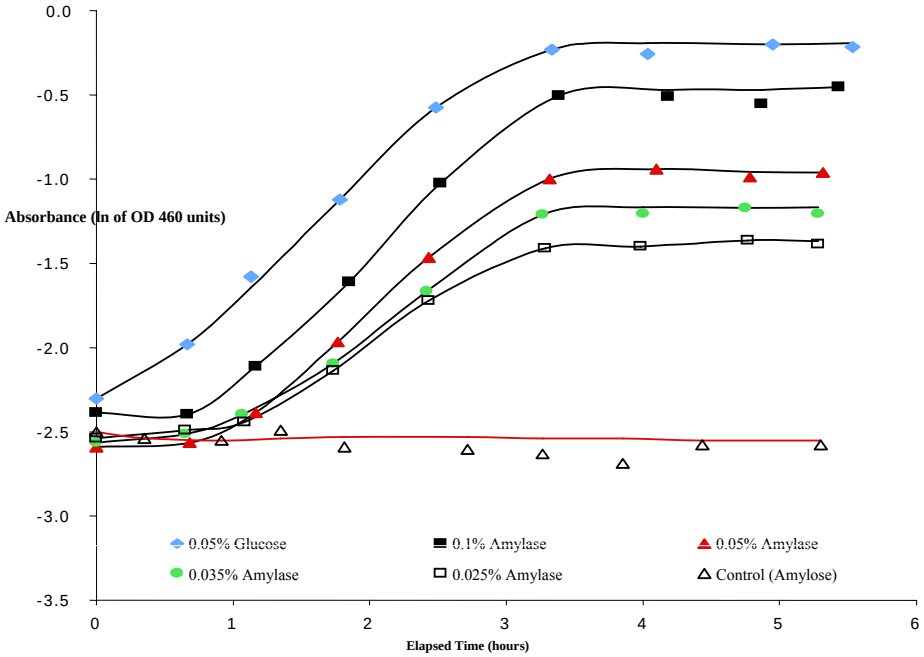

FIG. 2. Effect of changing the supplied amylase concentration on the growth of *E.coli* B23 incubated in M-9 minimal salts medium with a constant supply of amylase. The amylase varied in the test cultures. The amylase control culture was grown in the same conditions as the other cultures but lacked amylase. The glucose supplemented culture served as a growth control.

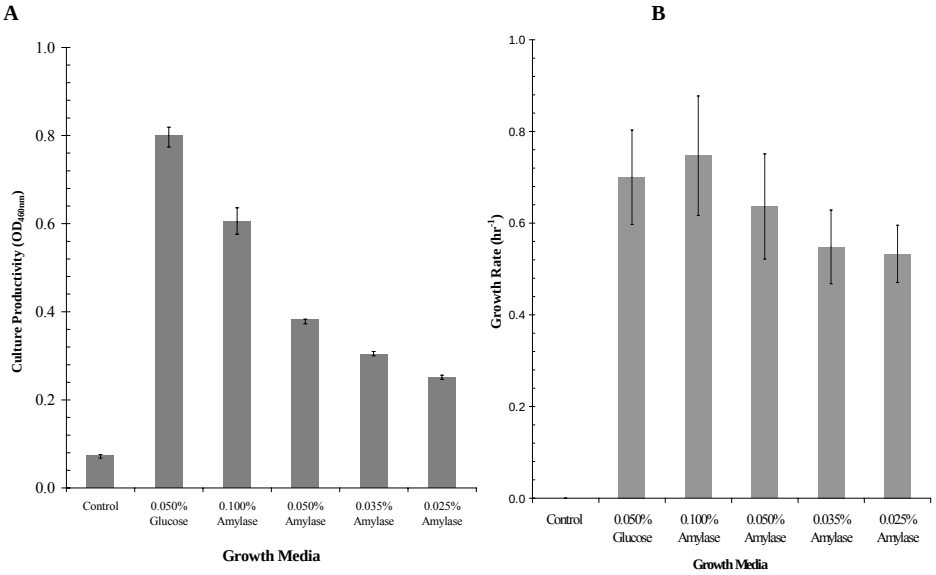

FIG. 3 (A) The productivity levels of the growing cells was measured based on the average of the turbidity of three points of the samples at stationary phase. (B) Turbidity-based growth rate of *E. coli* B23 in M9-broth supplemented with various levels of carbon source. Rates were calculated by taking the average of several semi-log slopes resulting from different combinations of data points from the exponential growth and constructing error bars based on the maximum and minimum slope values. Error bars were constructed based on the maximum and minimum values of the turbidity at stationary phase.

TABLE 1. Observed productivity of *E. coli* B23 after one and three day incubations in M9 minimal media containing either no carbon source, or 0.1% amylose as the carbon source with increasing concentrations of amylase. Cultures were aerated at 37 ˚C. Refer to FIG. 1 for a visual observation.

Growth Condition

TABLE 1. Observed productivity of *E. coli* B23 after one and three day incubations in M9 minimal media containing either no carbon source, or 0.1% amylose as the carbon source with increasing concentrations of amylase. Cultures were aerated at 37 ˚C. Refer to FIG. 1 for a visual observation.

Growth Condition	Growth Level*	
	Day 1	Day 3
No Carbon Source	-	-
No Enzyme (only Amylose)	-	-
0.1% Amylose + 0.001% Amylase	-	-
0.1% Amylose + 0.01% Amylase	-	-
0.1% Amylose + 0.025% Amylase	+	+
0.1% Amylose + 0.05% Amylase	++	++
0.1% Amylose + 0.10% Amylase	+++	+++
0.1% Amylose + 0.20% Amylase	++++	++++
0.1% Amylose + 0.50% Amylase	+++++	+++++
0.50 % Glucose (Data not shown)	+++++	+++++

*minus sign represents no visible growth. Plus signs represent increasing amounts of growth in cultures relative to one another. The culture turbidities were not measured quantitatively.

DISCUSSION

This experiment was carried out based on the hypothesis that a steady, yet limiting supply of maltose as the sole carbon source would control the growth rate of *E. coli*. As shown by Figures 3A, the different amylase concentrations did not result in significant differences in growth rates. A plausible explanation for this observation is the fact that the relationship between growth rate and nutrient concentration is analogous to Michaelis-Menten kinetics; at low maltose concentrations the growth rate is proportional to substrate concentrations but at higher concentrations of maltose the growth rate becomes independent of the concentration of this molecule (11). This is partly due to the fact that at high nutrient concentrations, the proteins involved in the transport of maltose, such as LamB and MBP, become saturated and the growth rate becomes

independent of nutrient concentration (10). Indeed, studies by Boos *et al.* (1) have shown that the maximal rate of maltose transportation, which is in part determined by the rate of diffusion through the LamB outer membrane porin, occurs at an external concentration of 0.1 mM. However, based on the theoretical conversion rate of the amylase used, a minimum maltose concentration of 15 mM (for 0.025% amylase culture) would have been liberated within the first thirty minutes of the experiment. This indicates that the maltose present in the cultures might have saturated the transport system if it is accumulated during the initial growth lag (FIG. 2).

Differences in the productivity of the cultures, determined by the different final turbidity measurements, were observed (FIG. 3B) and found to be associated with a decrease in the amylase concentrations. Similar trends were observed for the overnight cultures represented in Figure 1 and Table 1. This observation could be accounted for by the fact that the amylase may have become inactivated through degradation and denaturation early in the growth experiment. Since each culture contained a different concentration of amylase, assuming the same time of degradation, the amount of maltose liberated during the experiment would have been different but still at saturation levels for each culture. Therefore, the cultures with higher initial amylase concentrations (e.g. 0.1% amylase culture), would maintain a similar growth rate over a longer period of time compared to those with lower initial amylase concentrations (e.g. 0.025% amylase culture). This explanation is consistent with the observation that the 0.1% amylase culture reached the stationary phase slightly after the 0.025% culture, suggesting that the 0.025% culture might run out of the lower initial amylase concentration sooner (FIG. 2).

The fact that there were no appreciable changes in turbidity over three days for the amylase cultures (FIG. 1 and Table 1) provides further evidence for amylase inactivation. Indeed, amylase has been shown to be inhibited by acids, such as acetic acid, which may have been carried over from the large volume of the overnight inoculum used (9). Moreover, the overnight culture could also be a source of proteases. Lastly, excess maltose not taken up by the cells is known to competitively and non-competitively inhibit amylase present in the media (3).

In addition to differences in the amount of maltose produced before the enzyme became inactive, the differences in the duration of the lag phases could provide further explanation for the differences in productivity. The lack of a significant lag phase for the glucose culture can be explained by the fact that the growth conditions, including the media, for this culture were very similar to that of the overnight culture. Therefore, cells required minimal adaptation to the growth environment (8). However, in the case of the amylase cultures, cells needed additional time to synthesize new cytoplasmic enzymes such as amylomaltase, maltodextrin phosphorylase, and maltodextrin glucosidase to metabolize maltose into glucose and glucose-1-phosphate (1). In addition, the components of maltose transport system, such as LamB, MBP and membrane spanning subunits MalG and MalF, need to be up-regulated to sufficient quantities for the efficient uptake of external maltose (1). Furthermore, the external concentrations of maltose can indirectly affect the up-regulation of the expression of maltose transport system (1). In absence or at low concentrations of external maltose, MalK, the ATPase subunit of the transport system, can negatively inhibit the actions of MalT, thereby preventing it from activating the transcription of the maltose transport system (1). However, as maltose becomes available, MalK re-associates with the membrane spanning MalG and MalF, allowing MalT dependent up-regulation of the transport system (1). This suggests that cultures with lower amylase concentrations may require more time to liberate sufficient amounts of maltose to allow for the up-regulation of the maltose transport system, and hence the onset of exponential phase. Such differences in the timing of inducing the expression of maltose transport system may, therefore, explain the different lag phases associated with the amylase cultures.

In summary, our data showed that differences in growth rates could not be obtained through the designed system of amylose-amylase. This was most likely due to inappropriate enzyme concentrations that resulted in saturating levels of maltose in the different cultures. In addition, factors such as enzyme inactivitation and inconsistencies between the theoretical and actual concentrations of enzyme necessary to control growth of *E. coli* may have played major roles in the outcome of the experiment.

FUTURE EXPERIMENTS

To empirically provide evidence for the explanations of the results in this study, the activity of the amylase enzyme needs to be measured over time to determine if it is indeed being subjected to degradation. A possible method to determine amylase activity would involve

conducting an amylase assay on cultures containing bacterial cells and those lacking bacterial cells. This would distinguish if the amylase is being degraded by cell products or the conditions at which the experiment was carried out. Furthermore, an assay should be done to determine maltose concentrations over time. Such an assay would help to better understand the kinetics of maltose production via amylase or its consumption by bacterial cells. Lastly, to provide support for the hypothesis that the presence of lag phase for the amylase cultures is due to the time required for the expression of maltose utilization and transport system, the overnight culture used for inoculating amylase cultures can be grown in maltose instead of glucose as the carbon source.

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