Title: Effect of Glucose Concentration on the Biomass Yield of *Pichia pastoris*

Introduction:

In the course of a summer biotechnology internship at a university, a deep curiosity emerged for the intersection of biology, biotechnology, and sustainability. The experience served to fuel a passion for optimizing industrial processes in the production of bio-based products. Motivated by the metabolic mysteries of yeast cells, the goal is to contribute to eco-friendly and efficient bioprocessing methods, acknowledging the significance of reducing environmental impact across various biotechnology fields, from biofuels to pharmaceuticals. In the present work, the effect of varying glucose concentrations on the growth kinetics of the GS115 strain of *Pichia pastoris* is investigated. Through spectrophotometry, the OD is recorded thus depicting the biomass yield at each glucose concentration (2%, 10%, 20%, 30%). Through this experimentation, the main objective is to identify the threshold limit of the biomass growth of *Pichia pastoris*.

Research Question: How do varying concentrations of glucose (2%, 10%, 20%, 30%) affect the biomass yield of *Pichia pastoris* under control conditions as measured by optical density observed at 600nm?

Background information:

Pichia pastoris, also known as Komagataella phaffi, is a methylotrophic yeast (can thrive with methanol as the sole carbon source) part of the order Saccharomycetales, is currently recognized as one of the most versatile hosts for the production of heterologous recombinant proteins (*J L Cereghino, PubMed*). It has the ability to grow to high cell densities, thus being able to produce a high yield of secreted heterologous proteins while producing lower amounts of their endogenous proteins which reduces the interference of unwanted host proteins during protein production.

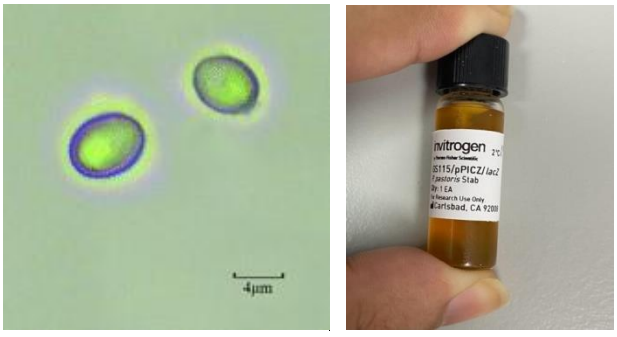


Figure 1 **(Left) Observation of *P. pastoris* GS115 under normal white light, Research Gate (Right) *Self-photographed***

Fermentation, a metabolic process, converts sugars into organic compounds (e.g., ethanol) by microorganisms like yeast in the absence of oxygen. Crucial for industrial applications, it's used in producing biofuels, pharmaceuticals, and recombinant proteins, such as those from *Pichia pastoris.*

Predictive microbiology is a field of study that focuses on using mathematical and statistical models (microbial cell growth curves) to predict the behavior of microorganisms in various environments such as incubation temperature, glucose concentration, pH, etc (*USDA*). Bio kinetic models predict microbial behavior, showing the link between factors and responses. They optimize growth conditions for maximum population densities and protein concentrations in minimal time. Medium composition influences protein yield and productivity in industrial fermentation, shaping efficient conditions for optimal outcomes.

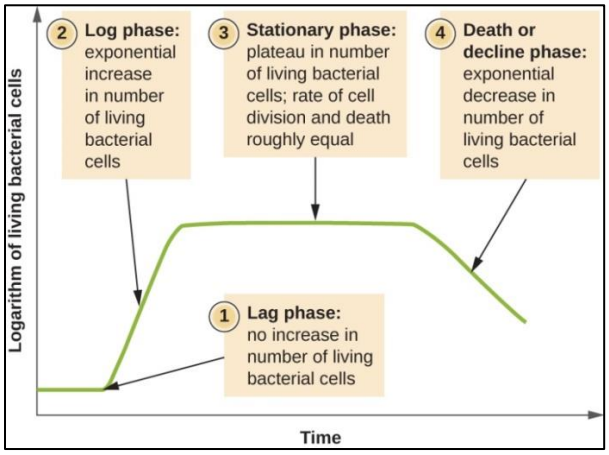


Figure 2 **Growth curve of a culture |** ***How Microbes Grow, Lumen Learning***

With a relatively short life span, rapid regeneration time, and cost-effective culture media that stimulate quick growth to high cell densities, *Pichia pastoris* simplifies experimental procedures making it a viable choice for numerous studies, and allows for great amounts of biomass for industries looking to produce proteins or enzymes on a commercial scale. One specific strain of *Pichia pastoris*, GS115 (Invitrogen), is derived from the wild-type strain NRRL-Y 11430. It is the most frequently used strain in biotechnology and medical fields, especially for heterologous protein production.

Optical density (OD) is the log to the base 10 of the reciprocal of transmittance. In spectroscopy, it is a measure of absorbance, indicating the ratio of the intensity of light falling upon a material to the intensity transmitted (*Photonics*). Optical density measurements are typically conducted at the maxima of absorbance spectra to ensure consistency and accuracy in quantifying cell concentration and biomass production (*BMC Biophysics*).

Hypothesis:

In response to the research question, I believe that as glucose concentration increases, there will be an improved yeast cell growth observed, thus increasing the change in OD600 of the yeast cells over the 24 hours observed. Based on this, 30% glucose (dextrose) concentration will observe the highest OD600 over the 24 hours, and thus the highest biomass yield.

H_1: There is significant difference of OD600nm between the four concentrations of glucose media used in the samples. Hence, glucose concentration has an effect on the biomass production of *Pichia pastoris*.

H_0: There is no significant difference of OD600nm between the four concentrations of glucose media used in the samples. Hence, glucose concentration has no effect on the biomass production of *Pichia pastoris.*

Independent variable:

Different glucose (dextrose) concentrations (2%, 10%, 20%, 30%) added to YPD medium during media preparation before inoculation from *Pichia pastoris* strain. For every 2.5 g of YPD extract in 50 ml of water, 10 g, 20 g, and 30 g of glucose is added.

This is done to potentially answer the research question posed, to observe the effects of glucose concentration on the biomass yield of the growth cultures. This particular range of concentrations covers both suboptimal and supra-optimal levels of glucose whose effects can be analyzed.

Glucose being a primary carbon and energy source, it is important to investigate how its availability affects the metabolism of *Pichia pastoris.*

Dependent variable:

Optical Density of sample observed at 600 nm in spectrophotometer initially, after 1 hour, 2 hours, 3 hours, 4 hours, 7 hours and 24 hours respectively, depicting cell biomass yield.

As cell biomass increases, the suspension becomes more turbid due to an increase in the number of cells showing higher OD values. Also, OD measurements allow repeated sampling of the same culture without disrupting cell growth, especially with such a long period of observation.

Control variables:

Variable	Impact if not controlled	Method of control
Ratio of YPD to water while preparing growth medium	Variations in the ratio of YPD to water would result in unequal nutrient availability. If the ratio is not the same for all samples, it can impact growth rates.	Constant ratio in each flask is maintained (2.5g YPD and 50 ml water)
Type of sugar added	Different sugars are metabolized at different rates by yeast. If various sugars are used, yeast cells will have varying growth patterns. The results could be skewed and not solely attributed to the varying glucose concentrations.	Varying concentrations of only one type of sugar Glucose (dextrose) is used for all samples.
Recording Time Intervals	Irregular intervals for recording OD would create data points that are not evenly spaced over time. This would lead to inaccurate observations.	OD is consistently recorded at specified time intervals (0th hour, 1 hour, 2 hours, 3 hours, 4 hours, 7 hours, and 24 hours) for each glucose concentration, ensuring that the same data points are collected at each time point.
Strain type and amount	Using different strain types and amounts can lead to variations in initial cell density and strain-specific characteristics, obscuring the impact of glucose concentration.	Each flask is inoculated with the same amount (1 mL) of the same strain (GS115) to maintain consistent starting conditions and to isolate the effects of glucose concentration.
Temperature	Yeast cells are sensitive to temperature changes, and variations can lead to inconsistencies in growth patterns. Uncontrolled temperature could cause cells to grow faster or slower depending on temperature increase or decrease respectively.	The incubator shaker is set and maintained at a constant temperature of 27°C for all samples.
RPM (Rotations per minute) in incubator shaker	In the absence of a constant RPM, yeast cells might experience settling at the bottom of the flask due to gravity. The data collected might not accurately represent the overall yeast growth, as some regions of the culture could exhibit different growth rates or phases than others.	A consistent shaking speed (120 rpm) is maintained in the incubator shaker for all flasks to provide uniform mixing and avoid sedimentation of cells.
Wavelength for Spectrophotometer	Different wavelengths for OD measurements would yield inconsistent readings. If wavelengths are not standardized, comparing the optical density data across samples accurately becomes challenging, as readings may not be directly comparable.	A fixed wavelength (600 nm) is used for all spectrophotometry measurements to ensure uniform and consistent OD readings across the experiment.

Materials:

- X4 50 ml Erlenmeyer flasks
- Measuring flask 500 ml
- Weighing Balance
- Cotton, Aluminum foil
- Water 200 ml
- YPD broth powder 10 g
- 2 ml Eppendorf tubes
- Invitrogen GS115 *Pichia pastoris* stab vials
- Micropipette
- Incubator shaker
- Freezer
- Centrifuge
- Tube tray
- Spectrophotometer, cuvettes
- Dextrose powder 60 g

Methodology:

After attempting to perform a preliminary experiment at the school lab, it was evident that the available equipment and resources were insufficient for the requirements of the study. Therefore, to ensure the necessary experimental conditions, a visit to an external university lab was arranged.

1. Media Preparation:

- A weighing balance is used to measure 10 g of YPD broth powder which is then added to a 500 ml measuring flask containing 200 ml of water, and the resulting mixture is divided into four 50 ml Erlenmeyer flasks.

2. To each flask, the corresponding amount of glucose (2 g, 10 g, 20 g, and 30 g) is added, based on the desired concentration, and after mixing, each flask is plugged with cotton and covered with aluminum foil to maintain sterility.
3. The autoclave machine is ensured to be turned on at least 20 minutes before use to reach sterilization temperature.
4. Each flask is inoculated with 1 ml of the GS115 Invitrogen strain using a micropipette.
5. 2 ml of the culture from each flask is collected in Eppendorf tubes inside the autoclave machine at specified time intervals: initially (at 0th hour), after 1 hour, 2 hours, 3 hours, 4 hours, 7 hours, and 24 hours.
6. After sterilization, the Eppendorf tubes are frozen to halt cell growth and to allow for simultaneous measurement of growth rates at different time intervals later for all samples collected.
7. The flasks are left in an incubator shaker at a constant temperature of 27°C and a shaking speed of 120 RPM which allows for growth under controlled conditions.

8. After all samples are collected into the Eppendorf tubes over the 24 hours, they are centrifuged to allow the yeast cells to settle at the bottom of the Eppendorf tubes, removing the liquid media.
9. The yeast cell pellets in each Eppendorf tubes are uniformly suspended in water with the help of a micropipette to reduce scattering of light in the spectrophotometer.
10. The wavelength of the spectrophotometer is set to 600 nm, and the spectrophotometer is initialized before the yeast cell solutions from the Eppendorf tubes are transferred one by one into the cuvette for readings.
11. The optical density (OD) readings for each sample are recorded using the Spectromanager app on the computer where they are automatically tabulated.

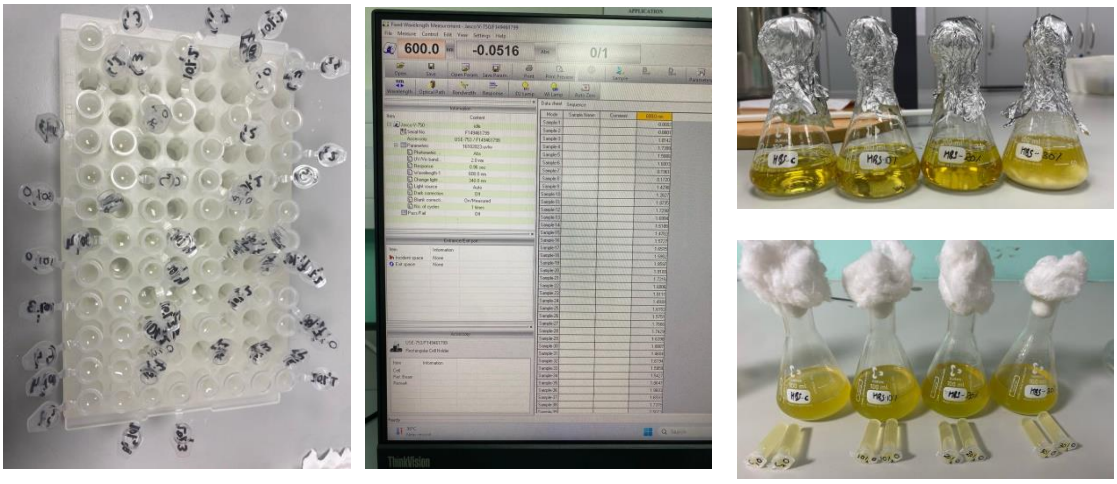


Figure 3 **Collage (Left to right, top to bottom) Resuspension of samples in water, *Spectromanager* specifications & raw data, YPD broth added to flasks, Clogged flasks containing inoculated strain**

* *Note that two samples were taken from each flask at every time interval for two readings.*

Precaution & Safety considerations:

To ensure aseptic conditions and prevent microorganism contamination, sample collection occurred within a laminar flow hood. Lab coats and gloves were worn consistently during laboratory work. The university laboratory personnel monitor and occasionally assisted in handling delicate equipment, such as the incubator shaker, autoclave, and centrifuge, to ensure safe usage.

Qualitative data: The clarity of the culture medium gradually diminished over time. As incubation continued, the medium lost clarity as it became increasingly cloudy. This cloudiness was consistently observed across all glucose concentrations and time intervals, depicting the accumulation of biomass and cellular growth in the culture medium.

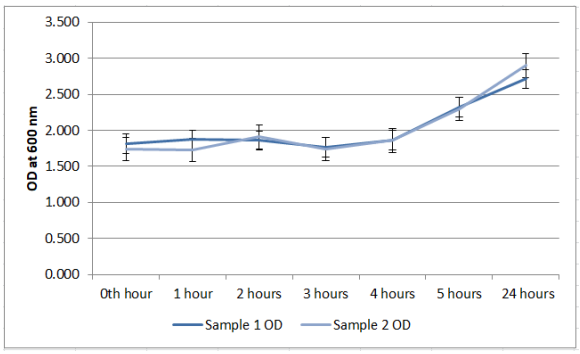
Quantitative data:

S. no	Time	Sample 1 OD	Sample 2 OD	Average	Standard deviation
1	0th hour	1.814	1.740	1.777	0.053
2	1 hour	1.874	1.730	1.802	0.102
3	2 hours	1.860	1.910	1.885	0.036
4	3 hours	1.767	1.743	1.755	0.017
5	4 hours	1.865	1.860	1.863	0.003
6	5 hours	2.324	2.296	2.310	0.020
7	24 hours	2.714	2.901	2.807	0.132

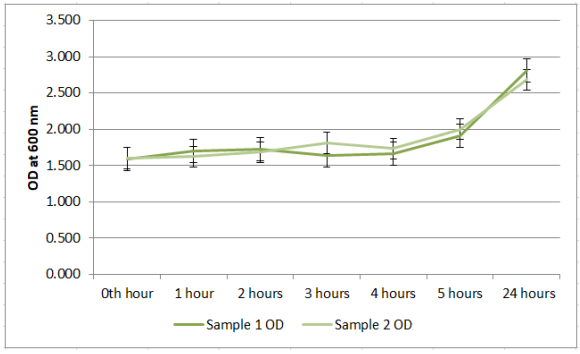
Table 1 **Control (2% glucose media)**

S. no	Time	Sample 1 OD	Sample 2 OD	Average	Standard deviation
1	0th hour	1.589	1.600	1.594	0.008
2	1 hour	1.699	1.619	1.659	0.057
3	2 hours	1.722	1.681	1.701	0.029
4	3 hours	1.640	1.809	1.724	0.119
5	4 hours	1.659	1.732	1.695	0.051
6	5 hours	1.906	1.998	1.952	0.065
7	24 hours	2.813	2.681	2.747	0.093

Table 2 **10% glucose media**



Graph 1 **Control (2% glucose media)**



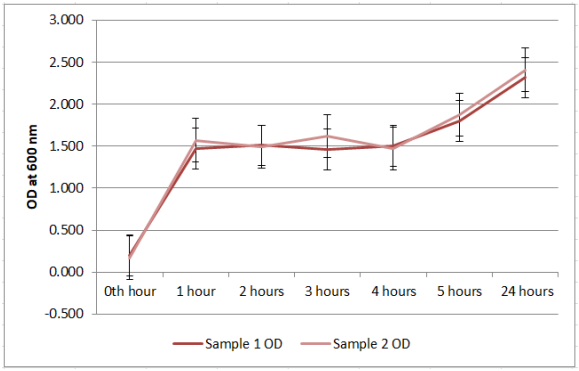
Graph 2 **10% glucose media**

S. no	Time	Sample 1 OD	Sample 2 OD	Average	Standard deviation
1	0th hour	0.196	0.172	0.184	0.017
2	1 hour	1.470	1.573	1.521	0.072
3	2 hours	1.511	1.494	1.502	0.012
4	3 hours	1.460	1.619	1.540	0.112
5	4 hours	1.502	1.469	1.485	0.024
6	5 hours	1.802	1.875	1.838	0.051
7	24 hours	2.316	2.410	2.363	0.066

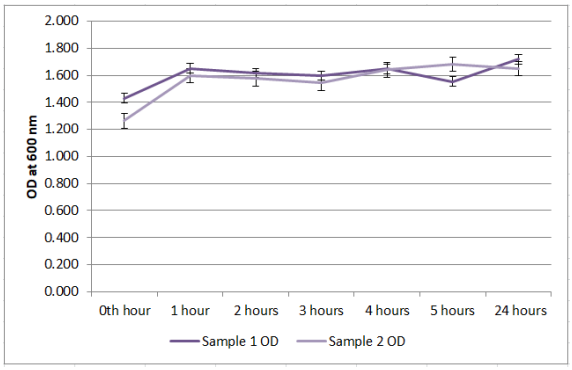
Table 3 **20% glucose media**

S. no	Time	Sample 1 OD	Sample 2 OD	Average	Standard deviation
1	0th hour	1.430	1.263	1.346	0.118
2	1 hour	1.652	1.595	1.623	0.040
3	2 hours	1.615	1.575	1.595	0.028
4	3 hours	1.596	1.543	1.569	0.038
5	4 hours	1.646	1.639	1.643	0.005
6	5 hours	1.555	1.683	1.619	0.091
7	24 hours	1.717	1.650	1.684	0.047

Table 4 **30% glucose media**



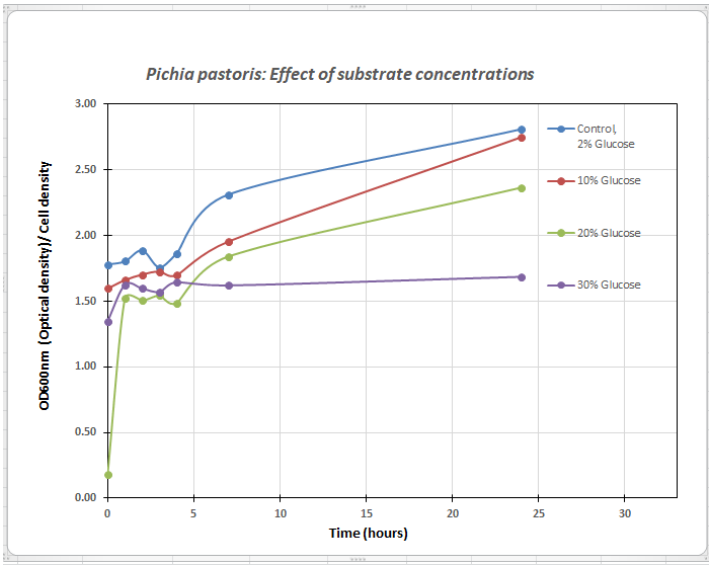
Graph 3 **20% glucose media)**



Graph 4 **30% glucose media**

Time (hours)	Control, 2% Glucose	10% Glucose	20% Glucose	30% Glucose
0	1.777	1.594	0.184	1.346
1	1.802	1.659	1.521	1.623
2	1.885	1.701	1.502	1.595
3	1.755	1.724	1.540	1.569
4	1.863	1.695	1.485	1.643
7	2.310	1.952	1.838	1.619
24	2.807	2.747	2.363	1.684

Table 5 **(Condensed) Average OD600nm readings of varying concentrations of glucose over time (hours)**



Graph 5 **(Condensed) Average OD600nm readings of varying concentrations of glucose over time (hours)**

Calculations:

1) Average Optical Density at 600nm of two sample readings

   The optical density at 600 nm for each of the two samples taken at each time interval and glucose concentration were summed up and divided by the number of concordant results in order to find an average OD600nm reading at a particular time with a certain glucose concentration.

2) Standard deviation for Optical Density at 600nm of two sample readings

   Standard deviation was calculated using the Excel function STDEV([@[Sample 1 OD]],[@[Sample 2 OD]])

   This helps quantify the variability or spread of data points around the mean, to help assess measurement consistency.

Statistical calculation:

An ANOVA test, specifically one-way ANOVA, is conducted on the data collected to compare the effect of different glucose concentrations on yeast sample growth after 24 hours. This test helps determine if there are statistically significant differences in OD600nm measurements among the different glucose concentration groups. The P value must be smaller than 0.05 for the value to be significant. Generally, the smaller the P value, the more significant it is and the bigger the F value, the more significant it is.

SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Glucose Concentration	4	0.62	0.155	0.014767		
OD600nm for sample after 24 hours	4	9.60115	2.400288	0.266792		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	10.08263	1	10.08263	71.62004	0.000149	5.987378
Within Groups	0.844677	6	0.140779			
Total	10.92731	7				

Table 6 **ANOVA one-way test calculations as performed with the help of Data Analysis ANOVA test feature on MS Excel**

Analysis:

Analyzing the data, it appears that as glucose concentration increases, yeast growth (as indicated by OD600nm) initially increases, but then decreases. In the first hour, yeast growth increases with higher glucose concentrations as glucose availability promotes initial yeast growth. However, as time progresses from 1 to 4 hours, the growth in the 20% and 30% glucose concentrations starts to decline, indicating that at these higher concentrations, the growth may be limited, and the yeast is not growing as effectively. At 7 hours, the pattern continues, with the 20% and 30% glucose concentrations showing lower growth compared to the 2% and 10% concentrations. By 24 hours, the trend is clear - the highest glucose concentration (30%) resulted in the lowest OD600nm value, indicating the least amount of yeast growth.

The one-way ANOVA results indicate that there is a statistically significant effect of glucose concentration on the OD600nm values after 24 hours of incubation. The F-statistic is 71.62, and the p-value is 0.0001, which is less than the typical significance level (0.05). This means there are statistically significant differences between the groups. This means that the different levels of glucose concentration (2%, 10%, 20%, and 30%) have a significant impact on the optical density (OD) of the yeast culture at

600nm. Thus, we can reject the null hypothesis and confirm the H_1 hypothesis; there is significant difference of OD600nm between the four concentrations of glucose media used in the samples. Hence, glucose concentration has an effect on the biomass production of *Pichia pastoris*.

Conclusion:

Data tables and data processing methods indicate that the collected and processed data can help answer the research question and establish a relation between the independent and dependent variable.

Overall, my hypothesis is only proven to be true to an extent. While the increase in yeast cell growth as glucose concentration increases can be seen as a trend from the 2% to the 20% glucose media, it is only observed until 4 hours when comparing the 20% and 30% growth media flasks. Thus, 30% glucose media does not observe the highest growth rate over the span of 24 hours.

The experimental data not only highlights a significant relationship between the increasing glucose concentration and yeast growth, but also helps us identify the threshold glucose limit of *Pichia pastoris* to be around 20% and 30% to be when growth begins to cease.

An osmotic effect is observed when the concentration of solutes in the surrounding environment becomes higher than that within a cell. In this experiment, as glucose concentrations increased, the osmotic effect is increased. Yeast cells, being surrounded by a hypertonic solution, have an efflux of water due to osmosis. This loss of water from the cells can lead to a reduction in turgor pressure and cellular volume, reducing the cells' growth and metabolic activity. The excessive presence of glucose might have led to the saturation of enzymes involved in glucose metabolism, causing an inhibition of metabolic pathways.

As the osmotic effect draws water out of the cells, *Pichia pastoris* cells are deprived of the water they require for enzymatic activity and cellular processes. Thus, the cells struggle to maintain their growth in an environment with excessive glucose, despite its nutrient value.

This understanding is applicable in biotechnological and fermentation techniques where precise control of nutrient concentrations is important for achieving optimal yeast growth and productivity.

Evaluation:

Error Analysis: The addition of error bars to the graphs suggests the potential scope of error during data collection. Another source of error could exist due to human error during data processing.

Strengths: There are various strengths in this experiment. The standard deviation was generally pretty low in the OD readings. This shows the reliability of the data in the investigation. All safety and ethical considerations were taken while heeding precautions.

Areas for improvement:

Area	Impact on results	Suggested improvement
Limited glucose concentration	The experiment only considered a limited range of glucose concentrations (2%, 10%, 20%, and 30%).	A range of concentrations especially between 2% and 10% will help determine exactly at which concentration growth decreases.
Single Carbon Source	The use of alternative carbon sources could be more cost-effective or yield improved results in certain applications.	Investigate the impact of various carbon sources, not limited to glucose, to identify alternative substrates that may enhance yeast growth under different conditions.
Less sample readings	A restricted number of sample readings can provide limited data points for analysis.	More sample readings at time points for a more detailed exploration of the yeast growth in response to varying glucose concentrations.
Lack of Protein-specific analysis	The study focuses on biomass yield but does not specifically address protein production levels.	The source of carbon or glucose concentrations could be focused to understanding protein production levels as well.

Further Extension & Improved Research Question:

How do varying concentrations of glucose (1%, 2%, 4%, and 8%) and alternative carbon sources (e.g., glycerol and lactose) influence the biomass yield of *Pichia pastoris* under controlled conditions?
This research investigates how varying glucose concentrations (1%, 2%, 4%, and 8%) and alternative carbon sources (glycerol, lactose) impact *Pichia pastoris* biomass yield. It aims to understand the yeast's response to nutrient conditions, identify optimal glucose concentrations for biomass production, and compare the effects of different substrates on growth and protein production. More frequent sampling (every 15 minutes) enhances capturing changes in yeast growth and protein expression.

References:

Special thanks to the university of BITS Pilani for granting access to their laboratory facilities, which greatly contributed to the successful completion of this IA. The support and resources provided by were invaluable in conducting the experiments and data collection presented in this investigation.

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