

## $\beta$ -Galactosidase Repression in *Escherichia coli* B23 Using Minimal Concentrations of Glucose and Sucrose

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**The synthesis of metabolic enzymes for poor carbon sources are repressed in the presence of glucose. It was investigated whether *Escherichia coli* cultured in various concentrations of glucose and sucrose would undergo  $\beta$ -galactosidase repression from the *lac* operon and to what extent. Cells were initially grown in 0.2% glycerol prior to the addition of one of the following nutrients: 0.08% glucose, 0.06% glucose, 0.05% glucose, 0.045% glucose, 0.08% sucrose, 0.06% sucrose, 0.05% sucrose and 0.045% sucrose. The data acquired indicates that glucose is more effective at catabolite repression than equal concentrations of sucrose as seen by a slowed induction rate compared to the glycerol control. No significant differences in repression were observed within the tested concentration range for either sugar suggesting that either the assay was not sensitive enough to differentiate between subtle changes in enzyme activity or that it may function in a discontinuous manner instead of a continuous trend, with all tested concentrations falling above a certain threshold value.**

The cell regulates the synthesis of transporter and initial metabolic enzymes of different carbon-containing compounds in response to the presence or absence of specific sugars [5]. For example, the presence of glucose in growth medium often inhibits catabolic enzymes of other sugars [2]. Carbon utilization by *E.coli* involves the global regulator CAP-cAMP complex and phosphoenolpyruvate-dependent carbohydrate:phosphotransferase (PTS) systems that act in concert to regulate catabolite repression and inducer exclusion. The PTS utilizes a protein phosphoryl transfer chain to transport and phosphorylate sugar substrates using phosphoenolpyruvate (PEP) as a phosphate donor. Extracellular glucose promotes a sequence of phosphoryl transfer events that begins with the glucose specific intermediate Enzyme IIA (EIIA<sup>Glc</sup>). EIIA<sup>Glc</sup> energizes glucose transport in *E.coli* and is also a regulatory protein. During glucose uptake, EIIA<sup>Glc</sup> becomes de-phosphorylated, and inhibits various catabolic enzymes and permeases, including the lactose permease [5]. However, without glucose present, EIIA<sup>Glc</sup> is predominately in its phosphorylated form and activates adenylate cyclase to make cAMP, which binds a catabolite activator protein (CAP) to activate transcription from the *lac* promoter. Therefore, the intracellular concentration of cAMP reflects the metabolic state of the cell. [2, 3, 5]

Sucrose, as compared to glucose, is a poor carbon and energy source. Sucrose transport is also dependent on EIIA<sup>Glc</sup>, and therefore should cause equivalent regulatory effects like the glucose PTS [5]. However, unlike glucose, sucrose is transported into the cell only by a single system. [5].

We report here a comparison of the effects of different glucose and sucrose levels on carbon catabolite repression by observing the minimal required levels to suppress  $\beta$ -galactosidase induction using IPTG. Knowledge of minimal sugar concentrations to suppress  $\beta$ -galactosidase induction could be used to economize experimental studies on *lac* operon regulation and induction. Additionally, it would allow one to manipulate the *E.coli lac* operon regulatory system in further experimental studies exploring catabolite repression.

### MATERIALS AND METHODS

Tests were done with *E. coli* in order to establish the purity of a culture. Sterile M9 media was made to a volume of 1 L for use in culture growth. Once the M9 media preparation had been completed, and isolated colonies of *E. coli* B23 obtained, 3 ml of M9 glycerol media were inoculated with a colony from the third glucose agar plate. This culture was then allowed to grow at 37°C for 24 hrs, in aerated conditions. Following this, 1 ml of the three was added to 2 ml of dH<sub>2</sub>O, and an OD<sub>460</sub> reading taken (the dilution was necessary to be able to take an accurate OD<sub>460</sub> measurement).

The total volume to be inoculated was 500 ml. The initial OD<sub>460</sub> was 0.906 (taken from the overnight 3 ml culture). The final desired OD<sub>460</sub> was 0.15. The volume of culture added to the 250 ml M9 starting volume was 2.62 ml. The two flasks were then set into a shaking water bath at 37°C until the culture reached the desired optical density of 0.15 (OD<sub>460</sub>).

Upon reaching the desired optical density, IPTG was added to a final concentration of 0.1 mM. This was deemed to be time zero (T=0), for the experiment.

At time equal to 3 min, 6 min, 9 min, and 12 min, 3 ml samples were removed from the culture. The optical density of the sample was taken and the sample then emptied into 10 ml glass test tubes containing 200  $\mu$ l of toluene. These test tubes were vortexed to ensure mixing of the cells from the culture with the toluene.

At time equal to 15 min, the initial culture was divided up into 40 ml volumes, and distributed into nine sterile 250 ml flasks. Each of the flasks held an additional volume of a specific concentration of nutrient (glucose or sucrose).

Prior to use, all flasks were pre-warmed in the 37°C water bath. Flask #1 served as the control vessel, and carried 40 ml of inoculated glycerol M9 media, the next four flasks held 0.08% glucose in 40 ml inoculated M9 media, 0.06% glucose in 40 ml inoculated M9 media, 0.05% glucose in 40 ml inoculated M9 media, and 0.045% glucose in 40 ml inoculated M9 media, respectively. The last four flasks held the same as the above, substituting equal percentages of sucrose where glucose was listed.

Sampling was started as soon as all nine 250 ml flasks were returned to the 37°C shaking water bath. 3 ml samples were removed from the 250 ml flasks, and the time recorded. The 3 ml samples were then decanted into spectrophotometer cuvettes, and the OD<sub>460</sub> taken, in order to measure the culture turbidity. The 3 ml samples were then decanted into non-sterile 10 ml glass test tubes, which already contained 200  $\mu$ l of toluene. The culture-toluene mix was vigorously vortexed for ten to twenty seconds. As the steps took a few minutes to complete, it was decided that a rotational sampling style was to be adopted: three ml samples were taken from each flask in a constant cycle, and the time the sample was taken simply recorded. Eight time points were sampled. The tubes containing the three ml samples mixed with toluene, taken from the flasks at the various time points, were then sealed with para-film. The tubes were stored in the 4°C fridge. Three days later, 0.4 ml of permeabilized cells from below the toluene layer were transferred to clean non-sterile tubes already containing 1.2 ml of Tris buffer (pH 8.0, 20 mM). All these tubes were then placed into a 25°C water bath to warm.

At this time, a test tube bearing a control sample collected late in the course of the experiment was subjected to ONPG treatment (0.2 ml, 5 mM), and watched for development of a clear yellow colour (2 ml of 0.6 M sodium carbonate was then added). The absorbance was measured. This tube then served as a reference for the intensity of colour development required for the rest of the enzyme assays.

Once warmed, a few tubes at a time had 0.2 ml of ONPG (5 mM) added. The time at which this was done was recorded, and the tubes then watched for the development of a yellow colour strong enough to match the control. The absorbance of the sodium carbonate treated samples was determined at 420 nm in a spectrophotometer zeroed with distilled water and blanks from the glycerol control (prepared using water in place of ONPG). Many of the steps in the above protocol were adapted from Experiment A4 of the Microbiology 421, Manual of Experimental Microbiology. [1]

## RESULTS

**Figures 1 and 3:** Growth was achieved with either glucose or sucrose, as shown in Figures 1 and 3. The doubling time under glucose conditions was about 50 minutes, and that of sucrose was approximately 60 minutes. The growth rate on glucose was similar to glycerol. Figure 3 showed slower growth on sucrose than glycerol, as shown by the smaller slope of the sucrose growth curve.

**Figures 2 and 4:** The data in Figures 2 and 4 shows obvious inhibition of enzyme induction rates by the addition of glucose or sucrose. Transient repression for all glucose concentrations lasted longer than for all sucrose concentrations, and varied slightly depending on the concentration. These trends are further explored in Figures 5-9.

**Figures 5 to 9:** Figure 5 compared the differences between glucose and sucrose at slowing enzyme induction rates and repression. Glucose displays stronger effects than sucrose, with transient repression lasting approximately 30 minutes and 10 minutes, respectively. This trend was also observed in Figure 6, in which glucose repression lasted approximately 3 times longer than sucrose (15 minutes versus 5 minutes).

At 0.05% glucose concentrations, repression occurred at time 15 minutes and lasted for about 10 minutes. Sucrose repression occurred 10 minutes after that of glucose, paralleling its rate of enzyme synthesis (Figure 7). Glucose showed significant repression in Figure 8, as did sucrose. However, sucrose showed earlier inhibition than glucose at this concentration. Concentrations of 0.045% and 0.08% showed the longest transient repression, lasting for approximately 20 and 30 minutes respectively (Figures 5 & 8). Figure 9 allowed comparison of both sugars at the extreme concentrations.

## DISCUSSION

To determine the basal concentrations of glucose or sucrose sufficient to affect catabolite repression, a range of 0.045-0.08% nutrient was analyzed for its effect on induction of the  $\beta$ -galactosidase enzyme of the *lac* operon in *E. coli*. The cell growth was similar at all sugar concentrations for a specific sugar (Figures 1 & 3). The observed growth rate for the glucose samples was normal while that of sucrose was slightly slow. This reduced growth would likely contribute to lower levels of enzyme induction in the system, though on a per cell basis induction may be similar. This complicates interpretation when comparing sucrose to the glycerol control as lower enzyme activity in the presence of sucrose may be due to either the effect of the particular sugar or the quantitative effects of having fewer cells contributing to enzyme synthesis. Similarly, when comparing between glucose and sucrose at similar concentrations there are fewer cells competing for uptake of sucrose, thereby potentially facilitating catabolite repression in the lower concentration range. In light of this potential advantage, glucose repression was observably more effective than sucrose as will be further discussed.

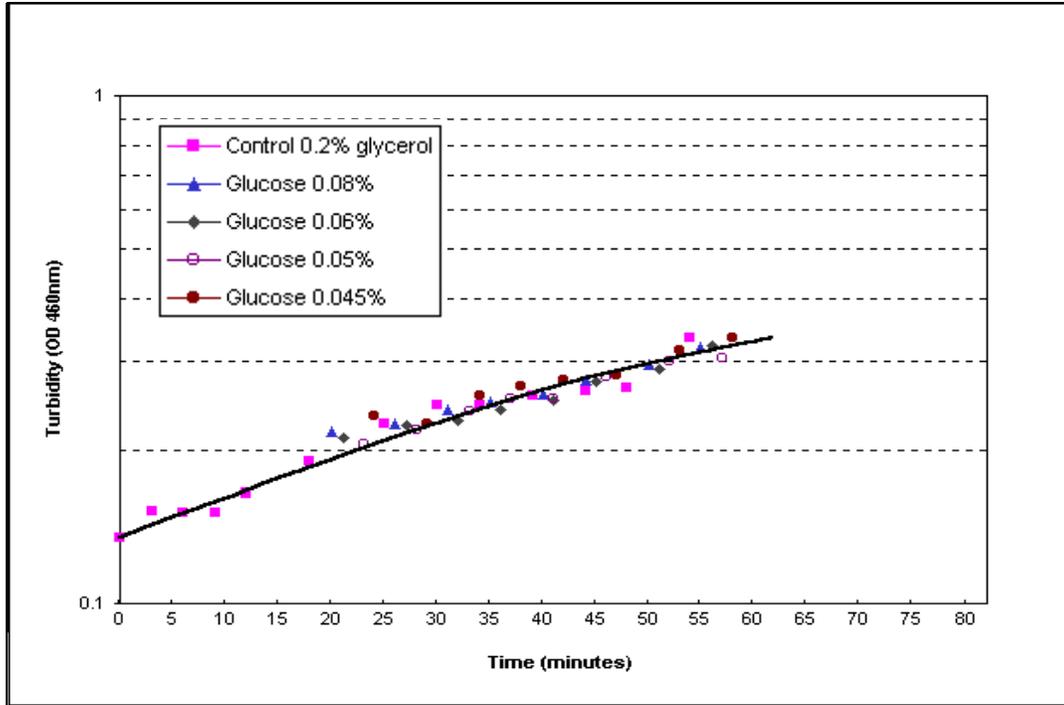


Figure 1: Growth of E.coli cultivated in Different Glucose Concentrations and Glycerol Control at 37°C with Aeration.

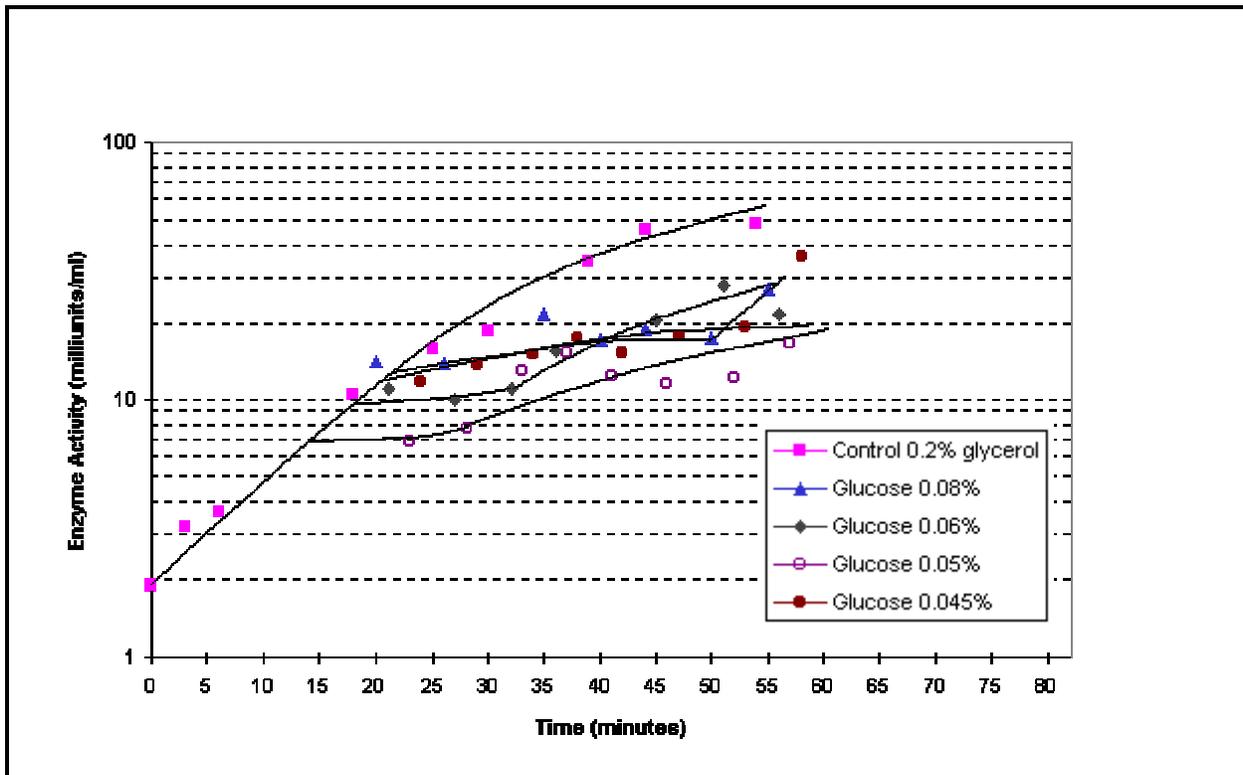


Figure 2: Beta-Galactosidase Induction of E.coli cultivated in Different Glucose Concentrations and Glycerol Control at 37°C with Aeration after Addition of IPTG

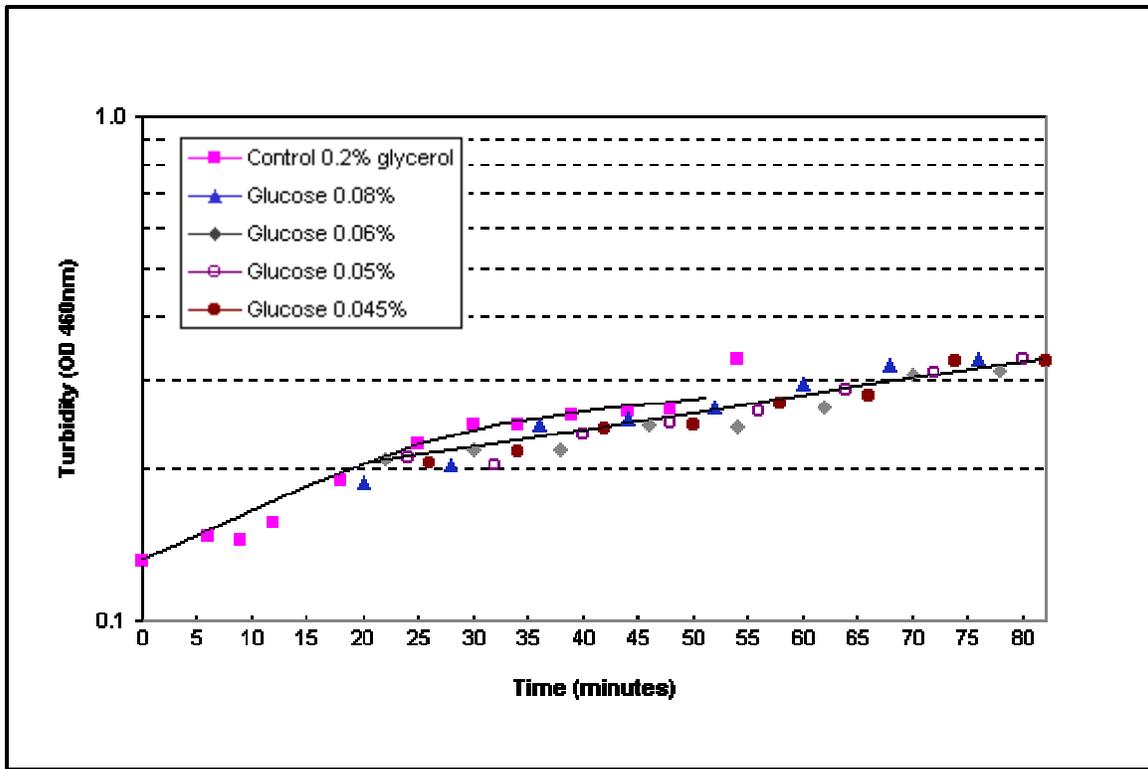


Figure 3: Growth of E.coli Cultivated in Different Sucrose Concentrations and Glycerol Control at 37°C with Aeration.

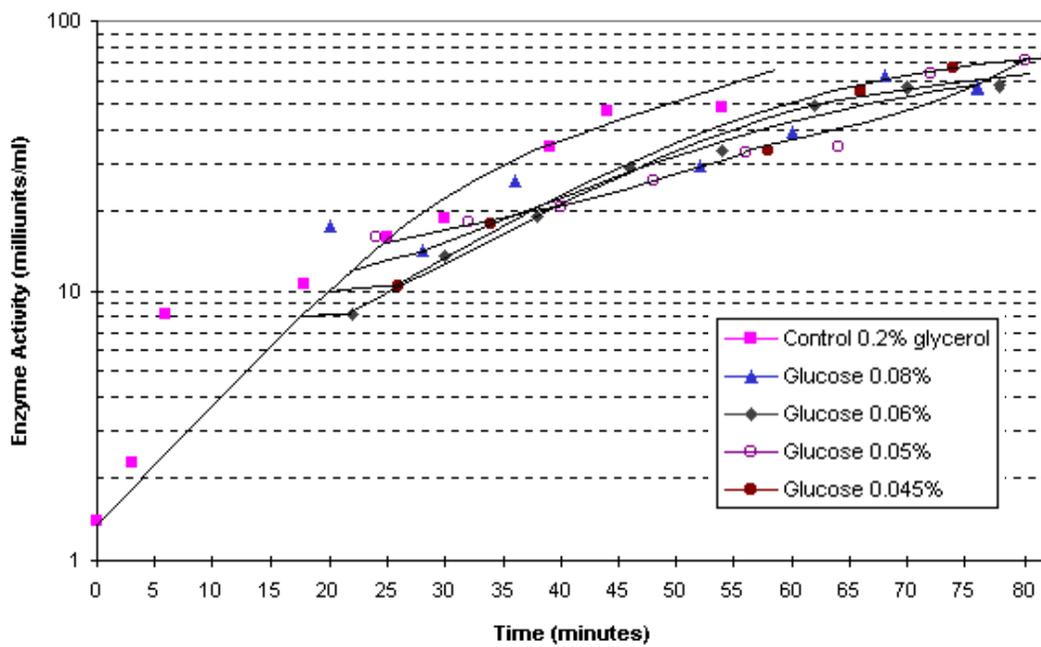


Figure 4: Beta-Galactosidase Induction of E.coli cultivated in Different Sucrose Concentrations and Glycerol Control at 37°C with Aeration after Addition of IPTG

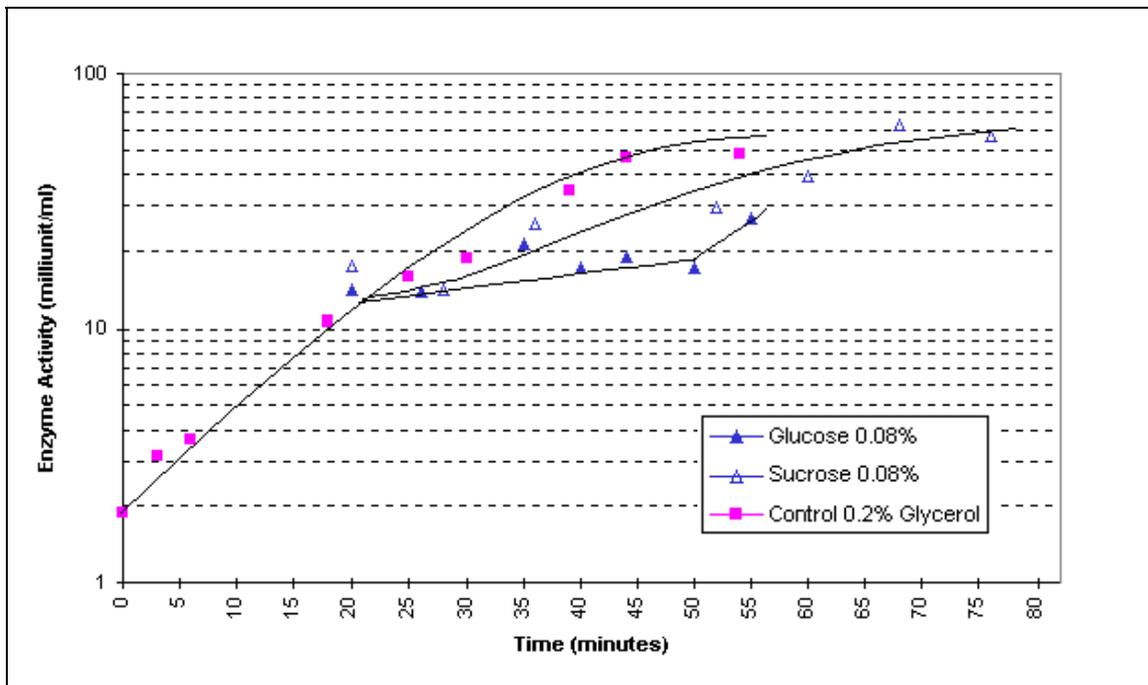


Figure 5: Comparison of the Effects of 0.08% Glucose and Sucrose on the Induction of Beta-Galactosidase

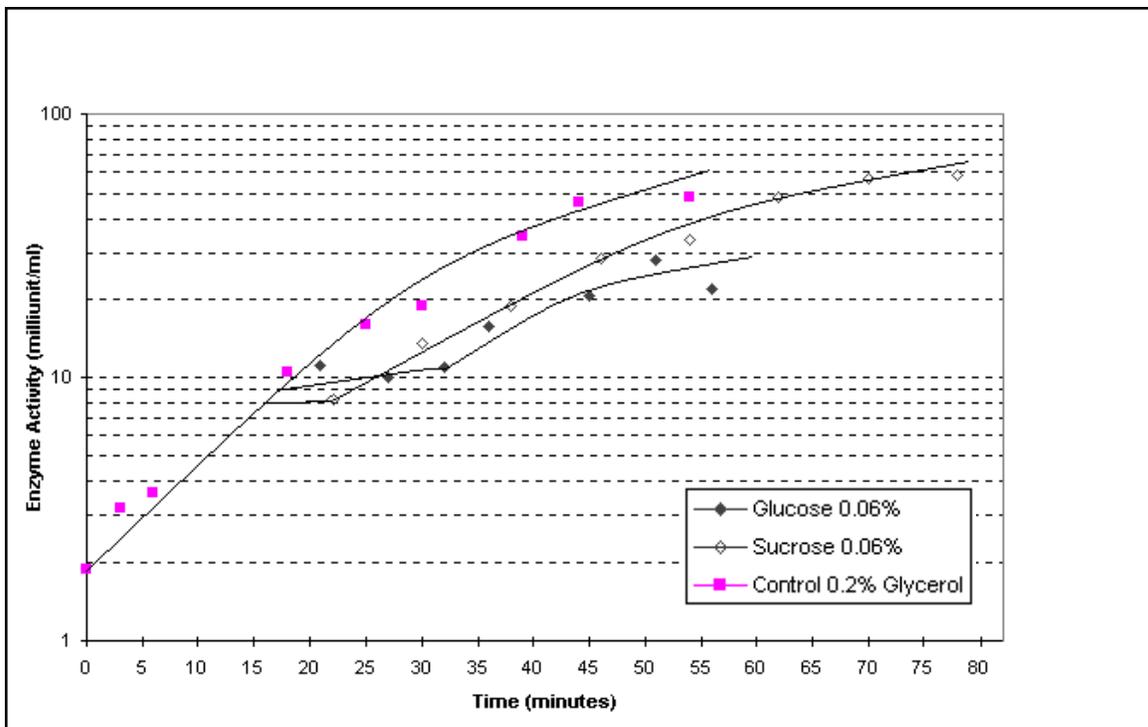


Figure 6: Comparison of the Effects of 0.06% Glucose and Sucrose on the Induction of Beta-Galactosidase

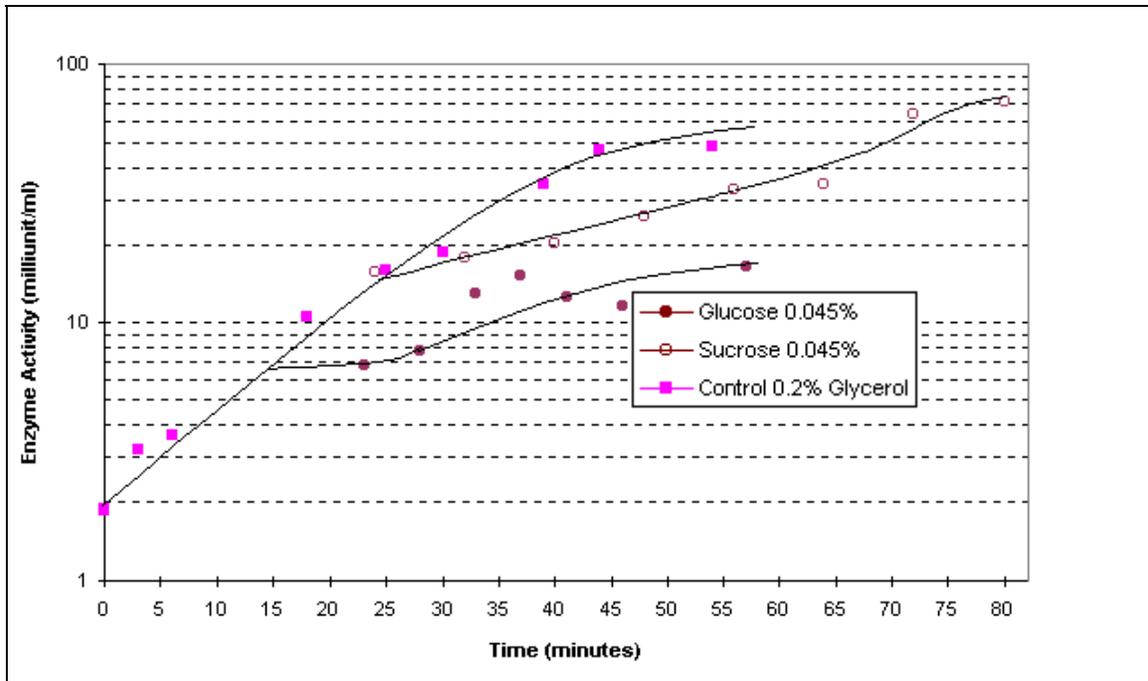


Figure 7: Comparison of the Effects of 0.05% Glucose and Sucrose on the Induction of Beta-Galactosidase

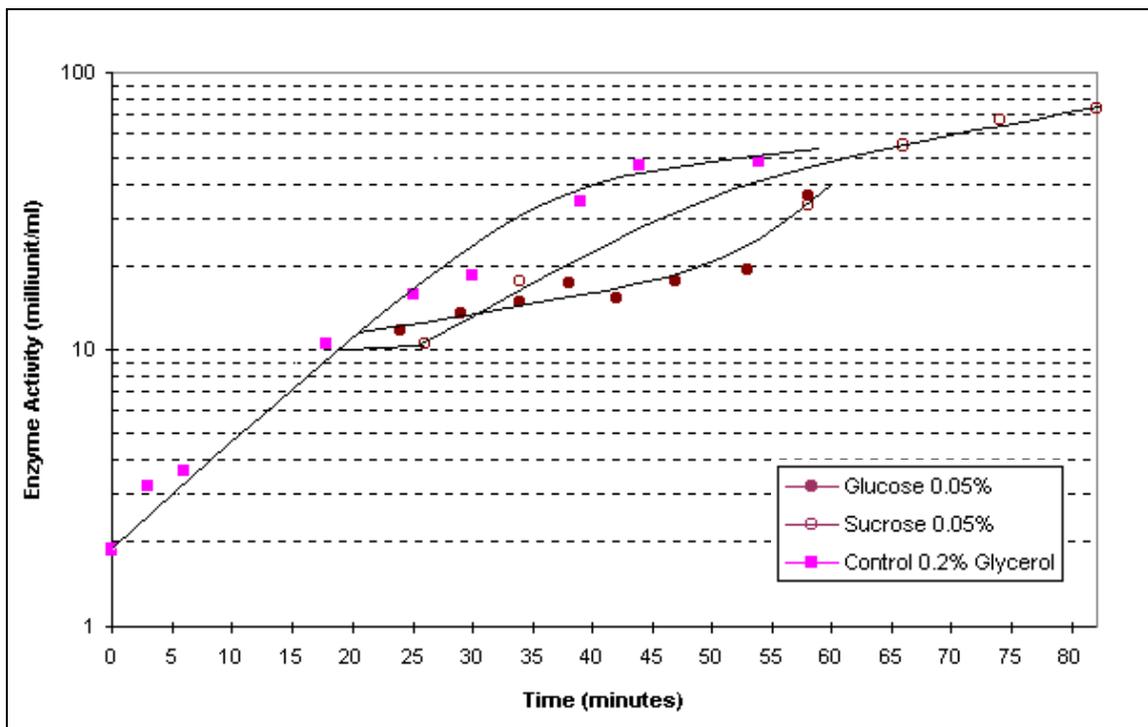
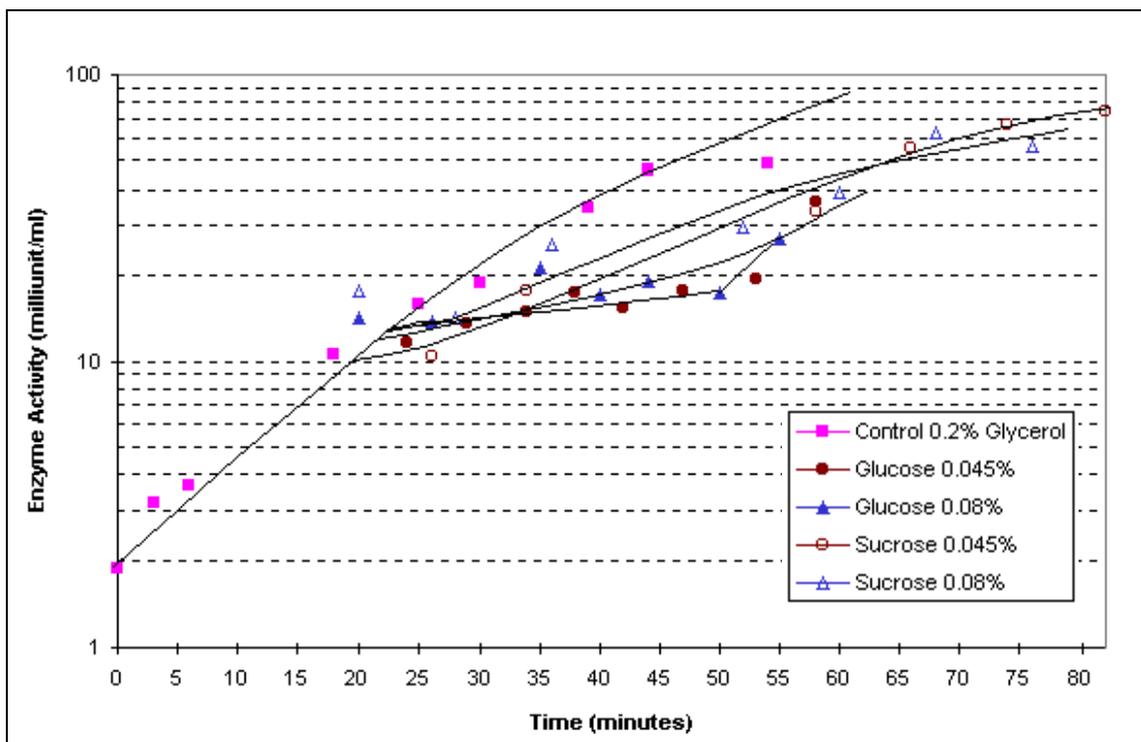


Figure 8: Comparison of the Effects of 0.045% Glucose and Sucrose on the Induction of Beta-Galactosidase



**Figure 9:** Beta-Galactosidase Induction of E.coli at 0.08% and 0.045% Glucose and 0.08% and 0.045% Sucrose

The glucose curves in Figure 2 indicate that the presence of glucose caused an initial lag in the induction rate of  $\beta$ -galactosidase, followed by a quick recovery. It should be noted, however, that the lower concentrations of the sugars displayed a stronger and earlier inhibition on induction rates. This was opposite to what was expected. Higher concentrations of the two sugars were expected to have slowed the enzyme induction rates more than the lower concentrations. This observed effect may have been due to sampling errors, or inaccurate spectrophotometric values. Figure 4 also reveals an initial lag enzyme induction occurring at lower concentrations of sucrose. Figure 9 further explores this observation and compares between the extremes of the concentrations under examination. Rather than seeing distinctive differences in enzyme activity, the curves display fairly similar trends though inhibition occurred earlier and lasted slightly longer at the lower respective sugar concentrations. It was assumed that as concentration decreased, catabolite repression would decrease and induction rate would increase in a proportional manner. Our data does not support this hypothesis, suggesting that catabolite repression may be governed by certain threshold values, as a discontinuous trait rather than being continuous in nature.

Figures 5-8 examine individual concentration revealing that glucose is more effective than sucrose at slowing enzyme induction when tested at equal concentrations. Generally, glucose shows patterns of longer transient repression followed by resumed enzyme synthesis at a slightly slower rate than the glycerol control. In comparison, sucrose shows shorter transient repression and gradual resumed synthesis at similar rates with the glycerol control, taking into consideration the overall slowed growth rate of the sucrose cultures.

The glucose curve showed obvious transient inhibition followed by quick recovery in Figure 5. This is as expected because glucose has been reported to be a better PTS sugar than sucrose. This trend is also observed in Figure 6 where glucose repression lasts approximately 2 times longer than sucrose repression. Rather than obvious repression, Figure 7 shows slower rate of synthesis rather than complete repression. The curve for 0.06% and 0.08% glucose respectively seem to display this behavior in the 20-34 minute time range, while no such effect is detectable for any of the sucrose concentrations. Comparisons between the effects of glucose or sucrose at the same concentrations are shown in Figures 5 through 8. This data reveals glucose to be slightly more effective at slowing induction than sucrose at equal concentrations.

Both sucrose and glucose are PTS-carbohydrates, sucrose being cleaved into glucose-6-phosphate and fructose-6-phosphate respectively upon entry into the cell. However, the first step of sucrose transport and phosphorylation is dependent on the same IIA enzyme as glucose and thus potentially able to cause equivalent regulatory effects [5]. Intracellular sucrose hydrolyzation to glucose and fructose as a secondary event should not then affect the inhibition rate. Instead, the above observations may be explained by differences in transporter availability rather than structural differences between the two sugars. Sucrose, unlike glucose, is transported into the cell only by a single transport system, while several transport systems exist for glucose. This uptake limitation may account for the slightly less efficient regulatory effect of sucrose as compared to glucose at the same concentration.

As mentioned above catabolite repression is a complex regulatory process involving many different components. While certain knowledge regarding its overall mechanism can be achieved via enzyme assays, deriving truly quantifiable data requires a more in depth approach. Further research could be done using exact methods to quantify intracellular changes at a molecular level, such as the levels of phosphorylated sugar or cAMP levels as a more direct correlation between minimal concentrations of necessary components of this regulatory system to achieve observable catabolite repression. Research in this area has begun, aimed at achieving a highly transparent biological model to truly verify and quantify the proposed model for this important regulatory process within *E.coli* [5].

#### REFERENCES

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## APPENDIX I: OD calculations:

Overnight culture preparations – calculations to determine volume of culture required to establish a culture of a specific optical density for a specified time.

$$\text{OD}_{460} \text{ dilution} = 0.302$$

$$\text{OD}_{460} \text{ culture} = 3 \times 0.302 \Rightarrow 0.906$$

$$\mu = \ln 2 / g \quad \text{where } \ln \text{ equals the growth rate}$$

$$\mu = (\ln x_f - \ln x_i) / \text{time}$$

$$0.69 = [\ln (0.15) - \ln x_i] / 4\text{hrs}$$

$$0.69 = [-1.8971 - \ln x_i] / 4\text{hrs}$$

$$-\ln x = 2.76 + 1.8971$$

$$x = e^{-4.6571}$$

$$x = 0.00949$$

Volume of culture required:

$$(x \text{ ml})(0.906) = (500 \text{ ml})(0.00949)$$

$$x \text{ ml} = 5.237 \text{ ml}$$

used 2.62 ml of culture per 250 ml of M9 media.

## APPENDIX II: Stock Solution Calculations

### MgSO<sub>4</sub>.H<sub>2</sub>O stock solution:

4g of the salt in the non-hydrated form were added to 20 ml of sterile dH<sub>2</sub>O

1 ml of this solution was then added to the M9 media under construction, this gave a final weight of 0.2 g of hydrated MgSO<sub>4</sub> per the final 1000 ml of M9 media.

### Glycerol stock solution – 40%

Volume made = 10 ml

$$\frac{40\% \text{ glycerol}}{10 \text{ ml}} \Rightarrow \frac{x \text{ ml glycerol}}{10 \text{ ml}} \Rightarrow 0.4 \Rightarrow 4.0 \text{ ml glycerol in } 6.0 \text{ ml dH}_2\text{O}$$

### Glucose stock solution – 10% (w/v)

Volume made = 100 ml

⇒ 10 g glucose dissolved in 100 ml dH<sub>2</sub>O

**Sucrose stock solution** – 10% (w/v)

Volume made = 100 ml

⇒ 10g sucrose dissolved in 100 ml dH<sub>2</sub>O

**APPENDIX III: Reagent Calculations**

**Tris Buffer** – 20 mM, pH8.0

Tris = 121.14 g/mol

⇒(0.03 moles Tris) x (121.14 g/mol)

⇒0.36342 dissolved into 150 ml dH<sub>2</sub>O

Volume needed = 150 ml

Concentration = 0.002 M

Actual weight used = 0.3641 g Tris powder

**Sodium Carbonate** – 0.6 M

Molecular weight of Na<sub>2</sub>CO<sub>3</sub> = 105.99 g/mol

⇒(0.250 L) x (0.6 mol/L)

⇒0.15 moles

⇒0.15 moles Na<sub>2</sub>CO<sub>3</sub> x 105.99 g/mol

⇒15.8985 g dissolved into 250 ml dH<sub>2</sub>O

Volume needed = 250 ml

Concentration = 0.6 M

Actual weight used = 15.8927 g

**ONPG** – 5 mM

Molecular weight = 301.3 g/mol

⇒0.025L x 0.005mol/L

⇒0.000125 moles ONPG

⇒0.000125 moles ONPG x 301.3 g/mol

⇒0.038 g dissolved into 25ml dH<sub>2</sub>O

Volume needed = 25 ml

Concentration = 5 mM

Actual weight used = 0.038 g

**IPTG** – 0.10 mM

Stock solution = 20 mM (provided by Nick)

Volume = 250 ml in each 500 ml flask

A total of 9 ml were removed from each flask to measure the starting turbidity (three different readings were taken over a the course of about one hour, 2:00 pm to 3:00 pm, the 29<sup>th</sup> of January 2002.

Adjusted Volume = 241 ml

Concentration = 0.10 mM

⇒ x moles = (0.0001 M)(0.241 L)

$\Rightarrow 2.41 \times 10^{-5}$  moles  
 $\Rightarrow (0.1 \text{ mM})(241 \text{ ml}) = (20 \text{ mM})(x \text{ ml})$   
 $\Rightarrow 1.205 \text{ ml IPTG } 20 \text{ mM stock solution added to the two } 500 \text{ ml flasks each carrying } \sim 241 \text{ ml of culture growing in M9.}$

### **Toluene**

A stock solution of toluene was obtained from Nick Cheng, the lab technician.

### **Glycerol Control—2% in 40 ml of M9 media**

Volume made = 1000 ml

Stock solution = 40% glycerol in 10 ml

$(1000 \text{ ml})(0.002) = (x \text{ ml})(0.40)$

$\Rightarrow 5 \text{ ml of the } 40\% \text{ glycerol stock solution needed for } 1000 \text{ ml of M9}$

### **Glucose and Sucrose Nutrient Addition**

0.08% nutrient

$(40 \text{ ml})(0.0008) = (x \text{ ml})(0.1)$

$\Rightarrow 0.32 \text{ ml nutrient stock solution into the } 40 \text{ ml of inoculated M9 media.}$

## **APPENDIX IV: Enzyme Activity Calculations**

$$\frac{\text{Enzyme Activity}}{\text{ml of enzyme}} = A \times \frac{1}{t} \times \frac{10^6}{15,000} \times \frac{N_v}{E_v} \times \frac{1}{E_v}$$

1 milliunit = 1 nmole of product formed per minute

A = difference in absorbance at 420nm in the enzyme and the corresponding blank assay  
after the elapsed time for the enzyme assay

t = time in minutes for the observed change in absorbance

15,000 = the molar extinction coefficient for o-nitrophenol in the Spectronic 20 tube

$10^6$  = a correction to change the molar part of the extinction coefficient from mole per litre to nanomole per millilitre

$N_v$  = total assay volume (ml) at the time of the absorbance reading

$E_v$  = volume of enzyme sample (ml) in the assay