BIOPROCESSING LABORATORY REPORT

ERYTHRITOL PRODUCTION ANALYSIS USING YEAST *MONILIELLA POLLINIS*

By:
Group 2
Alicia Cherie Chandra - 21010030
Gabriella Zevania Kaitlyn - 22010012
Kashka Ananggadipa Pradhana - 21010111
Mary Grace Carmelita Epifania Rahayu - 21010129
Priscilla Klaresza Adhiwijaya - 21010153

Submitted to

i3L – Indonesia International Institute for Life Sciences
School of Life Sciences

Jakarta, Indonesia
2024

ABSTRACT

As a four-carbon sugar alcohol, erythritol has been widely used as an alternative for sugar especially for those who are suffering from diabetes and obesity due to its low calorie nature. In accordance with the increasing demand, there are several ways of producing erythritol which involves chemical synthesis that is considered to be expensive and ineffective. Consequently, it has been found that erythritol could also be produced by yeast such as *Moniliella pollinis* upon the reduction of erythrose into erythritol. In this extensive experiment, the production of erythritol by *M. pollinis* was observed for 96 hours of cultivation upon the implementation of different conditions including the use of native *M. pollinis* strain with a varied Yeast Extract and NaCl concentration, as well as the use of mutant *M. pollinis* strain. During the cultivation, several testing methods such as OD600, Cell viability, pH testing, and biomass measurement were conducted to further observe the yeast's growth. At the end, the evaluation was done based on the HPLC analysis in order to observe the kinetics of erythritol production per substrate consumption, yield mass of the erythritol, and the volumetric productivity. The result shows that the addition of 5 g/L yeast extract as well as 25 g/L of NaCl concentration exhibited the highest erythritol yield and productivity. Additionally, the UV mutagenesis performed towards the *M. pollinis* strain did not generate a desirable result which might be due to the disruption effect towards the associated pathway. With that being said, an enhanced result could be obtained by implementing a better sterility of the experimental conduct, as well as to perform other mutagenesis methods such as the chemical-based in order to produce a more prominent mutant strain with an enhanced productivity.

Keywords: Erythritol; Moniliella pollinis; Glucose concentration; Yeast extract; Salt concentration; Mutagenesis

ACKNOWLEDGEMENTS

The authors would like to express their gratitude to Indonesia International Institute for Life Sciences (i3L) for the opportunity given to be enrolled and participated in the Bioprocessing Laboratory course during the 6th semester of their study. Specifically, the authors would also like to give appreciation for the guidance and knowledge given by Mr. Ihsan Tria Pramanda, S.Si., M.Sc., and Dr. Riahna Kembaren, B.S., M.Sc., as well as the teaching assistants Nethania Darmawan, Angellique Regina, and Jocelyne. Lastly, the authors would like to thank all students who are involved and contributed to the completion of this project course.

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LIST OF ABBREVIATIONS

CFU/mL	Colony Forming Units per Milliliter
C/N	Carbon-to-nitrogen
NaCl	Sodium Chloride
OD600	Optical Density 600 nm
PBS	Phosphate-Buffered Saline
HPLC	High-Performance Liquid Chromatography
YE	Yeast Extract
UV	Ultraviolet
YGM	Yeast Glucose Minerals
PDB	Potato Dextrose Broth
PDA	Potato Dextrose Agar
BSC	Biosafety Cabinet
RPM	Rotation per Minute
PPP	Pentose Phosphate Pathway
ER	Erythrose Reductase
GK	Glycerol Kinase
WCO	Waste Cooking Oil
KCl	Potassium Chloride
NH_4Cl	Ammonium Chloride
$MgSO_4.7H_2O$	Magnesium Sulfate Heptahydrate
KH_2PO_4	Potassium Dihydrogen Phosphate
K_2HPO_4	Potassium Hydrogen Phosphate
µL	Microliter
Nm	Nanometer
Q_{ERY}	Volumetric Productivity of Erythritol
Y_{ERY}	Yield Mass of Erythritol
RI Detector	Refractive Index Detector
mAU*min	Milli-Absorbance Units-minutes

I. INTRODUCTION

The growing need for healthier alternatives in food especially for diabetic or obese people has led to the introduction of substitutes to common ingredients such as sugar. One of the most promising substitutes that was discovered in 1852 and began to grow in popularity in the early 1980s is erythritol. This compound has been a popular substitute for sugar since it is 0.2 kcal/g and is comparable to sugar's taste profile (Carly, 2018). Erithyrol can be found in a variety of foods such as sugar free ice cream, candy, gum, cookies, cakes, and protein bars. Erythritol is primarily sourced from the fermentation of specific yeast organisms, The most commonly implemented microorganism is *Moniliella pollinis, Trichosporonoides megachiliensis*, *and recently*, *Yarrowia lipolytica* (Rzechonek, 2018).

However there are several issues with erythritol that is inhibiting it from being a mainstay in the food industry. First, Erythritol is produced through a fermentation process, chemical synthesis or through extraction which can be relatively expensive and inefficient (Rakicka, 2016). This experiment will explore possible improvements towards the production of erythritol using *Moniliella pollinis*. *M. pollinis* creates erythritol by fermenting glucose, sucrose, and glycerol under osmotic stress (Rzechonek, 2018). This yeast has the capabilities to produce 0.4 to 0.43 g/g of erythritol from glucose by the pentose phosphate pathway, and mutated strains can produce 0.44 to 0.57 g/g erythritol. This research will analyze the effects of different parameters such as yeast extract concentration, sodium chloride (NaCl) concentration and the process of UV mutagenesis.

1.2 Aim of the Research

1. Identifying the optimum growth conditions for erythritol production in the context of varying the yeast extract concentration (1 g/l, 3 g/l, and 5 g/l) that affects the C/N ratio, as well as the NaCl concentration (0 g/l, 25 g/l, and 50 g/l)
2. Creating a mutant strain of *Moniliella pollinis* and the identification of the best mutant strain.

1.3 Research Scope

1. The observation and the investigation on which yeast extract concentration and NaCl concentration have an impact towards the production of erythritol production using *Moniliella pollinis.*
2. Identification of the best composition of media for the production of erythritol
3. Analyze and collect data on the characteristics of erythritol and glucose consumption through use High-Performance Liquid Chromatography
4. To compare between normal and mutant strain of *Moniliella pollinis*

1.4 Research Question

1. What is the effect of varying yeast extract concentration on the production of erythritol?
2. What is the effect of varying NaCl concentration on the production of erythritol?
3. Does mutation of *Moniliella pollinis* through mutagenesis cause an increase in the production of erythritol?

1.5 Hypothesis

1. H_0: Varying yeast extract concentrations does not affect the production of erythritol
   H_1: Varying yeast extract concentration affects the production of erythritol
2. H_0: Manipulating osmotic pressure by varying NaCl concentrations does not affect the erythritol production
   H_1: Manipulating osmotic pressure by varying NaCl concentrations affects erythritol production
3. H_0: Random mutagenesis of *Moniliella pollinis* does not affect the production of erythritol
   H_1: Random mutagenesis of *Moniliella pollinis* would increase the production of erythritol

II. LITERATURE REVIEW

2.1 Erythritol

Erythritol ((2R,3S)-Butan-1,2,3,4-tetrol) is a sugar alcohol, also known as polyol, compound that is white in color and takes the form of an anhydrous crystalline powder. It is formed through the hydrolysation processes of the aldehyde or ketone group in various carbohydrates (Hong et al., 2019). Because erythritol only consists of four carbon atoms with the chemical formula of $C_4H_{10}O_4$ as shown in **Figure 2.1**, it is known to have the smallest molecular weight of all sugar alcohols and thus having a slightly different physician and chemical properties. Additionally, erythritol is a symmetrical molecule and exists only in the meso-form (Škoc et al., 2024).

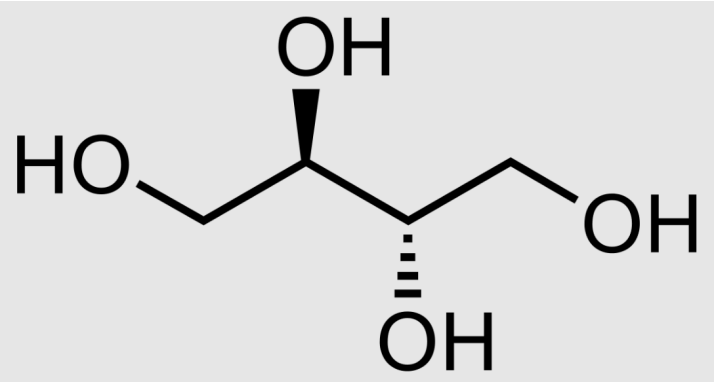

Figure 2.1. The structure of erythritol

Being a sugar molecule, the sweetness level of erythritol is approximately 60 - 80% compared to sucrose and it has a mild cooling effect on the mouth which is similar to menthol with no aftertaste. Additionally, it is naturally found on various fruits including melon, pears, watermelon, grapes; as well as fermented food such as soy sauce and cheese (Mazi & Stanhope, 2023). Erythritol is also a polyol that is non-caloric as it cannot be metabolized by the human body, does not provide any energy, and does not alter the level of glucose and insulin. Thus, it is not modified when excreted into the urine. This property allows it to be used as a sweetener for diabetic and obese people. Moreover, erythritol also has antioxidant properties due to its capability as a free radical scavenger while circulating the body before its secretion into the urine (Regnat, 2018).

2.2 Carbon Sources for Erythritol Production

In erythritol production, substrates such as glucose and glycerol that provide carbon sources are a major contributing factor to the amount of the produced bioproduct. According to Martău et al. (2020), glucose is considered to be the most widely used substrate for erythritol production due to its high availability and efficiency. Aside from glucose, glycerol also has a high potential to be used as a carbon source for bioprocesses although it contains high amounts of impurities (Carly & Flickers, 2018). A review by

Daza-Serna et al. (2021) stated that commercial carbon sources that are used to produce erythritol include glucose, xylose, and glycerol are capable to obtain the erythritol yields of 0.1 - 0.6 g/g, 0.26 g/g, and 0.66 g/g respectively using different yeast species (e.g. *Candida magnoliae*, *Trichosporonoides oedocephalis*, *Yarrowia lipolytica*, and *Aurebasidium pullulans*) under different cultivation medium and conditions. These obtained data provides further evidence that glucose and glycerol are suitable substrates for erythritol production.

In addition, non-conventional substrates like molasses and crude glycerol are also used in the context of circular economy. It is also summarized that erythritol yields obtained are 0.15 - 0.57 g/g for crude glycerol and 0.12 - 0.375 g/g for other non-convetional substrates using different yeast species (e.g. *Aurebasidium pullulans, Moliniella pollinis, Yarrowia lipolytica*) under different cultivation medium and conditions (Daza-Serna et al., 2021). Although these obtained yields are not as high as the conventional substrates, these values could be deemed decent and it needs to be considered that the usage of these substrates also provides solutions that reduce environmental waste. Moreover, they contain certain amounts of sugar as could be seen in **Table 2.1** below as stated by Khatape et al. (2023).

Table 2.1. Sugar composition of carbon substrates

Carbon	Sucrose (%)	Glucose (%)	Fructose (%)	Total sugar (%)
Glucose	Nil	99.8 ± 0.8	Nil	99.8 ± 0.8
Jaggery	78.5 ± 3.8	11.2 ± 3.1	5.8 ± 2.1	95.5 ± 9
Molasses	42.3 ± 4.2	2.9 ± 1.2	1.6 ± 1	46.9 ± 6.4
Sugarcane Juice	10.2 ± 1.2	2.27 ± 0.8	0.55 ± 0.5	13.02 ± 2.5

2.3 Erythritol Production

2.3.1. *Moniliella pollinis*

Moniliella pollinis is a yeast with a yellowish appearance. When observed on the surface of the agar plate, it develops a short closely wrinkled aerial hyphae with white to yellow color at the margin. Under the microscope, they could be seen as elongated rods that form a chain (Dooms et al., 1971; Khatape et al. (2023). Like many other yeast species, *M. pollinis* is capable to be used for erythritol production and currently it has been considered to be a commercial base for producing erythritol along with *Moniliella megachiliensis*, and *Yarrowia lypolitica* (Nakagawa et al., 2021).

2.3.2. Erythritol Production Pathway

In yeast, erythritol production is attained from the pentose phosphate pathway (PPP) within a two step reaction (**Figure 2.2**). Firstly, D-erythrose

4-phosphate, one of the final products of the non-oxidative phase of PPP, is dephosphorylated to erythrose by the enzyme polyol phosphatase PYP1. The erythrose is then reduced to erythritol by the enzyme NAD(P)H-dependent erythrose reductases (ERs) (Albillos-Arenal et al., 2023).

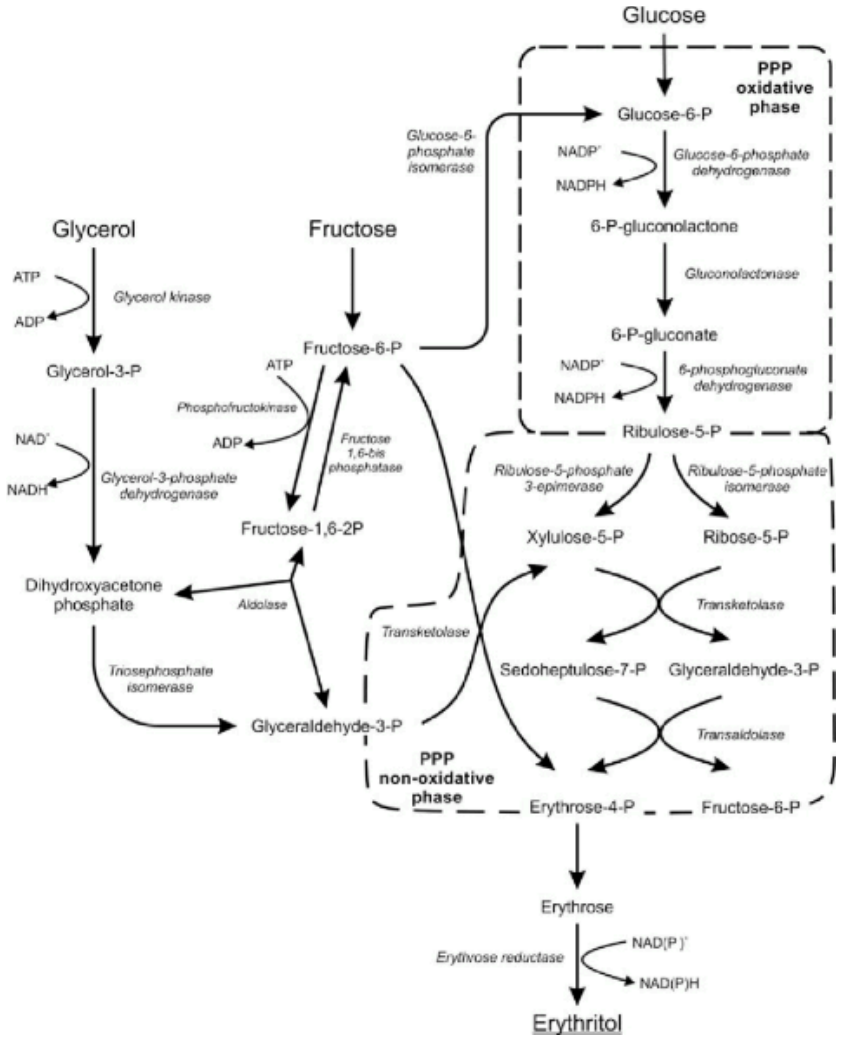

Figure 2.2. The biochemical pathways involved in erythritol production

Based on the **Figure 2.2** above, it could be seen that many carbon molecules including glucose, glycerol, and fructose are involved as the precursor compounds within the pathway. For instance, the glycerol is transformed into glycerol-3-phosphate by the enzyme glycerol kinase (GK) which is then converted into glyceraldehyde-3-phosphate through glycolysis and by the gluconeogenesis enzymes trio-sephosphate isomerase and glycerol-3-phosphate dehydrogenase. The glyceraldehyde-3-phosphate is then incorporated into the non-oxidative phase of the PPP by the transketolase enzyme. Meanwhile, glucose is incorporated to the PPP oxidative phase and then PPP non-oxidative phase pathways before the conversion reaction into erythritol (Rzechonek et al., 2018).

2.4 Strategy to Improve Erythritol Production by *M. pollinis*

2.4.1. Optimization of C/N Ratio

A major strategy involved to improve erythritol production by *M. pollinis* is applying high osmotic pressure as erythritol is a product of hyperosmotic response or oxidative stress of *M. pollinis*. One of the ways of increasing osmotic pressure is by using high glucose concentration with the range from 200 g/L to 400 g/L. Increasing glucose concentration also results in a higher C/N ratio as the relatively high ratio value is required to redirect the provided carbon sources towards erythritol production and away from biomass formation or other metabolic pathways. Manipulation of C/N ratio could also be achieved by manipulating the concentration of yeast extract of the media as the nitrogen sources where higher amounts of yeast extract would result in a lower C/N ratio (Carly & Flickers, 2018). However, it needs to be considered that high carbon content with insufficient nitrogen (nitrogen could be provided by the yeast extract) within the media would also hinder the fermentation reaction of the yeast. According to Hijosa-Valsero et al. (2021), the sufficient C/N ratio within the range of 150 - 180 are ideal for erythritol production. On the other hand, a study by Jagtap et al. (2021) which use the yeast *Yarrowia lipotylica* to produce erythritol from glycerol found the optimal C/N ratio is 5 and 15 as the higher ratio values would result in a lower erythritol production (Jagtap et al., 2021). As there are quite limited known studies which utilize the aforementioned approaches to manipulate the amount of erythritol production, this becomes the research gap that would be tested within the experiment as different yeast extract concentrations would be utilized as one of the variables.

2.4.2. Optimization of Salt Concentration

Aside from varying the C/N ratio, another way of inducing a hyperosmotic environment is through increasing the amount of salt within the media. According to a study conducted by Liu et al. (2018) which analyze erythritol production using *Yarrowia lipolytica* on waste cooking oil media (WCO), the highest amount of produced erythritol is achieved in the NaCl concentration of 80 g/l with the erythritol production found to be ± 20.3 g/L and erythritol yield to be ± 0.8 (**Table 2.2**).

Table 2.2. Effects of NaCl concentration on erythritol production

Parameters	Initial osmotic pressure (osmol/L)					
	0.08	0.75	1.42	2.09	2.76	3.43
NaCl (g/L)	0	20	40	60	80	100
Ery production(g/L)	2.6 ± 0.9	4.1 ± 1.1	10.9 ± 0.6	15.0 ± 1.2	20.3 ± 2.3	18.1 ± 1.9
$Y_{Ery\ P/S}$ (g/g oil)	0.11 ± 0.03	0.16 ± 0.04	0.40 ± 0.02	0.55 ± 0.04	0.80 ± 0.09	0.79 ± 0.06
CA production (g/L)	10.5 ± 0.8	12.2 ± 1.0	11.4 ± 0.9	9.1 ± 0.1	2.6 ± 0.0	1.7 ± 0.4
Residual WCO (g/L)	5.3 ± 0.1	4.4 ± 0.4	2.7 ± 0.6	2.9 ± 0.2	4.7 ± 0.3	7.0 ± 0.9
$Y_{CA\ P/S}$ (g/g oil)	0.43 ± 0.04	0.48 ± 0.03	0.41 ± 0.03	0.34 ± 0.00	0.10 ± 0.00	0.07 ± 0.01

Ery, erythritol; CA, citric acid; $Y_{Ery\ P/S}$, erythritol product yield on WCO; $Y_{CA\ P/S}$, citric acid product yield on WCO.

Additionally, another study by Li et al. (2017) that utilized *Trichosporonoides oedocephalis* under batch fermentation conditions found that erythritol production reached its maximum at the NaCl concentration of 5 g/L and KCl concentration of 5 g/L followed by the decline of production at higher concentrations of both salts. This decline of bioproduct production shows that higher salt concentration does not guarantee higher erythritol yields as this condition could also hinder the growth of the yeast cells. Overall, the usage of KCl also exhibits higher erythritol production compared to NaCl.

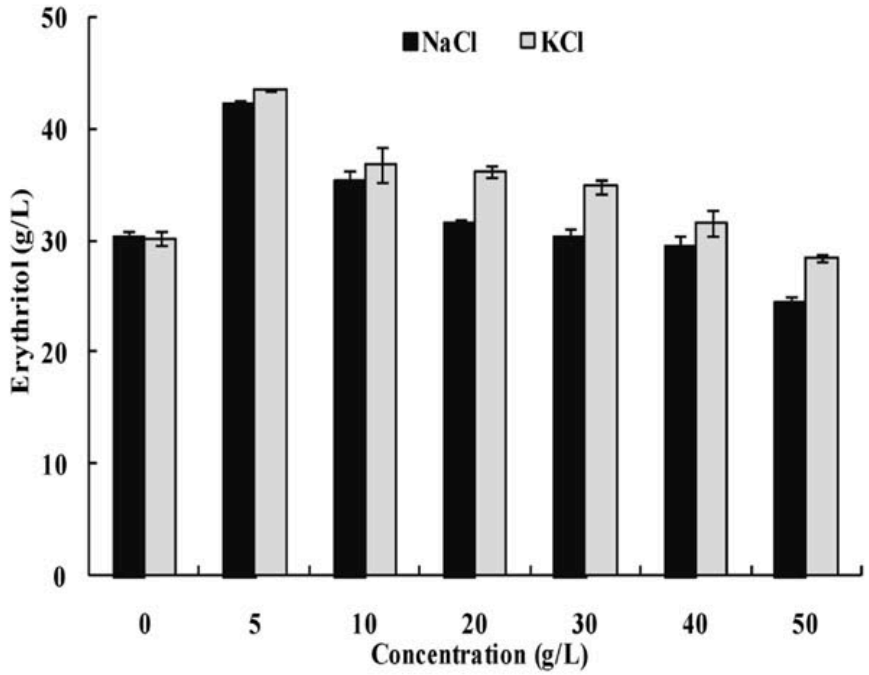

Figure x. The effect of different concentrations of NaCl and KCl on erythritol production

2.4.3. Strain Improvement through Genetic Modification

Genetic modifications could be considered as other innovative approaches for achieving optimal erythritol yields and improving production performances. Some of the desired superior characteristics are increased erythritol production capability, enhancing tolerance towards environmental stresses, and increased substrate consumption. The involved methods in genetic modifications include random mutations, mutant proteomic analysis, and genetic engineering which alters the genetic sequence of the yeast genome thus generating mutant yeast strains (Carly & Flickers, 2018). One of the most commonly used techniques is random mutagenesis which is used within this experiment and discussed further.

2.4.3.1 Random Mutagenesis

Random mutagenesis involves the generation of random mutant strains by UV or chemical treatment which has been successfully done within the last few years. A research conducted by Lin et al. (2010) managed to obtain a *M. pollinis* mutant strain capable of producing high erythritol yield of 59.5% from nitrosoguanidine (NTG) treatment. Another research by Rywninska et al. (2012) also obtained mutant *Y. lipolytica* Wratislavia 1.31 that also has enhanced erythritol production after exposure to UV radiation. However, UV radiation is preferred compared to mutation by chemicals as chemical mutagenesis could be hazardous which makes UV light to be safer. Additionally, the mutation procedure involving UV light is also considered to be easier and cost-effective (Liu et al., 2017). Thus, UV light is chosen as the random mutagenesis approach within the experiment.

2.5 High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is a technique that uses a column of liquid chromatography under high pressure to separate the constituents of a compound. As in other chromatography, the mobile phase is pumped and injected into the column, and the separation takes place in the column that has the stationary phase bound to it (Akash et al., 2020). The phases that are employed are as follows: the mobile phase is the solvent that is forced into the column at high pressure, while the stationary phase is made up of tiny porous granules that are used to selectively capture passing molecules based on affinity. Typically, the pump and injector are attached to a stainless steel capillary that serves as the column. The analyte's components pass through the column at varying rates because of their unique interactions with the stationary phase (Gritti et al., 2020). The column that was used was Shodex Sugar SP0810 7 µm, 8.0 mm I.D. x 300 mm which is polar, the column is designed to separate saccharides based on their molecular weight and charge interactions with the functional group sulfo (Pb2+) (Shodex, n.d).

III. MATERIALS & METHODS

3.1 Materials

The main materials used for this experiment include *M. pollinis* culture, yeast extract, D(+)-glucose monohydrate (Merck), erythritol, NH_4Cl (Merck), $MgSO_4.7H_2O$ (Merck), KH_2PO_4 (Merck), K_2HPO_4 (Merck), K_2HPO_4 (Merck), NaCl (Merck), bacteriological agar (Merck), PDB powder (Hi Media), PBS tablet (Merck), glycerol (Merck), type III water, and type I water.

3.2 Methods

3.2.1. Yeast Cultivation: Exploring the Growth Curve and Erythritol Production

3.2.1.1. Materials and Equipment Preparation

3.2.1.1.1. Preparation of Yeast Glucose Minerals (YGM)

In order to make the YGM media, the media components were weighted to make the total media volume of 250 mL. The components consist of yeast extract (Concentration: 1 gr/l for group 1 - 2; 3 gr/l for group 3 - 4; 5 gr/l for group 5 - 6), 75 gr of glucose (Concentration: 300 gr/l), 0.5 gr of NH_4Cl (Concentration: 2 gr/l), 0.25 gr of $MgSO_4.7H_2O$ (Concentration: 1 gr/l), 0.075 gr of KH_2PO_4 (Concentration: 0.3 gr/l), 0.025 gr of K_2HPO_4 (Concentration: 0.1 gr/l), and 6.25 gr of NaCl (Concentration: 25 gr/l).

The mixed components were then hydrated with 250 mL type III water inside a 500 mL erlenmeyer flask. Next, the flask was closed with aluminium foil, and a sterilization strap marker was placed on top of the foil. Lastly, the flask was put in an autoclave machine, and the autoclave was run at 121^{o}C for 15 minutes for sterilization.

3.2.1.1.2. Preparation of Potato Dextrose Broth (PDB) Media and Media Sterilization

In order to make the PDB media, 3.6 gr of PDB powder (Concentration: 24 gr/l) was weighted for a total media volume of 15 mL. The weighted PDB powder was then hydrated with 15 mL of type III water inside a 50 mL erlenmeyer flask. Next, the flask was closed with aluminium foil, and a sterilization strap marker was placed on top of the foil. Lastly, the flask was put in an autoclave machine, and the autoclave was run at 121^{o}C for 15 minutes for sterilization.

3.2.1.1.3. Preparation of Potato Dextrose Agar (PDA) Media and Media Sterilization

In order to make the PDA media, 7.2 gr of PDB powder (Concentration: 24 gr/l) and 4.5 gr of bacteriological agar (Concentration: 15 gr/l) were weighted for a total media volume of 300 mL. The weighted PDB powder was then hydrated with 300 mL of type III water inside a 500 mL erlenmeyer flask. Next, the flask was closed with

aluminium foil, and a sterilization strap marker was placed on top of the foil. The flask was then put in an autoclave machine, and the autoclave was run at 121°C for 15 minutes for sterilization.

After the autoclaving, the heated PDA was left to cool to 45 - 50°C. The cooled PDA was then poured into each plate and left on the sterile surface until the agar solidified. Lastly, the plates were stored in a refrigerator at 2 - 8°C.

3.2.1.1.4. Preparation and Sterilization of PBS buffer

In order to make the PBS buffer, one PBS tablet was dissolved in 100 mL of type III water in a 250 mL erlenmeyer flask. The PBS solution was then sterilized by autoclaving at 121°C for 15 minutes.

3.2.1.1.5. Preparation and Sterilization of 50% Glycerol Solutions

In order to make the 50% glycerol solution, 10 mL of glycerol and 10 mL of type III water (1:1 ratio) were mixed to make a total volume of 20 mL in a 50 mL erlenmeyer flask. The sterilization strap marker was then put on top of the bottle container. Lastly, the glycerol solution was sterilized by autoclaving at 121°C for 15 minutes.

3.2.1.1.6. Sterilization of Micropipette Tips

The micropipette tips were arranged on the box, and the sterilization strap marker was put on top of the micropipette box after the box was closed. After sterilization by autoclaving at 121°C for 15 minutes, the box of micropipette tips was put in the oven to dry.

3.2.1.1.7. Sterilization of Microcentrifuge Tube

The 1.5 mL microcentrifuge tubes (around 100 pieces for each group) were closed and put in a heat-resistant plastic bag. After the sterilization strap marker was placed on top of the plastic bag, the bag was sterilized by autoclaving at 121°C for 15 minutes and then dried in the oven.

3.2.1.1.8. Preparation of *M. pollinis* Pre-culture

The 1 mL sterile PDB was transferred to a sterile 1.5 mL microcentrifuge tube. This would be used as the blank for the subsequent OD measurement. After that, the *M. pollinis* cryo stock was transferred to 10 mL of sterile PDB using sterile ose aseptically to make the culture. The culture was incubated at 30°C, 120 rpm for 2 - 3 days. This pre-culture would be used to make another cryo stock for the glycerol stock preparation step.

3.2.1.2. Preparation of *Moniliella pollinis* glycerol stock

3.2.1.2.1. Preparation of Preculture Glycerol Stock

Before preparing the glycerol stock, 200 µl of samples including the blank and pre-culture were taken and dispensed into separate wells of a 96-well plate for optical density (OD) measurement at the wavelength of 600 nm (OD_{600}). This procedure was repeated in triplicates. After that, 500 µl of the *M. pollinis* preculture was added to 500 µl of the 50% glycerol within a 2 mL labeled cryovial followed by gentle mixing. The glycerol stock cryovial was then stored at -80^{o}C. The sterile preculture was finally kept for streaking to PDA and random mutation purposes.

3.2.1.2.2. Streaking the Preculture *M. pollinis* to PDA

The stored *M. pollinis* preculture was used to streak the PDA media in a Four Quadrant Streak pattern using a sterile loop or ose. The media was finally incubated at room temperature for 3 - 4 days.

3.2.1.3. Random Mutagenesis of *M. pollinis* using Ultraviolet (UV) Light

About 30 µl of the *M. pollinis* preculture grown on PDB was transferred and spread to the center of the PDA. The agar was then exposed to UV light at different periods, 10 mins for group 1 - 3 and 15 mins for group 4 - 6. The number of grown colonies was expected to be reduced significantly to 3 - 5 colonies. The preculture spread in PDA as the negative control was not exposed to UV light. After the UV radiation, the agar plate was put in an incubator and incubated for 4 days at room temperature. The colonies that grew after irradiation were called mutant strains.

3.2.1.4. Determination of the Growth Phase of *M. pollinis* in YGM media

Firstly, 1 mL of sterile YGM media was transferred into a sterile 1.5 mL microcentrifuge tube. This will be used as the blank for the OD measurement. Using sterile tips, 10 mL of *M. pollinis* pre-culture was transferred into the sterile prepared YGM media and mixed well. A 4 mL aliquot of the obtained culture was transferred using a 10 mL serological pipet into a sterile 15 mL falcon tube. This aliquot would be used as the 0 hours data for the OD600 measurement, CFU/mL measurement (using Miles-Misra), pH measurement (using pH universal indicator), as well as biomass and supernatant collection.

After the measurement at 0 hours, the culture was incubated at 30^{o}C, 150 rpm for 4 days. During this period, the aforementioned measurements were also performed daily, and the obtained values were taken as 24 hours (T1), 48 hours (T2), 72 hours (T3), and 96 hours (T4). After 4 days of incubation, the cells were harvested by transferring the cell culture to the falcon tube and applying centrifugation at 5000 rpm for 20–25

minutes. The obtained supernatant was collected for the subsequent HPLC measurement to measure erythritol production, and the biomass was put in the oven for 2 - 3 days to obtain the dry cell mass.

The obtained values from the previously conducted measurements were used to plot four types of graphs: one graph with the time in hours as the X-axis and OD600 as the Y-axis, one graph with the time in hours as the X-axis and cell viability in CFU/mL as the Y-axis, one graph with the time in hours as the X-axis and mass of biomass in grams as the Y-axis, and one graph with the time in hours as the X-axis and pH measurement as the Y-axis. Lastly, the growth rate and doubling time of the *M. pollinis* were calculated.

3.2.1.4.1. Optical density (OD) measurement

Firstly, 200 µl of samples (Blank and pre-culture) were taken and dispensed into separate wells of a 96-well plate. This procedure was repeated in triplicates. Using a plate reader, optical density (OD) measurement at the wavelength of 600 nm (OD_{600}) was performed.

3.2.1.4.2. Miles-Misra method

The serial dilutions of the yeast suspension were prepared by diluting the yeast culture suspension using a sterile PBS buffer. Then, 100 µl of *M. pollinis* culture sample was transferred and mixed with 900 µl of sterile PBS buffer in one sterile microcentrifuge tube as the initial 10^{-1} dilution. The contents were then mixed by shaking. Next, 100 µl of the cell suspension was transferred to another microcentrifuge tube containing 900 µl of PBS buffer as the 10^{-2} dilution. The procedure was repeated for the subsequent dilutions until all desired dilutions were obtained.

As for the PDA in the Petri dish, the dish was divided into 6 sectors, each sector for one type of dilution. From the selected dilution, the dilated cell suspension was deposited as drops of 10 µl onto a sector on the PDA. Each sector on one plate received 3 separate 10 µ drops of the sample. The procedure was repeated as duplicates on a different PDA plate. The PDA plate was incubated for 4 - 5 days, and then the obtained colonies of each drop were counted for the CFU/mL calculation.

3.2.1.4.3. Biomass and Supernatant Collection

Before placing the sample, the empty 1.5 mL microcentrifuge tube or 50 mL falcon tube was weighed. 1 mL of the *M. pollinis* culture was then transferred into the microcentrifuge tube. Next, the sample was centrifuged at 5000 rpm for 4 - 8 minutes if using a microcentrifuge tube and at 5000 rpm for 20 - 25 minutes if using a falcon tube. The supernatant was collected in a sterile container and stored at 4°C for the subsequent HPLC analysis. As for the obtained biomass, the microcentrifuge tube was put in the

oven for 2 - 3 days to dry the cell biomass. These procedures were repeated in duplicates when using microcentrifuge tubes and several falcon tubes during harvesting.

3.2.1.4.4. pH measurement

Using a sterile tip, 200 µl of samples were transferred into the top of the pH universal indicator paper and left for some seconds for the indicator color to change. Finally, the color of the universal indicator papers was compared to the color chart to determine the pH of the sample. These procedures were repeated in duplicates for each measurement.

3.2.2. Production of Erythritol With A Different Salt Concentration In The Medium

3.2.2.1. Materials and Equipment Preparation

3.2.2.2.1. Prepare Yeast Glucose Minerals (YGM) with Different NaCl Concentration

In order to make the YGM media, the media components were weighted to make the total media volume of 250 mL. The components consisted of 0.25 gr yeast extract (Concentration: 1 gr/l), 75 gr of glucose (Concentration: 300 gr/l), 0.5 gr of NH_4Cl (Concentration: 2 gr/l), 0.25 gr of $MgSO_4.7H_2O$ (Concentration: 1 gr/l), 0.075 gr of KH_2PO_4 (Concentration: 0.3 gr/l), 0.025 gr of K_2HPO_4 (Concentration: 0.1 gr/l), and NaCl (Concentration: 0 gr/l for group 1 - 2; 25 gr/l for group 3 - 4; 50 gr/l for group 5 - 6).

The mixed components were then hydrated with 250 mL type III water inside a 500 mL erlenmeyer flask. Next, the flask was closed with aluminium foil, and a sterilization strap marker was placed on top of the foil. Lastly, the flask was put in an autoclave machine, and the autoclave was run at 121^{o}C for 15 minutes for sterilization.

3.2.2.2.2. Preparation of Potato Dextrose Broth (PDB) Media and Media Sterilization

In order to make the PDB media, 7.2 gr of PDB powder (Concentration: 24 gr/l) was weighted for a total media volume of 30 mL. The weighted PDB powder was then hydrated with 30 mL of type III water inside a 50 mL erlenmeyer flask. Next, the flask was closed with aluminium foil, and a sterilization strap marker was placed on top of the foil. Lastly, the flask was put in an autoclave machine, and the autoclave was run at 121^{o}C for 15 minutes for sterilization.

3.2.2.2.3. Preparation of Potato Dextrose Agar (PDA) Media and Media Sterilization

The steps for preparing PDA media were the same as the procedure mentioned in section 3.2.1.1.3.

3.2.2.2.4. Preparation and Sterilization of PBS Buffer

The steps for preparing PDA media were the same as the procedure mentioned in section 3.2.1.1.4.

3.2.2.2.5. Sterilization of Micropipette Tips

The steps for preparing PDA media were the same as the procedure mentioned in section 3.2.1.1.6.

3.2.2.2.6. Sterilization of Microcentrifuge Tube

The steps for preparing PDA media were the same as the procedure mentioned in section 3.2.1.1.7.

3.2.2.2.7. Preparation of *M. pollinis* Pre-culture

One *M. pollinis* colony was picked up with sterile tips or ose and transferred to PDB media. The culture was incubated at 30^{o}C, 120 rpm for 2 - 3 days. After the incubation, the mutant pre-culture was streaked on PDA media followed by 3 - 4 days of incubation.

3.2.2.2. Erythritol Production in Media with Different Salt Concentrations

Firstly, 1 mL of sterile YGM media was transferred into a sterile 1.5 mL microcentrifuge tube. This will be used as the blank for the OD measurement. Using sterile tips, 10 mL of *M. pollinis* pre-culture was transferred into the prepared YGM media having different salt concentrations (0 gr/l, 25 gr/l, and 50 gr/l). After mixing well, a 4 mL aliquot of the obtained culture was transferred using a 10 mL serological pipet into a sterile 15 mL falcon tube. This aliquot would be used as the t=0 for the OD_{600} measurement, CFU/mL measurement (using Miles-Misra), pH measurement (using pH universal indicator), as well as biomass and supernatant collection.

After the t=0 measurement, the culture was incubated at 30^{o}C, 150 rpm for 4 days. During this period, the aforementioned measurements were also performed daily, and the obtained values were taken as T1, T2, T3, and T4. After 4 days of incubation, the cells were harvested by transferring the cell culture to the falcon tube and applying centrifugation at 5000 rpm for 20 - 25 minutes. The obtained supernatant was collected for the subsequent HPLC measurement to measure erythritol production, and the biomass was put in the oven for 2 - 3 days to obtain the dry cell mass.

The obtained values from the previously conducted measurements were used to plot four types of graphs: one graph with the time in hours as the X-axis and OD600 as the Y-axis, one graph with the time in hours as the X-axis and cell viability in CFU/mL as the Y-axis, one graph with the time in hours as the X-axis and mass of biomass in grams as the Y-axis, and one graph with the time in hours as the X-axis and pH measurement as the Y-axis. Lastly, the growth rate and doubling time of the *M. pollinis* were calculated.

After the HPLC running was finished, the standard curves for the glucose and erythritol standards were made with concentration in mg/mL as the X-axis and peak area in mAU*min as the Y-axis. Using the standard curve, the erythritol and glucose concentrations were calculated. Additionally, the yield mass of erythritol (Y_{ERY}) in g/g and erythritol volumetric productivity (Q_{ERY}) in g/L.h as the fermentation parameters were calculated.

3.2.2.3. Substrate Consumption and Erythritol Production Analysis Using HPLC

3.2.2.3.1. Standard solution preparation

In order to make the stock solution for the glucose standard with a 300 mg/mL concentration, 1.5 grams of glucose was transferred into a microcentrifuge tube containing 5 mL of type III water. 750 µl of the stock solution was then transferred into another microcentrifuge tube containing 750 µl of type III water to make a glucose standard solution of 150 mg/mL. Next, 750 µl of the 150 mg/mL standard solution was transferred into another microcentrifuge tube containing 750 µl of type III water to make another standard solution of 75 mg/mL. The procedure was repeated until the concentrations of the glucose standard of 37.5 mg/mL and 18.75 mg/mL were also obtained.

As for the erythritol standard solution, 40 mg grams of erythritol was transferred into a microcentrifuge tube containing 2 mL of type III water to make the stock solution with a 20 mg/mL concentration. 1 mL of the stock solution was then transferred into another microcentrifuge tube containing 1 mL of type III water to make a glucose standard solution of 10 mg/mL. Next, 1 mL of the 10 mg/mL standard solution was transferred into another microcentrifuge tube containing 1 mL of type III water to make another standard solution of 5 mg/mL. The procedure was repeated until the concentrations of the erythritol standard of 2.5 mg/mL and 1.25 mg/mL were also obtained.

3.2.2.3.2. HPLC Analysis

The prepared standard solutions and samples were filtered using syringes and put inside HPLC vials. The total volume of the solutions for each vial must be more than 500 µl. The type I water was also filtered as the mobile phase of the HPLC. The filled HPLC vials were then put on the sample tray of the HPLC machine. Next, the HPLC was run with the settings shown in **Table 3.1**.

Table 3.1. HPLC settings

HPLC Conditions and Specifications	Description
Column	Shodex Sugar SP0810 7 µm, 8 mm I.D. x 300 mm

Running time	30 minutes
Upper limit	40 bars
Mobile phase	Type I water 100%
Temperature column	80^{o}C
Detector	Refractor index (RI) detector
Temperature detector	50^{o}C
Injection volume	2 µl
Flow rate	0.7 mL/min

After the HPLC running was finished, the standard curves for the glucose and erythritol standards were made with concentration in mg/mL as the X-axis and peak area in mAU*min as the Y-axis. Using the standard curve, the erythritol and glucose concentrations were calculated. Additionally, the yield mass of erythritol (Y_{ERY}) in g/g and erythritol volumetric productivity (Q_{ERY}) in g/L.h as the fermentation parameters were calculated.

3.2.3. Screening the mutant stains for their potential for Erythritol production

3.2.3.1. Materials and Equipment Preparation

3.2.3.1.1. Prepare Yeast Glucose Minerals (YGM) for Mutant Screening

In order to make the YGM media, the media components were weighted to make the total media volume of 250 mL. The components consist of 0.25 gr of yeast extract (Concentration: 1 gr/l), 75 gr of glucose (Concentration: 300 gr/l), 0.5 gr of NH_4Cl (Concentration: 2 gr/l), 0.25 gr of $MgSO_4.7H_2O$ (Concentration: 1 gr/l), 0.075 gr of KH_2PO_4 (Concentration: 0.3 gr/l), 0.025 gr of K_2HPO_4 (Concentration: 0.1 gr/l), and 6.25 g of NaCl (Concentration: 25 gr/l).

The mixed components were then hydrated with 250 mL type III water inside a 500 mL erlenmeyer flask. Next, the flask was closed with aluminium foil, and a sterilization strap marker was placed on top of the foil. Lastly, the flask was put in an autoclave machine, and the autoclave was run at 121^{o}C for 15 minutes for sterilization.

3.2.3.1.2. Preparation of Potato Dextrose Broth (PDB) Media and Media Sterilization

The steps for preparing PDB media were the same as the procedure mentioned in section 3.2.2.2.2.

3.2.3.1.3. Preparation of Potato Dextrose Agar (PDA) Media and Media Sterilization

The steps for preparing PDA media were the same as the procedure mentioned in section 3.2.1.1.3.

3.2.3.1.4. Preparation and Sterilization of PBS buffer

The steps for preparing PDA media were the same as the procedure mentioned in section 3.2.1.1.4.

3.2.3.1.5. Sterilization of Micropipette Tips

The steps for preparing PDA media were the same as the procedure mentioned in section 3.2.1.1.6.

3.2.3.1.6. Sterilization of Microcentrifuge Tube

The steps for preparing PDA media were the same as the procedure mentioned in section 3.2.1.1.7.

3.2.3.1.7. Preparation of *M. pollinis* Pre-culture

One mutant *M. pollinis* colony was picked up with sterile tips or ose and transferred to PDB media. The culture was incubated at 30^{o}C, 120 rpm for 2 - 3 days. After the incubation, the mutant pre-culture was streaked on PDA media followed by 3 - 4 days of incubation.

3.2.3.2. Selection of the Best Mutant Strain for Erythritol Production

Firstly, 1 mL of sterile YGM media was transferred into a sterile 1.5 mL microcentrifuge tube. This will be used as the blank for the OD measurement. Using sterile tips, 10 mL of *M. pollinis* mutant pre-culture was transferred into the sterile prepared YGM media and mixed well. A 4 mL aliquot of the obtained culture was transferred using a 10 mL serological pipet into a sterile 15 mL falcon tube. This aliquot would be used as the t=0 for the OD_{600} measurement, CFU/mL measurement (using Miles-Misra), pH measurement (using pH universal indicator), as well as biomass and supernatant collection.

After the t=0 measurement, the culture was incubated at 30^{o}C, 150 rpm for 4 days. During this period, the aforementioned measurements were also performed daily, and the obtained values were taken as T1, T2, T3, and T4. After 4 days of incubation, the cells were harvested by transferring the cell culture to the falcon tube and applying centrifugation at 5000 rpm for 20 - 25 minutes. The obtained supernatant was collected for the subsequent HPLC measurement to measure erythritol production, and the biomass was put in the oven for 2 - 3 days to obtain the dry cell mass.

The obtained values from the previously conducted measurements were used to plot four types of graphs: one graph with the time in hours as the X-axis and OD600 as the Y-axis, one graph with the time in hours as the X-axis and cell viability in CFU/mL as the Y-axis, one graph with the time in hours as the X-axis and mass of biomass in grams as the Y-axis, and one graph with the time in hours as the X-axis and pH measurement as the Y-axis. Lastly, the growth rate and doubling time of the *M. pollinis* were calculated.

3.2.3.3. Substrate Consumption and Erythritol Production Analysis Using HPLC

3.2.3.3.1. Standard solution preparation

In order to make the stock solution for the glucose standard with a 300 mg/mL concentration, 1.5 grams of glucose was transferred into a microcentrifuge tube containing 5 mL of type III water. 2.5 mL of the stock solution was then transferred into another microcentrifuge tube containing 2.5 mL of type III water to make a glucose standard solution of 150 mg/mL. Next, 2.5 mL of the 150 mg/mL standard solution was transferred into another microcentrifuge tube containing 2.5 mL of type III water to make another standard solution of 75 mg/mL. The procedure was repeated until the concentrations of the glucose standard of 37.5 mg/mL and 18.75 mg/mL were also obtained.

As for the erythritol standard solution, 60 mg grams of erythritol was transferred into a microcentrifuge tube containing 3 mL of type III water to make the stock solution with a 20 mg/mL concentration. 1.5 mL of the stock solution was then transferred into another microcentrifuge tube containing 1.5 mL of type III water to make a glucose standard solution of 10 mg/mL. Next, 1.5 mL of the 10 mg/mL standard solution was transferred into another microcentrifuge tube containing 1.5 mL of type III water to make another standard solution of 5 mg/mL. The procedure was repeated until the concentrations of the erythritol standard of 2.5 mg/mL and 1.25 mg/mL were also obtained.

3.2.3.3.2. HPLC Analysis

The prepared standard solutions and samples were filtered using syringes and put inside HPLC vials. The total volume of the solutions for each vial must be more than 500 µl. The type I water was also filtered as the mobile phase of the HPLC. The filled HPLC vials were then put on the sample tray of the HPLC machine. Next, the HPLC was run with the settings shown in **Table 3.2**.

Table 3.2. HPLC settings

HPLC Conditions and Specifications	Description
Column	Shodex Sugar SP0810 7 µm, 8 mm I.D. x 300 mm

Running time	30 minutes
Upper limit	50 bars
Mobile phase	Type I water 100%
Temperature column	70^{o}C
Detector	Refractor index (RI) detector
Temperature detector	50^{o}C
Injection volume	2 µl
Flow rate	0.7 mL/min

After the HPLC running was finished, the standard curves for the glucose and erythritol standards were made with concentration in mg/mL as the X-axis and peak area in pa*min as the Y-axis. Using the standard curve, the erythritol and glucose concentrations were calculated. Additionally, the yield mass of erythritol (Y_{ERY}) in g/g and erythritol volumetric productivity (Q_{ERY}) in g/L.h as the fermentation parameters were calculated.

IV. RESULTS AND DISCUSSION

Erythritol is known as one alternative sweetener which is low in calories and glycemic index but able to provide a comparable level of sweetness to sucrose (Mazi & Stanhope, 2023). Considering its benefits, erythritol now has an increasing demand especially for those who suffer from obesity or diabetes. In order to fulfill the markets' demand, many studies including this extensive experiment have been conducted in order to produce erythritol through microbial fermentation such as *M. pollinis* yeast.

Different conditions were implemented in order to find the optimum growing conditions which consequently resulted in an optimized production of erythritol. From the results, a standard curve can be generated from the log CFU/mL against time, and the OD600 against time graphs were plotted in order to analyze the change in number of viable colony-forming units per milliliter of microbial sample as well as the growth of the microbial sample over time, respectively. During the experimental conduct, the *M. pollinis* yeast was supplemented with Yeast Growth Medium (YGM) as the nutrient source. Within the YGM, several compounds are contained including glucose, yeast extract (YE), ammonium chloride, and monopotassium phosphate, all of which would provide essential nutrients needed to sustain various metabolic activities. Additionally, NaCl or table salt may also be added to promote erythritol production, due to the bioproduct's role as an osmoprotectant (Rzechonek et al., 2018). Likewise, salt may also function as a nutrient and mineral source, in which the presence of sodium ions may help maintain the membrane potential of yeast cells (Yenush, 2016).

4.1 Erythritol production of *M. pollinis* at varying YE concentrations

Tao et al. (2023) stated that YE functions to support the growth and development of yeast by providing several nitrogen-rich nutrients, such as amino acids and proteins, that may aid the synthesis of essential amino acids involved in the cellular metabolism process of *M. pollinis* itself. Meanwhile, glucose serves as a source of carbon for the production of carbon-rich compounds, such as erythritol, and a source of energy that may be obtained from the process of aerobic respiration (Maślanka & Zadrąg-Tęcza, 2021). As essential nutrients with their own functions, the two may be joined into a single parameter, C/N ratio, depicting the proportion of carbon and nitrogen available within a medium. Altering the C/N ratio of a medium may affect various organisms differently, depending on whether the organism requires the manufacture of more C-rich or N-rich compounds to sustain its survival (Zhang et al., 2020). Therefore, the assessment of what C/N ratio may work best for the growth and erythritol production of *M. pollinis* may be investigated by employing different yeast extract concentrations. In this experiment, the concentration of yeast extract within the YGM varied from 1 g/L, 3 g/L, and 5 g/L across six groups.

4.1.1. Growth curve of *M. pollinis* at varying YE concentrations

In order to obtain a better understanding regarding the growth of a certain microorganism, a growth curve is the most commonly used empirical model which provides information regarding one's growth or evolution over a period of time (Krishnamurthi et al., 2021). Specifically, in the field of microbiology, the growth curve often represents the growth of a microorganism upon the implementation of certain conditions starting from the lag, exponential, stationary, and the death phases (Ughy et al., 2023). Following the previous explanation, the growth curve presented in **Figure 4.1** below shows the increasing trend of *M. pollinis* according to the OD600 (optical density) absorbance result measured over four different time periods, namely 24, 48, 72, and 96 hours. The use of OD is generally aimed to roughly calculate the density of cells contained in a liquid culture. As the name suggests, this method works upon the measurement of light absorbance that is proportional with the concentration value of absorbing particles contained by the sample at the wavelength of 600 nm (Beal et al., 2020). Furthermore, the choice of 600 nm as the wavelength has several advantages during the analysis of yeasts' growth where first this specific wavelength number is directly related to the OD of the yeast culture where higher OD represents higher cell concentration. Other than that, the 600 nm absorbance is known to be a good indicator for cell growth and viability since it has sensitivity towards changes in the concentration of yeast cells. Lastly, the 600 nm absorbance also provides high specificity towards yeast cells, which enables easy differentiation between yeast cells and other occurring contaminants (Bondareva et al., 2021; Fukuda, 2023).

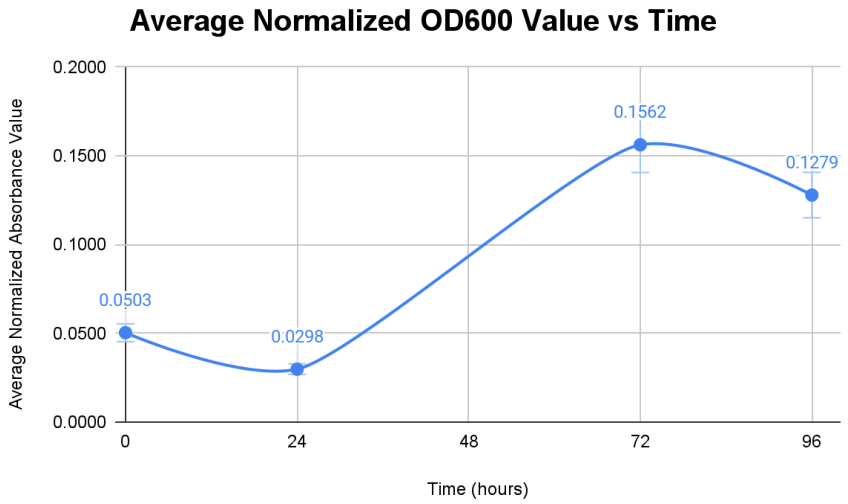

Figure 4.1. Growth curve of OD600 vs time of native *M. pollinis* in 1 g/L YE-containing YGM medium

As seen in **Figure 4.1**, the lag phase can be indicated from 0 to 24 hours which is then continued from 24 to 72 hours while from 72 to 96 hours shown to be in the

stationary phase. The cell density absorbance value each day has a value of 0.0503 at 0 hours, 0.0298 at 24 hours, 0.1562 in 72 hours, and 0.1279 in 96 hours. Reflecting on a study done by McBirney et al. (2016), it is expected that the increase in the time period should have resulted in an increase in the cell density which therefore increases the OD600 measurement result as well. Regardless of the limitation from the OD600 absorbance method, the increase in the cell density might be caused by the presence of other components such as death cells as well as metabolic byproducts (Duedu & French, 2017). However, the result shown by the graph shows a fluctuating trend with the major increase of the cell density appearing within the 24 hours to 96 hours time period. Not much is known regarding the cause of the result instability. One possibility may be due to a pipetting error while loading the sample to the 96-well plate which caused an uneven distribution of the cell inside of the sample aliquot itself.

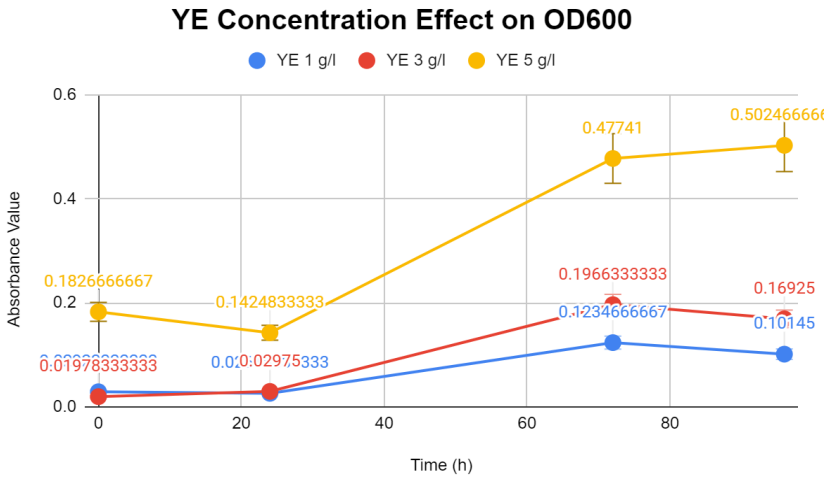

Figure 4.2. Different yeast extract concentrations effects on OD600 vs time of native *M. pollinis* in YGM media of varying YE concentration

Figure 4.2 above shows the impact of a varied yeast extract concentration on the growth of *M. pollinis* within the time period mentioned previously. It can be seen that the increasing yeast extract concentration is proportional to the increase of *M. pollinis* cell density. From the trendline, the time point 0 hour to 24 hours represents the lag phase, or the initial phase during which the yeast cells are adjusting to the current environment prior to the growth. Continuing that the exponential growth phase was identified to occur starting from the time point 48 hours to 72 hours, with the highest and most distinct growth shown by 5 g/L yeast extract concentration. As for the last time period (48 hours to 72 hours), one notable difference was shown where *M. pollinis* grown under 5 g/L yeast extract underwent another increasing growth trendline, while the remaining two concentrations showed a decreasing growth trendline.

Yeast extract has a main component of mannose oligosaccharides and β-glucans that contains glycogen and chitin which make up as much as 83% of total carbohydrate

content of yeast cells (Tao et al., 2023). Additionally, yeast extract may also act as a great nitrogen supplementation, given that it is high in amino acids, vitamins, and minerals that are an ideal nutrient growth medium in the lab. This finding is aligned with a study conducted by Malik et al. (2021) which discovered that the increase of yeast extract concentration resulted in a shorter lag phase which then followed by a rapid increase in the yeast growth. From there, it can be summarized that the prime C/N ratio can be achieved upon the addition of 5 g/L yeast extract within the YGM.

However, the OD600 absorbance value could only determine the stage of cell growth by analyzing the optical density or cell density but it could not differentiate the living and dead cells (Duedu & French, 2017). Further methods of viable cell detection should be done to know the estimated viable cell in the culture from each day. Miles and Misra method is a microbiological technique that could be used to determine the number of colony forming unit (CFU) amounts in a solution where only viable cells would grow in the media (Puchkov, 2014).

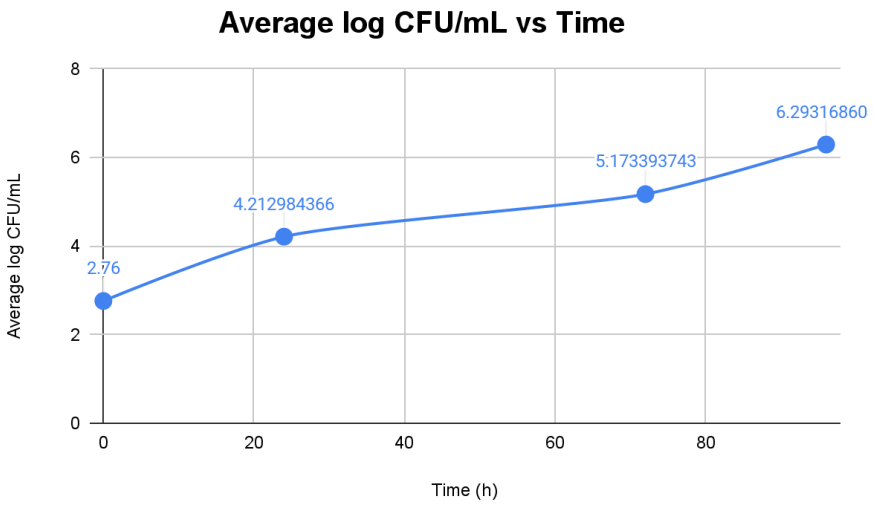

Figure 4.3. Growth curve of log CFU/mL vs time of native *M. pollinis* in 1 g/L YE-containing YGM medium

From **Figure 4.3**, the plotted Miles-Misra result was done in four timepoints of 0, 24, 72, and 96 hours where it can be seen that the colony formed from Group 2 increases overtime. The cell grows at a linear rate where point 0 to 24 hours the cells are still in the lag phase to adjust with the environment and allow maturation but have not started to divide and increase in population. Within 24 to 72 hours, it can be indicated that the log phase has happened and the cells started to divide and increase in population as seen by the increase in CFU. At hour 72 to 96, the cells have started to enter the stationary phase where the cell growth starts to cease but they could still maintain their metabolic activity (Joers & Tenson, 2016).

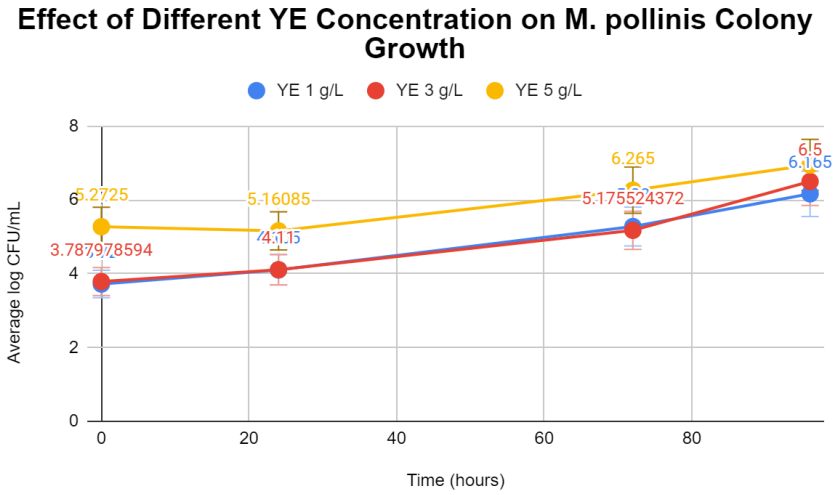

Figure 4.4. Different yeast extract concentrations effects on log CFU/mL vs time of native *M. pollinis* in YGM media of varying YE concentration

In **Figure 4.4** above, the CFU from 1 g/L, 3 g/L, and 5 g/L yeast extract are indicated in blue, red, and yellow color respectively. The three treatments of different yeast extract concentration used showed a slight difference in the colony growth between the *M. pollinis* treated with 1 g/L and 3g/L, while for the yeast treated with 5 g/L yeast extract are significantly different than the other concentration. At 0 hours, the CFU differences are also significant where the values are 3.72, 3.78, and 5.27 for 1 g/L, 3 g/L, and 5 g/L yeast extract sequentially. For the concentration of 1 g/L and 3 g/L, the treated *M. pollinis* has similar starting point and overtime only differs only in the 96th hour, where the CFU in 3 g/L yeast extract are 6.5 CFU/mL which is slightly higher than 1 g/L with a value of 6.165 CFU/mL. A balanced carbon and nitrogen source is assimilated with the longer lifespan of the cell's viability in yeast (Santos et al., 2016). This aligns with the OD600 growth curve where *M. pollinis* in 5 g/L yeast extract has a shorter lag phase than the other concentration and has the highest value of colony grow with the value of 6.948 CFU/mL due to the abundance food source available for the yeast to proliferate.

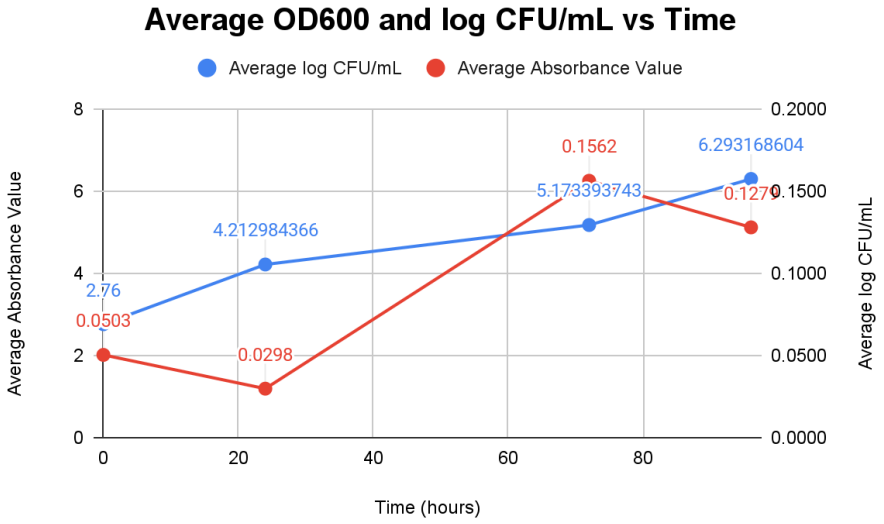

Figure 4.5. Growth curve of OD600 and log CFU/mL vs time of native *M. pollinis* in 1 g/L YE-containing YGM medium

Based on **Figure 4.5,** the blue line represents the CFU/mL value and the red line represents the OD600 concentration. The curve depicts that the concentration of CFU increases overtime which means that the *M. pollinis* is still in the growth phase and has not reached the death phase. However, the OD600 value showed a decrease value at hour 24 and 96 which could be caused by uneven spread of the culture when pipetting to the 96 well-plate from the YGM culture. This can be avoided by resuspending or shaking the flask before pipetting the cell culture (McBirney et al., 2016).

4.1.2. Doubling Time of *M. pollinis* at varying YE concentrations

The generation time or also known as the doubling time can be defined as the duration required for the strain to be double in number (Small et al., 2020). From the obtained results, the doubling time was calculated using the data from 0 hour and 24 hours time points. The determination of these time points was made during which the yeast was at the log phase or the phase where an exponential growth is detected upon the presence of increasing trendline in the growth curve. This is done since cells that are in the log phase exhibit a persistent growth rate as well as a uniform metabolic activity. From those two time points, the values represented as N_t and N_0 were taken from the CFU/mL value of the chosen time point, and more specifically at a certain dilution concentration. Since the miles misra was conducted in duplicate, thus the value was calculated from the average CFU/mL value of plate A and plate B. In this calculation, both of the N_t and N_0 value are referred to the average CFU/mL value of 24 hours and 0 hour respectively, sourced from the -2 dilution. From there, the doubling time calculation of *M. pollinis* native strain was done according to the following equation:

$$G = \frac{Specified\ Time\ (t)}{3.3 log N_t / N_0}$$

Doubling time of the native *M. pollinis* strain grown in media with 1 g/L yeast extract:

$$G = \frac{(24-0)}{3.3log(16666.66667/55000)}$$

$$G = 14.03\ hours$$

Unfortunately, not much information could be obtained regarding the expected doubling time of *M. pollinis*. However, considering the function of yeast extract as a crucial nutrient source for yeast, it can be said that logically the decrease of yeast extract concentration within the growth medium may lead to a decrease in the cell growth rate including the doubling time itself.

4.1.3. Biomass of *M. pollinis* at varying YE concentrations

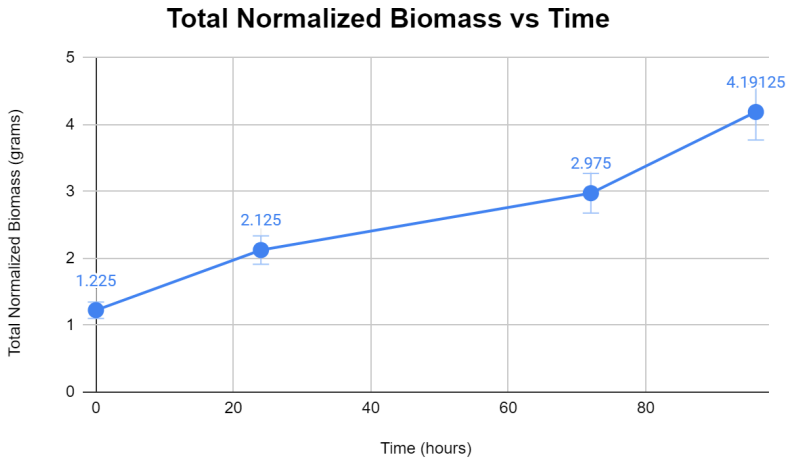

Figure 4.6. Total normalized biomass vs time of native *M. pollinis* in 1 g/L YE-containing YGM medium

The separated and dried biomass should consist of very-insoluble components previously suspended within the media, one of which would be the *M. pollinis* cells themselves (Al-Faqheri et al., 2017). Therefore, the measurement of total biomass should reflect the number of cells present within the medium. As expected, the increasing trend of the graph in **Figure 4.6** indicates that cells in the medium continued to proliferate even until the end of the 96 hours, yielding around 4.19 grams-worth of cells.

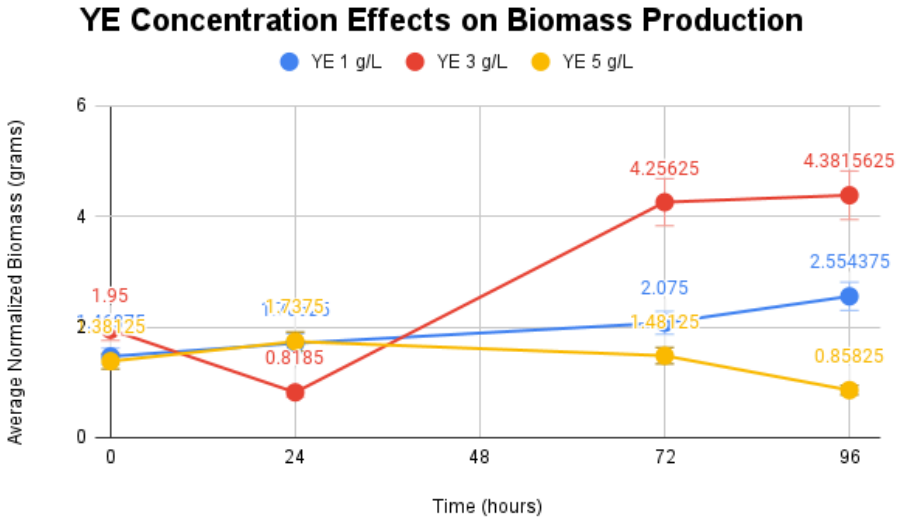

Figure 4.7. Different YE concentrations effects on total biomass production vs time of native *M. pollinis* in YGM media

Strangely, the varying amount of YE added to the different YGM media did not show a clear trend. While the addition of YE supposedly helps with cell growth and proliferation by providing nitrogen-rich compounds for protein production, the biomass produced in YE 5 g/L seems to be the lowest among the rest, indicating the lowest amount of cells present, only possessing a total of 0.86 g cells at 96 hours. This is closely followed by YE 1 g/L, which follows a similar trend but ends up with 2.55 g cells at the end of the 96 hours. The YE 3 g/L media abnormally starts out with the highest mass of 1.95 g at 0 hours, and ends with the highest mass of 4.38 g at 96 hours, when it theoretically should be in the middle of YE 1 g/L and YE 5 g/L. Even weirder, the graph dips down at 24 hours, displaying 0.82 g, the lowest mass recorded throughout the entire time frame.

However, considering that the biomass measured should reflect the generated growth curves in **Figures 4.2** and **4.4**, which do follow appropriate trends, it is likely that the biomass measurement was done incorrectly, generating multiple errors.

4.1.4. pH of *M. pollinis* medium at varying YE concentrations

Upon growing and proliferating, the consumption of substrates and production of metabolites by cells would typically start to alter the composition, and hence pH level, of the medium that they are in (Bordet et al., 2020). For instance, the accumulation of carbon dioxide (CO_2), a product of aerobic respiration, in a medium would start to bring its pH down. This is because when dissolved in a solution, CO_2 would start to form carbonic acid (H_2CO_3) with water (H_2O), which would then rapidly dissociate into hydrogencarbonate (HCO_3^-) and the strongly-acid hydrogen ion (H^+) (Wang et al., 2016). The reactions are also depicted below:

$$CO_2 + H_2O \rightarrow H_2CO_3$$
$$H_2CO_3 \rightarrow HCO_3^- + H^+$$

While this process is natural, excess acidification of a medium may actually endanger the cells living within it when its pH starts to fall below the optimum pH allowing the growth of the species. In fact, cells should even start to die when its medium gets too acidic, as its enzymes would not be able to sustain their functions upon acidic degradation, where their active site's 3D conformation would start to change (Guzik et al., 2014).

In the case of *M. pollinis*, the desired medium pH range should fall between 5–6, with 6 being its optimum pH (Khatape et al., 2023). Further acidification would start to compromise the survival of the cells.

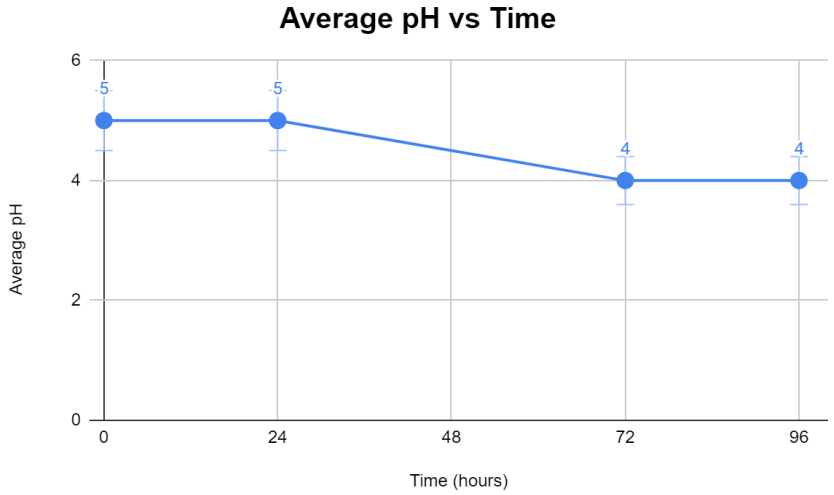

Figure 4.8. pH vs time of native *M. pollinis* in 1 g/L YE-containing YGM medium

As seen in **Figure 4.8**, the average pH level derived from duplicate measurements did change over the course of four days. An overall downward trend may be seen, where the medium initially started at a pH of 5 at 0 hours to a pH of 4 at 96 hours. This would mean that by 96 hours in a 1 g/L yeast extract-containing YGM medium, *M. pollinis* should not be living within its favorable pH range anymore.

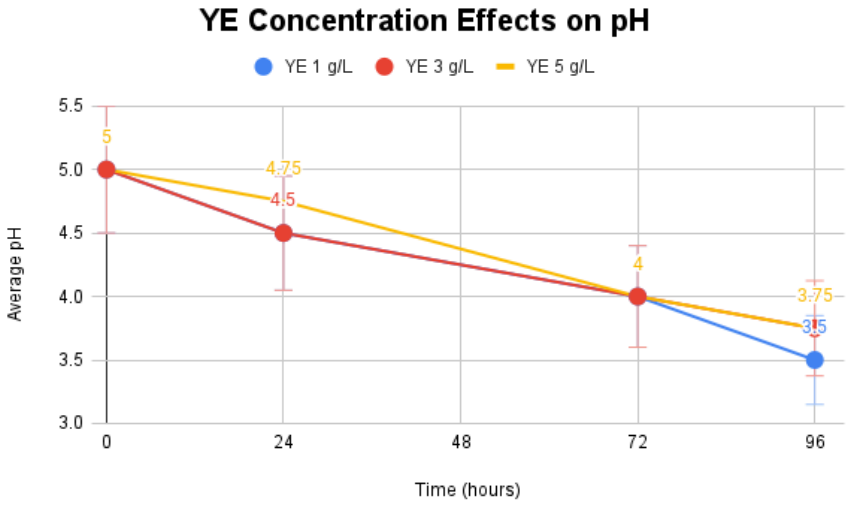

Figure 4.9. Different YE concentrations effects on pH vs time of native *M. pollinis* in YGM media

Figure 4.9 depicts the effects of different yeast extract concentrations on the change in pH levels observed within the media. It can be seen that all three of the media follow very similar trends, only with slight variations of 0.25 between YE 1 g/L and YE 3 g/L from YE 5 g/L at 24 hours, as well as another different of 0.23 between YE 1 g/L from YE 3 g/L and YE 5 g/L at 96 hours. Nonetheless, given the striking similarities between the three generated graphs, it is likely that the intervals of the three differing concentrations in this experiment was not sufficient to produce any observable change in pH level. Further testing with larger differences among the yeast extract concentration independent variable may need to be done in order to hopefully see discrepancies in the change of pH over time.

4.1.5. Substrate consumption of *M. pollinis* at varying YE concentrations

In order to evaluate the efficiency of erythritol production by *M. pollinis* under different YE concentrations, one measurement method is the High Performance Liquid Chromatography (HPLC) analysis which will provide data required to calculate the substrate consumption as well as the yield produced. Generally, the amount of substrate consumed/used within a reaction is always proportional to the amount of product generated (Mazzeo & Piemonte, 2020). Within this experiment, it is known that glucose served as the substrate, which activity can be observed by looking at the chromatogram result of the HPLC analysis. From the result, a standard curve of the glucose standard was depicted by plotting the glucose concentration (mg/mL) as the X axis, followed by the peak area as the Y axis. Furthermore, the standard curve generated a linear equation as follows, which was then used to calculate the concentration of glucose.

$$y = 0.305x + 0.932$$

To the linear equation, the y variable was substituted with the value of the peak area and the calculation resulted in the x value of 152.996 as the glucose concentration in mg/mL. Following that, the glucose consumption value of the experiment can be calculated by subtracting 300 mg/mL as the initial concentration of glucose provided, with the previous glucose concentration value. From here, it is revealed that the amount of glucose consumed during the fermentation is 147.003 mg/mL.

4.1.6. Erythritol production of *M. pollinis* at varying YE concentrations

Continuing that, the determination of the erythritol concentration was also done by following the similar procedure. A standard curve was schemed with the concentration as the X axis and peak area as the Y axis. From the curve, it was a generated a linear curve as below:

$$y = 0.241x - 7.21 * 10^{-4}$$

By substituting the y variable with the area value of the peak occurred in the chromatogram. Upon calculation, it is found that under 1 g/L YE concentration, *M. pollinis* generates an erythritol concentration of 13.289 mg/mL. Furthermore, the concentration value can be further used to calculate the yield mass as well as the volumetric productivity. Starting from the mass yield, the value was calculated by dividing the erythritol concentration by the value of glucose consumed found in Section 4.1.5. The yield mass was shown to have a value of 0.09. Following that, the volumetric productivity was also calculated by dividing the concentration of erythritol with the total cultivation duration which was 96 hours and generated a value of 0.138 g/L.h. (citation) stated that the volumetric productivity is a measure of the amount of product produced per volume of fermentation, per time which in this experiment is in hour. Theoretically, Mahé et al. (2022) stated that a high volumetric productivity value is desirable where it can be achieved by elevating the number of cell density and in some cases, the productivity of the bioreactor which eventually causes an increase in the product concentration as well as the overall yield. In addition to that, a high volumetric productivity value cannot be specified as there are several affecting factors including the application field.

4.1.7. HPLC comparison of Different Yeast Extract Concentration

Table 4.1. The yield mass and productivity of erythritol in different yeast extract concentration

	Yeast extract	Yield mass ($g_{ery}/g_{glucose}$)	Productivity yield (g/L hrs)
Group 1	1 g/L	0.118	0.073

Group 2	3 g/L	0.090	0.138
Group 3		0.127	0.121
Group 4		0.159	0.136
Group 5	5 g/L	0.080	0.137
Group 6		0.172	0.159

HPLC results of different yeast extract can be seen in **Table 4.1.**, where the increase in yeast extract concentration results in an increase in both yield mass and productivity yield. According to Al-Juthery et al. (2020), the increase in yeast extract affects the lag phase and means more yield of mass could be collected and the erythritol yield would be higher. This is in accordance with the group's data where the lowest value of yield mass and productivity yield can be seen from 1g/L concentration and the highest from 5 g/L.

4.2 Erythritol production of *M. pollinis* at varying NaCl concentrations

In the case of erythritol production, Yang et al. (2014) had found that an appropriate level of high osmotic pressure is able to enhance the yield of erythritol while also increasing the productivity of the yeast. This phenomenon is very commonly observed in various cells, including yeasts, as increasing NaCl levels would typically mean increasing osmotic stress and ion toxicity (Boumaaza et al., 2020). In osmotic stress, water from the cells would start to leak out their semi-permeable plasma membranes by osmosis into the medium in response to the imbalance in osmotic pressure, leaving less water inside the cells to carry out metabolic processes necessary for survival like aerobic respiration. Meanwhile ion toxicity refers to the forceful involvement of ions, especially Na^+ in this case, into an organism’s metabolic processes, which may disrupt the natural established homeostasis of cells (Hashem et al., 2018). With that, the impact of different salt concentrations on the overall erythritol production was evaluated through several testing methods including OD600 absorbance, Miles Misra (for cell viability), pH, biomass production, and HPLC, which result will be shown in the following subsections.

4.2.1. Growth curve of *M. pollinis* at varying NaCl concentrations

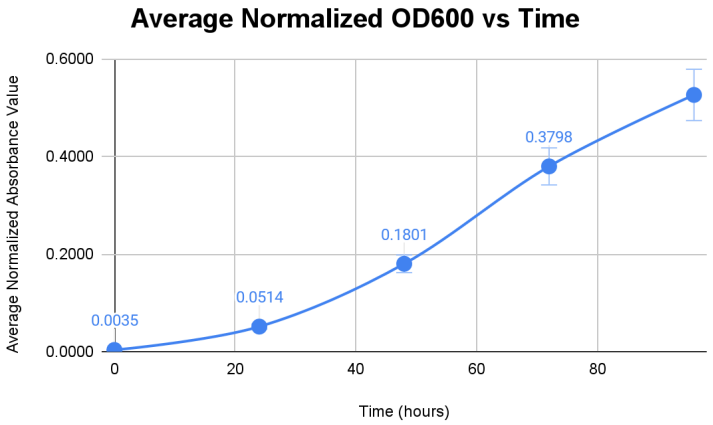

Figure 4.10. Growth curve of OD600 vs time of native *M. pollinis* in 0 g/L NaCl-containing YGM medium

Based on the OD600 absorbance data, the growth curve of *M. pollinis* grown under 0 g/L NaCl concentration was plotted. The trendline in the graph shows a continuous increase starting from 0 hour to 96 hours of growth period. Despite the stable growth of the yeast, there are three trends that can be observed within the curve. First, within the first 24 hours of the growth, it can be seen that the yeast is still undergoing its lag phase where the increase of the absorbance level is still not that distinct. Following that, between the time period of 24 hours to 72 hours, the yeast exhibits a significant growth starting from an OD600 absorbance value of around 0.0514 and ends at around 0.3798 - indicating the exponential phase of its growth. Lastly, the final growth time period shows a slight decline on the trend which might indicate the start of the lag phase. This can be said as previously, the yeast shows a growth by roughly 0.3 points, while from the time point of 72 hours to 96 hours, the growth increased by only around 0.2 points.

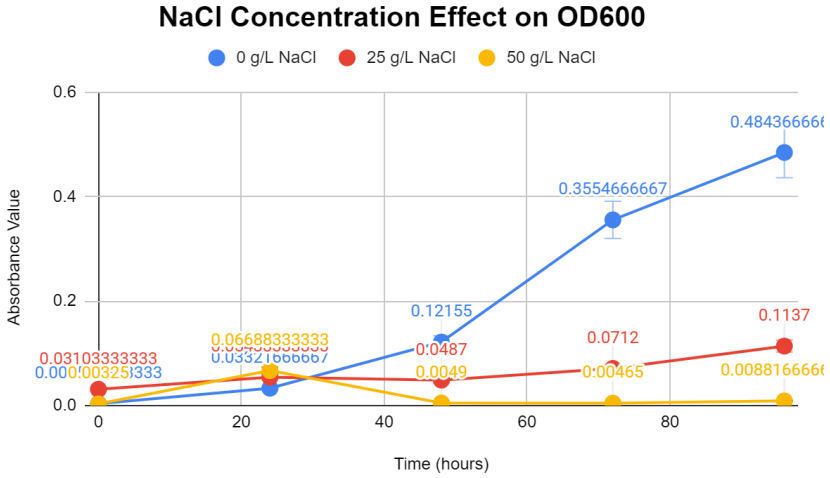

Figure 4.11. Different NaCl concentrations effects on OD600 vs time of native *M. pollinis* in YGM media

In comparison to the growth of yeast under two higher salt concentrations, - be it 25 g/L and 50 g/L, it is evident that the highest salt concentration exhibited the lowest cell density value. This result is in accordance with the previous finding where the level of osmotic pressure is negatively correlated with the growth rate of the yeast which means that the higher the salt concentration, the less time required by the yeast to double themselves (Postaru et al., 2023). Since a 0 g/L concentration of salt was applied during this experiment, it can be seen how the exponential growth has just started during the middle phase of the overall growth period. Regardless of the relatively slow growth, the yeast experienced a distinct and highest increase in the cell density. However, this phenomenon needs further confirmation which can be done upon the analysis of CFU/mL (viable cell) data later on.

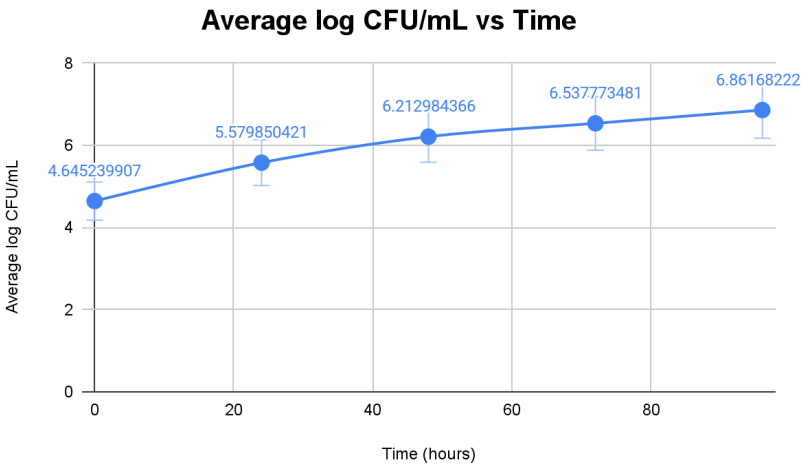

Figure 4.12. Growth curve of CFU/mL vs time of native *M. pollinis* in 0 g/L NaCl-containing YGM medium

Figure 4.12, growth curve of cell viable can be seen to increase overtime and is in accordance with the absorbance values in **Figure 4.10**. This indicates that the cells are still actively dividing and growing in the growth media. Moreover, the high CFU value tells that the viable cells content inside the growth medium is high which aligns with the finding that there are still sufficient amounts of cells alive in the media (Sankaranayanan et al., 2014).

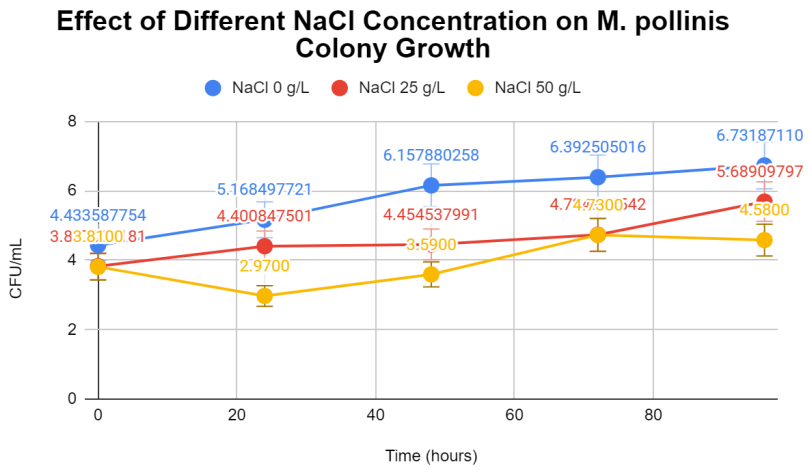

Figure 4.13. Different NaCl concentrations effects on log CFU/mL vs time of native *M. pollinis* in YGM media

In **Figure 4.13**, growth curve of cell viable can be seen to be affected by the different NaCl concentrations where in 0 g/L concentration of NaCl, the cell growth is higher than 25 g/L and 50 g/L respectively. This is due to the osmotic pressure from the NaCl where higher concentrations means to stress the cell more and limits the population growth by inducing longer lag phase before the *M. pollinis* could produce erythritol as an osmoprotectant (Wood, 2015).

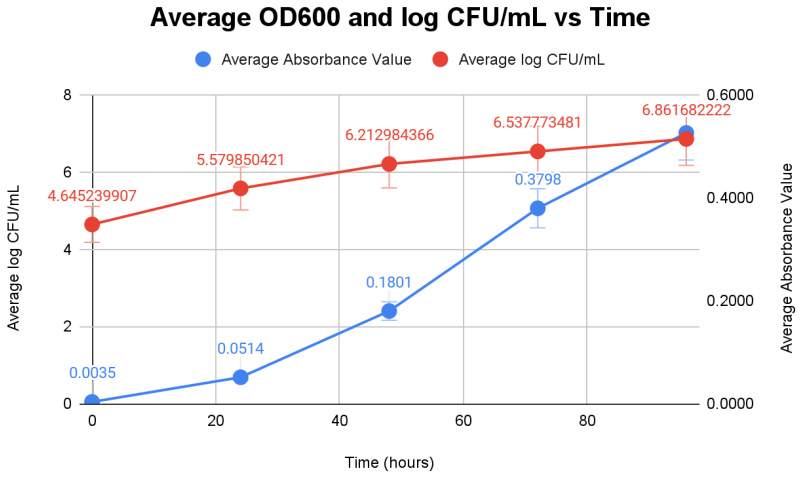

Figure 4.14. Growth curves of OD600 and log CFU/mL vs time of native M. pollinis in 0 g/L NaCl-containing YGM medium

In **Figure 4.14**, Group 2's cell growth is seen to be high which is aligned with the absorbance value and OD600 that increases overtime. From this curve, the cells have a short lag phase from 0 to 24 hours, whereas it has an exponential growth from hour 24 to 72. Moreover, at hour 96 the cell growth starts to slow down and it starts to enter the stationary phase.

4.2.2. Doubling Time of *M. pollinis* at varying NaCl concentrations

Using the formula that has been stated above, the doubling time of *M. pollinis* growth under the influence of 0 g/L NaCl concentration was observed. For this particular experiment, the doubling time calculation was done according to the average CFU/mL data of 0 hour and 48 hours. Similar as before, the determination of both time points were made when the *M. pollinis* yeast underwent its exponential growth phase. Using the same formula, the N_t and N_0 values were also calculated from the average CFU/mL value for each 48 hours and 0 hour time point, specifically at the dilution of -4 and -1 respectively.

Doubling time of *M. pollinis* under 0 g/L NaCl concentration:

$$G = \frac{(48-0)}{3.3log(1666666.67/44333.33)}$$

$$G = 9.2\ hours$$

In comparison to the previous doubling time value, it can be seen that the 0 g/L of NaCl concentration resulted in a lower doubling time of the yeast. Regardless of the varied yeast concentration in the previous section, the yeast was initially grown under the NaCl concentration of 25 g/L. A study by Nepal & Kumar (2020) revealed that the salt concentration also has a positive correlation with the population doubling time. This means that an elevated salt concentration will lead to the inhibition of cell growth which eventually causes an increase in the doubling time. This finding aligns with the generated doubling time of this experiment where it is reduced by approximately ¾ of yeast growth doubling time with 25 g/L NaCl concentration which is due to the absence of salt that maintains the osmotic balance as well as provides the main minerals for yeast (Carcea et al., 2020).

4.2.3. Biomass of *M. pollinis* at varying NaCl concentrations

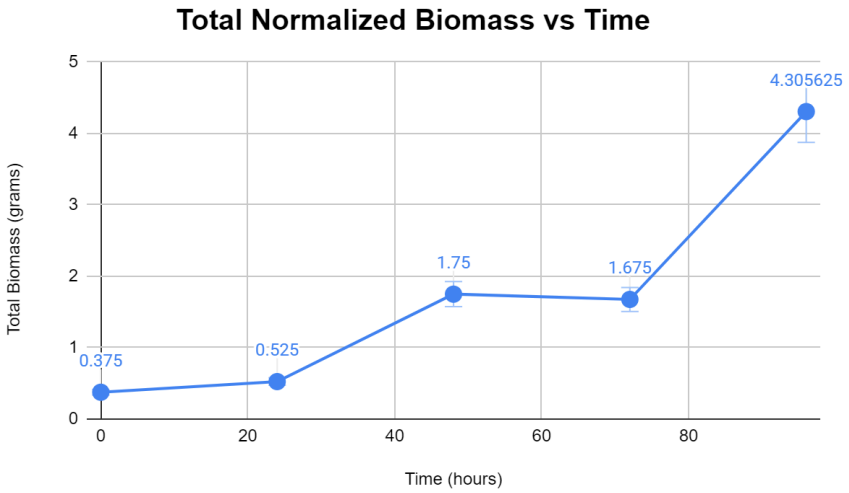

Figure 4.15. Total biomass production vs time of native *M. pollinis* in 0 g/L NaCl-containing YGM medium

The biomass growth curve from *M. pollinis* culture media that contains no salt showed to have a steady cell growth. From 0 to 24 hours, the cell population is still small as it is still in the lag phase where the cells are adapting to the media. The increase at 48 to 72 hours indicates that the population has entered the early stages of log phase, while at 72 to 96 hours they have reached the exponential phase. Biomass contains both living and dead cells, the fluctuation at 96 hours indicates an increase of cell population in the media which is correlated with the cell stage that the yeast is in.

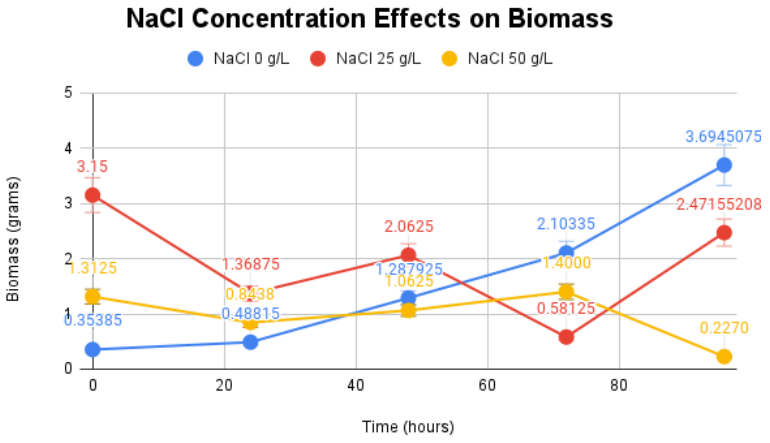

Figure 4.16. Different NaCl concentrations effects on total biomass production vs time of native *M. pollinis* in YGM media

As seen in **Figure 4.16**, the biomass resulting from different salt concentrations has a high fluctuating yield for 0 g/L whereas 50 g/L concentration has the lowest yield. This fluctuating biomass production is associated with the prolonged lag phase due to high osmotic pressure, while decrease in the biomass is caused by the characteristic of cells which is autolysis in a stressful environment (Chu et al., 2015; Srivastava & Sahoo, 2021). This is especially noticeable at higher concentrations of 50 g/L NaCl that has the lowest peak at hour 96 compared to the other concentrations due to higher osmotic stress, this decrease could imply that the cultured cell viability has lowered as it could no longer survive the pressure. However, the stable and lack of production in 0 g/L salt concentration could indicate that there is low to no interaction between yeast extract and salt to assist the population growth of *M. pollinis* yeast (Zhao et al., 2021).

4.2.4. pH of *M. pollinis* media at varying NaCl concentrations

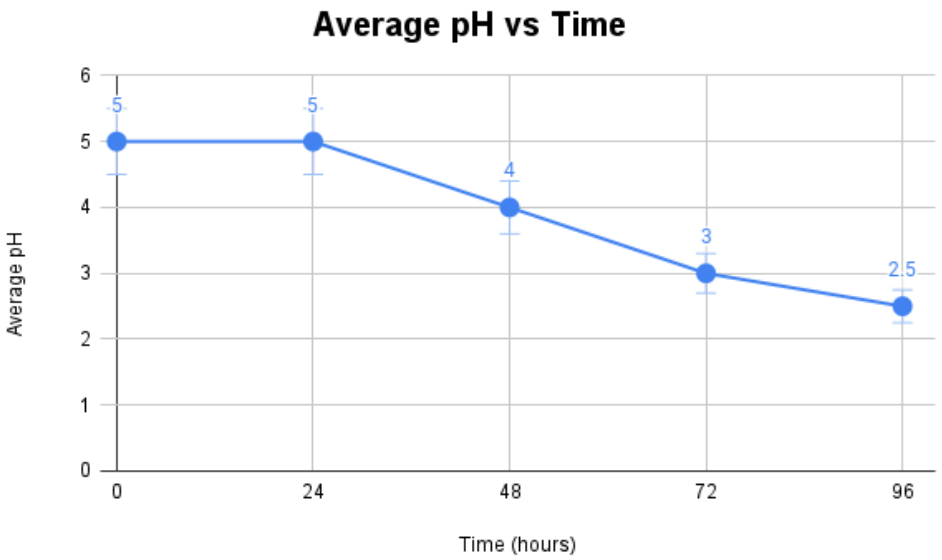

Figure 4.17. pH vs time of native *M. pollinis* in 0 g/L NaCl-containing YGM medium

Similar to the previous case of cultivating *M. pollinis* in 1 g/L yeast extract concentration, the pH level of the medium was also observed to follow a decreasing trend as seen in **Figure 4.17**. However, the pH level here did reach lower values, achieving the final level of 2.5 at 96 hours, while starting from a similar value of 5 at 0 hours. This suggests that the medium was slightly more acidic at the end than the one used before. The yeast extract concentration used in this case was also 1 g/L, leaving the only difference between the two to be the usage of 25 g/L NaCl in the previous batch, which was expected to alter pH this way.

NaCl itself is a neutral salt, as its constituents Na^+ and Cl^- should not interact with water once dissolved, meaning that its addition or absence itself should not alter a medium's pH level (Nyachwaya, 2016). However, as demonstrated by steeper

increases in cell viability and biomass measurements in the case of using 0 g/L NaCl compared to 25 g/L NaCl, NaCl does seem to have toxic properties against *M. pollinis*. Therefore, having 0 g/L NaCl in the medium may have actually enhanced the growth and increased the metabolism of *M. pollinis*, which may correlate to more CO_2 produced in respiration and a faster rate of medium acidification.

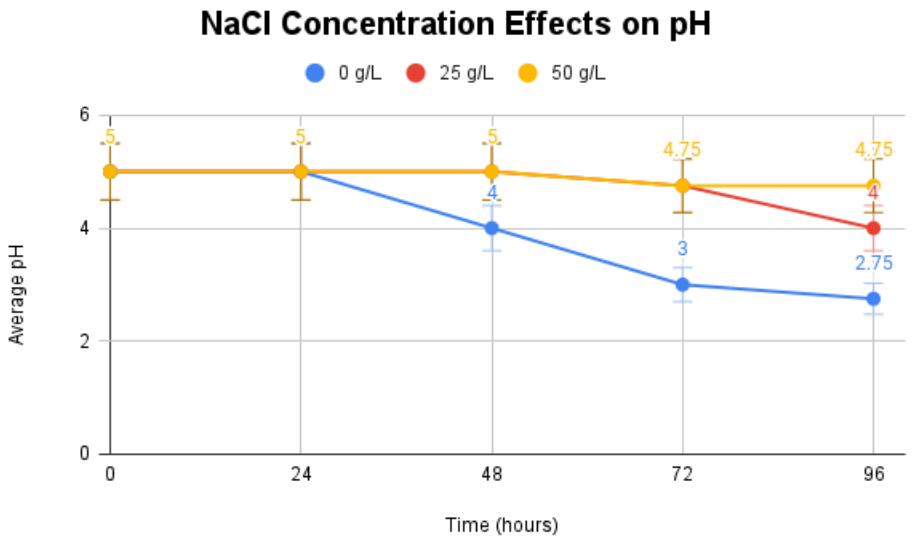

Figure 4.18. Different NaCl concentrations effects on pH vs time of native *M. pollinis* in YGM media

Fortunately, the aforementioned trend was also reflected in the media of varying NaCl concentrations, as shown in **Figure 4.18**. All three concentrations started at a pH level of 5, but the steepest decrease in pH may be observed in the media with 0 g/L, where it ended up with an average pH level of 2.75 at 96 hours and only took 48 hours to experience a pH decrease. This is followed by the media with 25 g/L NaCl, which had a pH level of 4 by the end of the 96 hours and required 72 hours for its pH to finally drop by a value of 0.25. Finally, the media with the 50 g/L NaCl followed a similar pattern to the 25 g/L NaCl media, but possessed a pH of 4 at the end of the 96 hours. Nonetheless, It may be seen that the increase in NaCl concentration from 0 g/L to 25 g/L yielded much more drastic alterations in pH change than the increase from 25 g/L to 50 g/L.

4.2.5. Substrate consumption of *M. pollinis* at varying NaCl concentrations

Similar to the HPLC analysis in the previous section, the determination of *M. pollinis'* productivity was done upon the analysis of the consumed substrate. The chromatogram result shows a peak with the retention time of 12.558 and a peak area of 43.081. Under 0 g/L NaCl concentration, the glucose standard curve made upon the result of HPLC analysis generated a linear equation as follows:

$$y = 0.305x + 0.932$$

From the linear equation above, the y variable was substituted with the peak area that has been stated previously, and the x variable remained to be the variable to be calculated, which is the glucose concentration. The calculation generated a glucose concentration value of 138.193 mg/mL which from there, the value of glucose consumed can also be calculated. If 300 mg/mL as the initial concentration is subtracted with 138.193 mg/mL as the concentration value, the glucose consumed value is found to be 161.806 mg/mL.

4.2.6. Erythritol production of *M. pollinis* at varying NaCl concentrations

The linear equation obtained from the erythritol standard curve was:

$$y = 0.241x - 7.21 * 10^4$$

Upon the substitution of the Y variable with the peak area of the erythritol chromatogram result, it was observed that the erythritol concentration under 0 g/L NaCl is 16.260 mg/mL. For further analysis, the erythritol concentration is then involved in the calculation of yield mass using the same formula from the previous section, and it resulted in the value of 0.1. Lastly, upon the division of erythritol concentration with 96 hours as the total cultivation period, it is obtained 0.169 g/L.h as the volumetric productivity of *M.pollinis* in producing erythritol at 0 g/L NaCl concentration.

4.2.7. HPLC comparison of different NaCl Concentration

Table 4.2. The yield mass and productivity of erythritol in different NaCl concentration

	NaCl	Yield mass ($g_{ery}/g_{glucose}$)	Productivity yield (gr/L.h)
Group 1	0 g/L	0.26	0.17
Group 2		0.10	0.16
Group 3	25 g/L	0.36	0.14
Group 4		0.09	0.09
Group 5	50 g/L	0	0
Group 6		0	0

HPLC results of different NaCl can be seen in **Table 4.2**, where the osmotic pressure plays a big role in the mass yield and bioproduct production productivity yield. A sufficient amount of NaCl could induce the production of erythritol, but a

higher concentration of NaCl could cause excess stress to the cells and result in less mass yield and bioproduct (Rzechonek et al., 2017). As seen from the table above, the higher erythritol produced is obtained by group with 25 g/L concentration. This finding is aligned with previous research that states an increase in sufficient salt concentration would increase the production of erythritol but in a higher osmotic stress it could inhibit the growth rate of cells as the cells undergo physiological and molecular changes (Bremer & Kramer, 2019).

4.3 Erythritol production of mutant *M. pollinis* strain

A different strain of *M. pollinis* was tested to observe the amount of erythritol production. To achieve the mutated strain a non-mutated *M. pollinis* was subjected to UV radiation to induce random mutagenesis, each group subjected their own culture of *M. pollinis* to UV bombardment for 10 minutes, then the mutant colonies were cultured and measured with several parameters. In accordance with the previous literature, the UV light exposure towards the sample caused a fewer growing colonies on the PDA (Tan et al., 2021). However, in comparison to the negative control, the morphology of the colonies forming is similar to those growing in the negative control plate From the data collected it is possible to generate a standard curve from log CFU/mL against time, biomass, erythritol production, substrate usage, and the OD600 absorbance.

In this experiment, data from Group 3 was eliminated from further analysis due to the culturing of a contaminating microorganism instead of *M. pollinis*. This contamination likely occurred during the development of the pre-culture, allowing a different organism to grow in the medium instead. Plating of the unknown organism yielded morphological properties distinguishable from those of *M. pollinis*. Upon further investigation, the unknown contaminant was deemed to be likely a bacterial species from a nearby experiment.

4.3.1 Growth Curve of Mutant *M. pollinis* strain

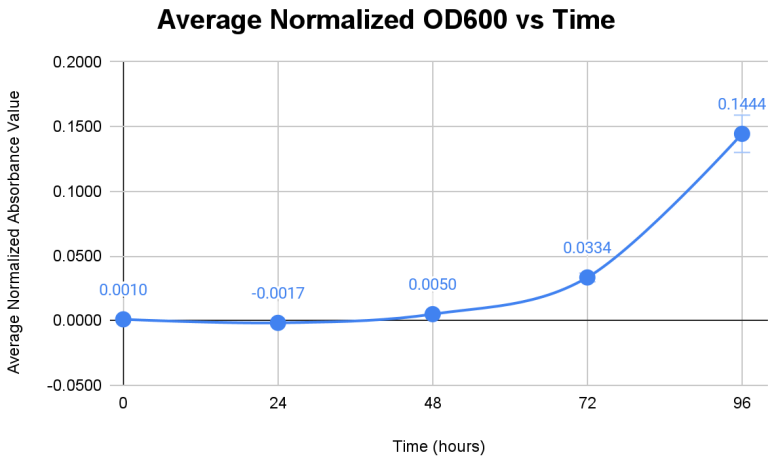

Figure 4.19. Growth curve of OD600 vs time of mutant *M. pollinis* in YGM medium

As seen in **Figure 4.19**, the absorbance value can be seen to be aligned with the cell availability in the media. From 0 to 72 hours, the cells are depicted to be still in the lag phase. This might occur due to the mutation where random mutagenesis cause lower viability because there is an unstable DNA condition by the alterations through negative mutation (Mao et al., 2017). From **Figure 4.20**, the best mutant strains are from Group 2 and 4 with the value of 0.1444 and 0.2180. Furthermore, seen from all groups OD600, these mutants could be a great option for fast-paced cultivation as the cell density is already high within 4 days of incubation. Considering the limitation of the UV mutagenesis method, the result shown by the curve may not provide a highly accurate data as the mutagenesis appeared randomly. In order to ensure the impact of mutagenesis on the enhanced erythritol-production productivity, further research could be conducted by implementing another type of mutagenesis method such as the chemical-based one which is known to provide a more even mutation on the exposed strain.

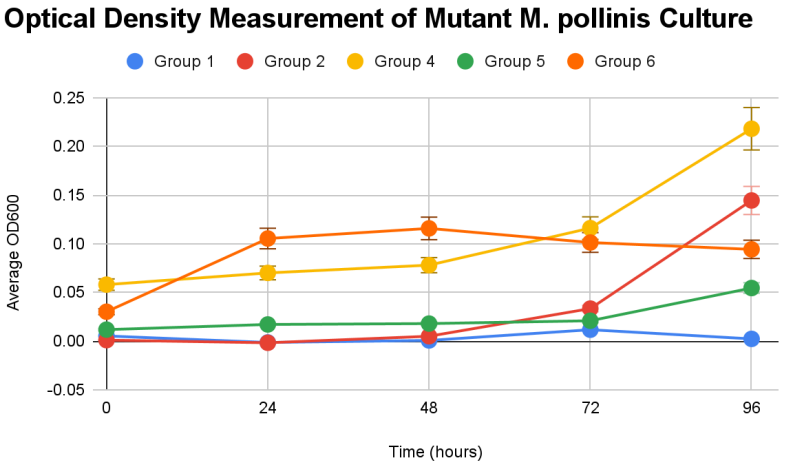

Figure 4.20. Different mutations effects on growth curve of OD600 vs time of different mutant *M. pollinis* strain in YGM medium

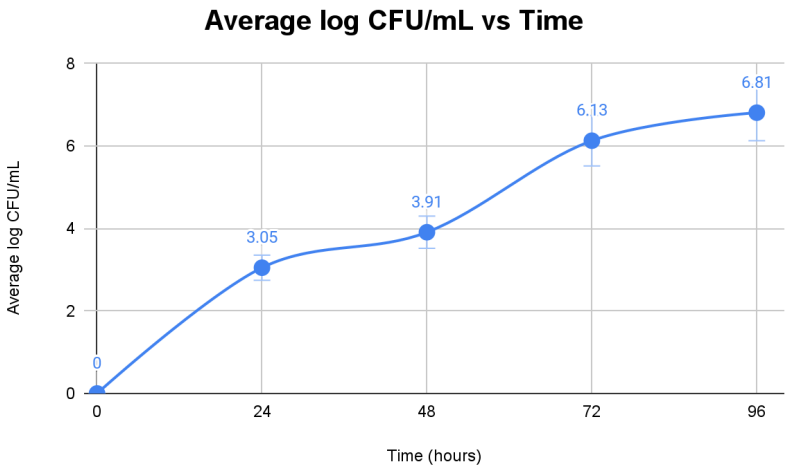

Figure 4.21. Growth curve of OD600 vs time of mutant *M. pollinis* strain in YGM medium

Figure 4.21 represents the CFU/mL of the mutated strain over 96 hours. Much like the non-mutated *M. pollinis*, the CFU/mL of the strain was calculated and plotted into a graph as can be seen by the figure above. An overall increase may be seen in the progression of log CFU/mL over the course of the four days, starting from 0 at 0 hours to 6.81 at 96 hours. However, unlike the previous log CFU/mL graphs, this one appears to be less smooth, having a slight dip at 48 hours. This is also the first time a log CFU/mL graph from Group 2 started from 0. This might correlate to a longer lag phase, in which longer time is needed for the cells' growth and proliferation to even be noticeable.

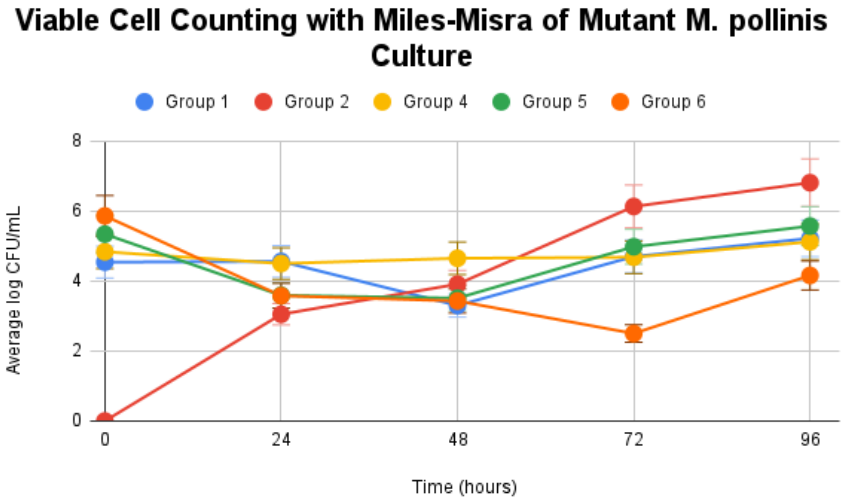

Figure 4.22. Different mutations effects on growth curve of log CFU/mL vs time of different mutant *M. pollinis* strain in YGM medium

Figure 4.2 shows the comparison of different groups' log CFU/mL graphs. Weirdly, only Group 2 showed an overall increase in log CFU/mL over the four days, while the others seemed to be either relatively stable or experience a decrease. This may be an indication that Group 2's mutant strain displays the best potential in terms of growth.

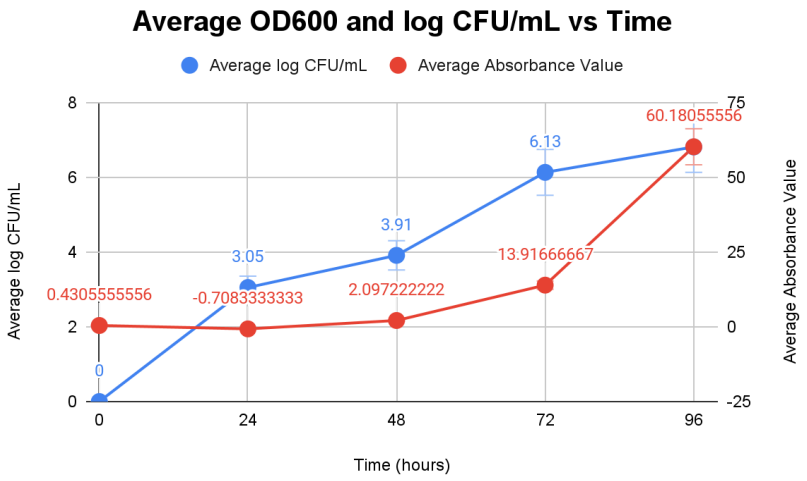

Figure 4.23. Growth curve of OD600 and CFU/mL vs time of mutant *M. pollinis* strain in YGM medium

4.3.2. Doubling Time of mutant *M. pollinis* strain

Briefly, the mutation of the yeast was done by exposing the *M. pollinis* strain to a UV light for a certain period of time, which in this case was 10 minutes. As has been stated earlier, the mutation functions to selectively determine mutants within the strain that have rapid growth rates which eventually leads to a significant reduction in the population doubling time (Hawary et al., 2019). Based on that understanding, it can be seen how the doubling time value of the mutant strain is significantly reduced to 2.49 hours. This value was obtained using the formula stated in Section 4.1.2 above where the data used was based on the exponential growth period observed between the 72 hours and 24 hours time points. Additionally, the average CFU/mL calculation was based on the values from -3 and -2 dilution of the respective time point.

Doubling time of mutant *M. pollinis* strain:

$$G = \frac{(72-24)}{3.3log(1466666.667/633333.3333)}$$

$$G = 2.49\ hours$$

4.3.3. Biomass of mutant *M. pollinis* strain

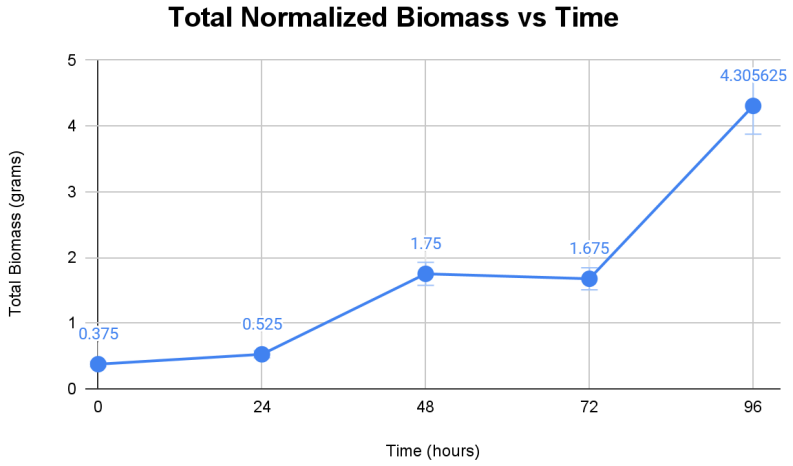

Figure 4.24. Total biomass production vs time of mutant *M. pollinis* in YGM medium

At the end of the 96 hour-period, the amount of biomass produced in the culture had reached 4.31 g, which is comparable to the final biomasses recorded in **Figure 4.6** and **Figure 4.15**. However, **Figure 4.24** especially bears a striking resemblance to **Figure 4.15**, where growth is very slow up until the 72 hour mark. It is likely that the mutation induced by the UV radiation may have caused the strain to experience a longer duration of lag phase than normal, mimicking the conditions of using 0 g/L NaCl inside the YGM medium. This could have happened due to alterations in genes expressing proteins important in the progression of the strain's lag phase towards its exponential phase, yielding slower rates of reactions in its metabolic pathways due to defective proteins (Adkar et al., 2017).

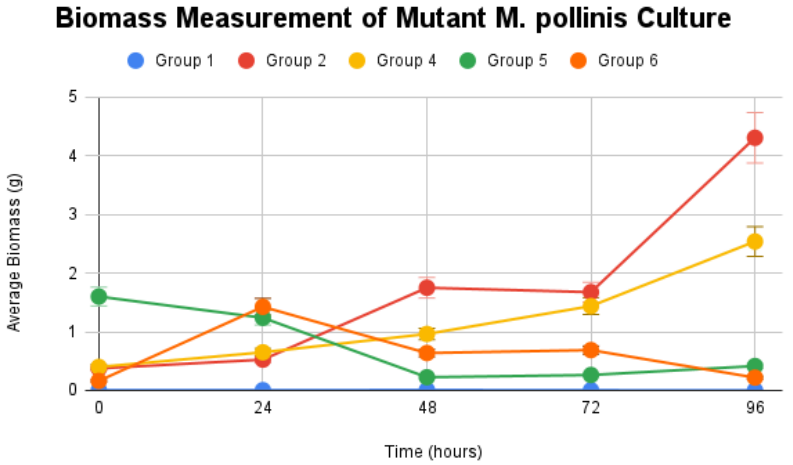

Figure 4.25. Different mutations effects on total biomass production vs time of mutant *M. pollinis* in YGM media

Despite the slow lag phase, the mutant strain from Group 2 seemed to have produced the highest amount of biomass after 96 hours compared to the others. In tandem with Group 2's promising rate of OD600 and log CFU/mL increase, it may be considered that Group 2's mutant strain contains genotypes that may be favorable for faster cell growth and proliferation. Therefore, further experimentation may be done to Group 2's strain in hopes of identifying the gene responsible for this desirable trait.

Interestingly, several groups experienced decreases in biomass instead, namely Groups 1, 5, and 6. Normalized biomass indicates the total mass of cells accumulated in a medium at a point in time, and it should be impossible for cells to disappear or decrease in mass that drastically, hence it is likely that these strange results are attributed to wrongful measurements. For example, the usage of cells from the stock culture without adequate resuspension may mean that less or more cells than wanted were being observed that day, meaning a poor representation of the actual stock was used for measurements.

4.3.4. pH of mutant *M. pollinis* strain media

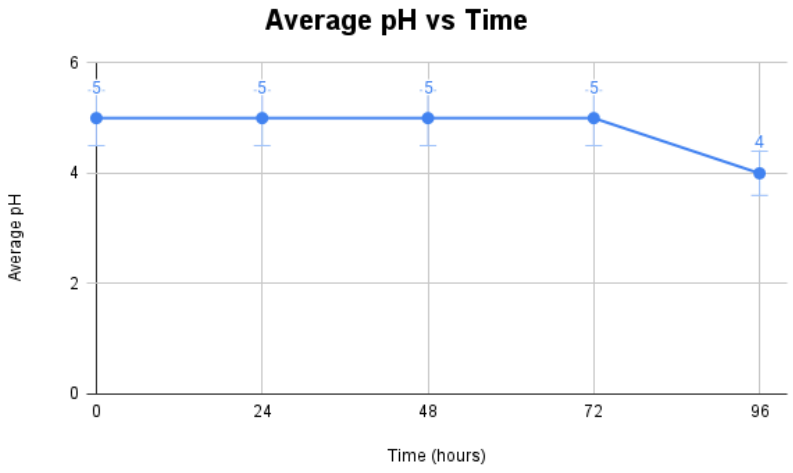

Figure 4.26. pH vs time of mutant *M. pollinis* in YGM medium

As seen in **Figure 4.26**, the change in pH over time follows a downwards trend, similar to all of the previous patterns of pH change. However, in this case, it took 96 hours for a decrease in 1 pH value to be noticeable, suggesting that the acidic metabolites are produced at a much slower rate than usual. This ties in with the OD600 growth curve in **Figure 4.19** and graph of biomass production in **Figure 4.24**, where a steep increase in OD600 (indicating cell density) and biomass production (indicating amount of cells) only occurs after the 72 hour mark. However, despite sharing similar patterns in the change of total biomass over time with the medium

containing 0 g/L in Section 4.2, it seems that this mutant strain does not release as much acidic compounds into the media.

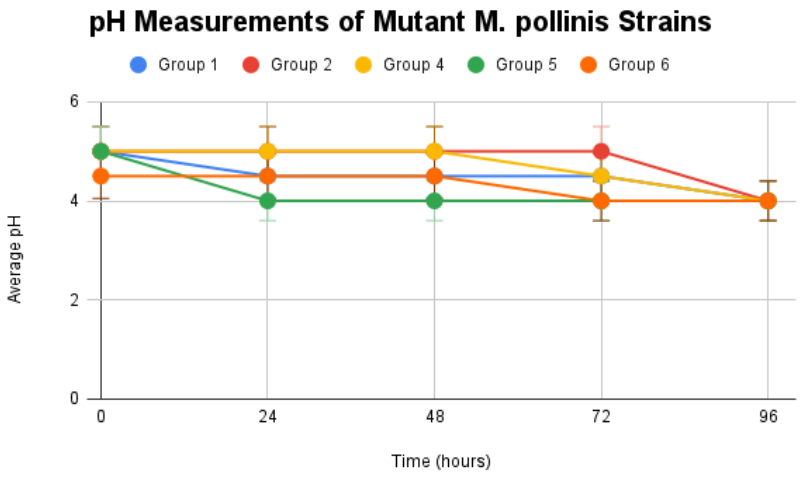

Figure 4.27. Different mutations effects on pH vs time of mutant *M. pollinis* in YGM media

Almost all of the mutant strains developed by the other groups followed a similar trend in pH decrease, with all of them ending up with a pH value of 4. Almost all of the groups also started with a pH value of 5 at 0 hours, except for Group 6, which started with a pH of 4.5. Differences in pattern may be observed in the time it took for the strains to decrease in pH. For instance, Group 5 experienced a decrease of 2 levels within the first 24 hours of growth, likely indicating that it had faster metabolic activity and growth initially.

4.3.5. Substrate consumption of mutant *M. pollinis* strain

Using the same formula as stated in Section 4.1.2, the amount of glucose consumed during the fermentation period is calculated using the linear equation obtained from the standard curve which consists of the concentration of glucose and peak area from the chromatogram result of mutant *M. pollinis* strain. Specifically, the curve generated a linear equation of:

$$y = 0.166x + 3.61$$

Same as the previous ones, when the peak area replaced the y variable, the glucose concentration represented by the x variable exhibited a value of 609.73 mg/mL. If this value is subtracted to 300 mg/mL as the initial glucose concentration, thus the substrate consumption in this laboratory conduct would have a value of -309.73 mg/mL. According to the value obtained from the standard equation, the glucose concentration could not be found as well as the glucose consumed. This might be due to the mutation that occurs, where the desired characteristics of the *M. pollinis*

to produce erythritol is disrupted. Moreover, instead of the glucose being consumed, the amount of glucose available in the media doubled from 300 mg/mL to 609.73 mg/mL. From a research by Sarkar & Gaddameedhi (2020), this condition is possibly caused by the effect of UV irradiation which causes the disruption of glucose-sensing and signaling pathways to convert glucose into erythritol.

4.3.6. Erythritol production of mutant *M. pollinis* strain

The chromatogram result from the HPLC analysis of *M. pollinis* mutant strain specifically for the erythritol standard generated a peak with the retention time of around 24.252 and a peak area of 1.7217. Other than this peak and the following retention time, the other detected peaks were gathered and plotted into the erythritol standard curve from which a linear equation was obtained as follows:

$$y = 0.166x + 3.61$$

Based on this equation, the erythritol concentration was calculated to be 5.906 mg/mL. For the mass yield and the volumetric productivity, the value could not be obtained as the glucose consumed and the erythritol produced is not aligned. This would result in a negative yield of erythritol, and due to the disrupted pathway of mutant *M. pollinis* there would be only little to no erythritol produced. Moreover, there would be no volumetric productivity available as there was no production occurring through the signaling pathway in the first place.

4.3.7. HPLC comparison of random UV mutagenesis of *M. pollinis*

Table 4.3. Yield mass of erythritol production in mutant strain

	Glucose			Erythritol	
Group Number	Area (pa*min)	Conc. (mg/mL)	Glucose increase	Area (pa*min)	Conc. (mg/mL)
Group 1	128.6203	743.85	443.85	n/a	-
Group 2	104.8252	606.30	306.30	1.7217	6.80
Group 3	115.4773	667.88	367.88	n/a	-
Group 4	108.9217	629.98	329.98	n/a	-
Group 5	114.0613	659.69	359.69	n/a	-
Group 6	140.5807	812.98	512.98	n/a	-

From previous analysis of different treatments for *M. pollinis*, the erythritol produced by the mutant has the lowest value. As seen in **Table 4.3**, none of the group has a detected erythritol produced except for Group 2 with a peak area of 1.72

at the erythritol retention time. Thus, mutant strains from UV exposure are highly unreliable as a method to increase the yield of erythritol.

V. CONCLUSION

The experiment demonstrated how different treatment of *M. pollinis* could affect the production of erythritol. The different C/N ratios, NaCl concentrations, and random mutation in the media affects the production of erythritol of *M. pollinis.* From the data that was collected the yeast extract had an effect on the erythritol production with the best concentration of 5 g/L meaning the second hypothesis is accepted. The results show that 25 g/L NaCl concentration had the highest erythritol yield. In contrast, concentrations above 25 g/L had worse erythritol yield. Meaning the second hypothesis is accepted. From all the mutated strains only one group detected the production of erythritol produced by the mutant of 1.72 at the erythritol retention time. Therefore the first hypothesis is accepted since mutation is highly unreliable. For future experiments rational design would be far more efficient than directed evolution since rational design does not rely on random mutation instead it uses direct modification of the genomic sequence making it far more precise and efficient and not as time consuming.

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APPENDIX

Turnitin checking: