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Development Of Glucose-independent *Yarrowia Lipolytica* Processes

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Development Of Glucose-independent *Y. Lipolytica* Processes

Abstract

As the global population continues to expand, there is a rising demand for animal meat production. However, the consumption and production of animal-based protein have detrimental effects on human health and the environment. Furthermore, challenges such as zoonotic diseases, climate change, and land scarcity underscore the need for exploring alternative meat sources.

The study aims to investigate the feasibility of using *Yarrowia lipolytica* in mycoprotein research, focusing on enhancing its growth rate on environmentally friendly carbon sources that mitigate industrial CO_2 emissions. This involves employing molecular and genetic approaches to optimize natural metabolic pathways and utilizing adaptive laboratory methods for fermentation.

This study, a part of the Yummowia project, advances scientific understanding in synthetic biology and food science and holds promise in shaping the future landscape of sustainable and appealing meat alternatives. The collaborative and multidisciplinary nature sets the stage for continued innovation, with the potential to positively influence the broader food industry.

Keywords

Yarrowia Lipolytica, Yummowia Project, Carbon Sources, Meat Alternatives, CO_2

CERCS: B230 Microbiology, Bacteriology, Virology, Mycology, T430 Food and Drink Technology, T490 Biotechnology

Glükoosist sõltumatute *Y. Lipolytica* protsesside arendamine

Lühikokkuvõte

Maailma elanikkonna jätkuva kasvu tõttu suureneb pidevalt nõudlus loomaliha tootmise järele. Loomse valgu tarbimine ja tootmine on aga kahjulikud nii inimese tervisele kui ka keskkonnale. Lisaks tõstavad zoonootilised haigused, kliimamuutus ja maade nappus esile vajaduse uurida alternatiivseid lihaallikaid.

Käesoleva magistritöö eesmärk on uurida *Yarrowia lipolytica* kasutamise võimalikkust mükoproteiinide uurimisel, keskendudes selle kasvukiiruse suurendamisele keskkonnasõbralikel süsinikuallikatel, mis vähendavad tööstuslikke CO_2 heitkoguseid. Selleks kasutati molekulaarseid ja geneetilisi lähenemisviise, et optimeerida looduslikke ainevahetusradasid ja kasutada kääritamiseks kohandatud laboratoorset meetodit.

See uurimustöö, mis on osa Yummowia projektist, edendab teaduslikku arusaamist sünteetilise bioloogia ja toiduteaduse valdkonnas ning tõotab kujundada jätkusuutlike ja atraktiivsete lihaalternatiivide tulevikku. Koostöö ja multidistsiplinaarsus loovad eeldused pidevale innovatsioonile, millel on potentsiaal mõjutada positiivselt toiduainetööstust laiemalt.

Võtmesõnad:

Yarrowia Lipolytica, Yummowia projekt, süsinikuallikad, lihaalternatiivid, CO_2

CERCS: B230 Mikrobioloogia, Bakterioloogia, Viroloogia, Mükoloogia, T430 Toiduainete ja Jookide Tehnoloogia, T490 Biotehnoloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

AcCoA	Acetyl Coenzyme A, Acetyl-CoA
ALE	Adaptive Laboratory Evolution
BII	BioInnovation Institute, Denmark.
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization
FBS	Fetal Bovine Serum
FDA	U.S. Food and Drug Administration
GK	Glycerol Kinase
GLA	γ-linolenic Acid
GMF	Genetically Modified Foods
GMO	Genetically Modified Organisms
MUFAs	Monounsaturated Fatty Acids
non-oxPPP	Non-Oxidative Pentose Phosphate Pathway
oxPPP	Oxidative Pentose Phosphate Pathway
PCR	Polymerase Chain Reaction
PDC	Pyruvate dehydrogenase complex
PDH	Pyruvate Dehydrogenase
SCO	Single-Celled Fat
TAGs	Triacylglycerides
TCA	Tricarboxylic Acid
VFAs	Volatile Fatty Acids
YPD	Yeast Extract-Peptone-Dextrose Media

INTRODUCTION

The need to increase meat production to adapt to a growing global population has raised concerns about the difficulty of increasing the amount of traditional meat production as well as the environmental impacts associated with the processes involved. These include greenhouse gas emissions, soil and water depletion, animal welfare issues, antibiotic residues and the risk of zoonotic diseases. In addition, meat consumption is associated with health problems such as heart diseases, obesity and some types of cancer. There is growing interest in developing alternative protein sources to overcome these challenges.

Research in this area explores a variety of meat substitutes, including plant-based alternatives, cultured meat, algae, edible insects, and mycoprotein, as potential solutions to alleviate environmental, ethical, and health concerns associated with conventional meat production. A central component of this research is the Yummowia project, which aims to revolutionize the food industry by exploiting the unique properties of *Yarrowia lipolytica*. The project aims to develop *Y. lipolytica* yeast into a sustainable and nutritious meat alternative product that will rival or surpass conventional meat in sensory properties. Unlike traditional meat production, this alternative will be both animal-free and environmentally sustainable.

Yarrowia lipolytica stands out as a promising yeast strain for alternative protein production due to its versatility. It is an ideal candidate for biotechnological applications thanks to its ability to adapt to a wide range of substrates and environmental conditions.

Adaptive laboratory evolution and metabolic engineering techniques for overexpression of the acetyl-CoA synthetase (ACS) pathway were used to optimize the use of these carbon sources. The ability of the resulting strains to consume acetate and mannitol were compared to glucose and glycerol utilization.

The experiments conducted for this research were carried out at Yeti Foods, BioInnovation Institute, Denmark.

1 LITERATURE REVIEW

1.1 MEAT CONSUMPTION AND SUBSTITUTES

The human population may increase to 8.5 billion in 2030 and to 9.7 billion in the following 20 years (United Nations Department of Economic and Social Affairs, 2022). Accordingly, a significant increase in global meat demand is expected. The Food and Agriculture Organization (FAO) estimates that, meat consumption reaches 455 million tons, which is 150 million tons more than the consumption in 2013 (*Alternative Pathways to 2050*, 2018).

Meat is one of the most valuable sources of protein, contains vitamin B complex, vitamins A and D, abundant iron, zinc and other mineral substances, while producing sensory pleasure, identity, status for consumers and is also closely linked to numerous cultural traditions (Baltic & Boskovic, 2015; Font-i-Furnols & Guerrero, 2022).

However, due to high meat consumption, especially in regions where high-income groups live, the intake of fiber and plant-based foods is often inadequate. Besides, excessive meat consumption can cause health problems such as heart diseases, excess weight, and some certain types of cancer (Battaglia Richi et al., 2015; Phillips, 2020).

Despite the nutritional benefits of meat consumption, meat production processes associated with meat have substantial environmental impacts. These include the emission of greenhouse gases, extensive land and water use, adverse effects on animal welfare, the presence of antibiotic residues in animals, and the potential for the spread of zoonotic disease (Van Der Weele et al., 2019; Zhang et al., 2022).

The food industry is exploring emerging non-traditional meat and protein products, including cultured meat and genetically modified organisms, to address the challenges of animal welfare, health and sustainability issues amid growing demand. However, currently available artificial meat is not sufficient to replace traditional meat. Plant-based protein and mycoprotein are the main substitutes for traditional meat, and alternative protein sources such as insects and algae are also gaining ground in the market (Bonny et al., 2015; Van Der Weele et al., 2019). These meat substitutes are comparable to traditional meat products in taste, texture and nutritional profiles, while reducing environmental impact and addressing ethical and health concerns. The development and adoption of meat substitutes represents a promising path to promote sustainable food systems and improve public health. The development and adoption of meat

substitutes represents a promising path to improve public health and animal welfare by promoting sustainable food systems (Caputo et al., 2023; Costa-Catala et al., 2023).

1.1.1 Plant-Based Meat Alternatives

The main sources of alternative protein are soybeans, wheat, oilseeds and legumes, and are available in grocery stores as tofu, seitan and plant-based burgers, sausages and nuggets (Bonny et al., 2015; Fehér et al., 2020; Lynch et al., 2018).

Plant-based alternatives have lower resource use, waste and greenhouse gas emissions associated with their production than traditional meat (Lynch et al., 2018). They also have lower cholesterol content and lower incidence of foodborne illnesses and can be produced on an industrial scale (Bonny et al., 2015).

The production of plant-based meat substitutes has expanded as a result of marketing campaigns and product development initiatives, but novel formulations and processing technology advancements aimed at enhancing meat-like qualities are still in their early stages and face numerous technical challenges. A number of additives are added to food to provide it the texture, juiciness, mouthfeel, and flavor of meat, but this raises concerns regarding customer confidence, clean label, cost, and nutrition (M. Ahmad et al., 2022). Additionally, the perception of plant-based products as highly processed foods may deter health-conscious consumers from incorporating them into their diets (Lynch et al., 2018).

Consumer acceptance and sensory properties are central obstacles to the widespread use of plant-based meat alternatives. The taste and texture compared to conventional meat are different for non-vegetarian consumers, reducing their desire to switch to plant-based options (Hoek et al., 2004).

The mission of the plant-based meat industry is to create plant-based alternatives that closely mimic the sensory experience of traditional meat, even with every challenge it faces. This is achieved through innovations in food technology, such as the use of new ingredients and processing techniques, and by consistently improving its products to increase consumer appeal and market penetration (Szenderák et al., 2022).

1.1.2 Cultured Meat

Cultured meat, also known as laboratory-grown or cell-based meat, is made from animal cells cultured in growth medium in a bioreactor, rather than derived directly from killed animals (Thavamani et al., 2020; Treich, 2021).

Lab-grown meat reduces the risk of contaminants and foodborne pathogens because it is produced under highly controlled conditions (Chriki & Hocquette, 2020).

Consumers can find in cultured meat the appearance, taste, smell and aroma they are familiar with in conventional meat. This may increase their likelihood of trying and purchasing a product similar to traditional meat. Additionally, cultured meat is very similar to conventional meat in terms of nutritional value, probably in both positive and negative ways (Bonny et al., 2015; Smetana et al., 2015).

Cultured meat has a high potential to reduce environmental impacts from conventional animal husbandry (Post, 2012). Although it has the highest environmental impact of all meat substitutes, it uses fewer resources than conventional meat. It also has the potential to reduce waste and greenhouse gas emissions (Bonny et al., 2015).

The process of culturing cells for meat production presents several challenges, especially regarding the choice of culture medium. Fetal bovine serum (FBS) has been an important ingredient due to its ability to potently stimulate cell proliferation. However, FBS derived from the blood of slaughtered calves is not only ethically problematic, but also prevents its acceptance by certain religious and ethical communities such as vegetarians, vegans, and Muslims. In addition, the fact that FSB is expensive and unsustainable, and that serum-free media still cannot be designed due to the complexity of reconstructing the nutrient and growth factor profiles found in FBS, continues to be a significant bottleneck (Chriki & Hocquette, 2020; Hamdan et al., 2018; Martins et al., 2024).

In order for cultured meat to be commercialized, both technological and legislative problems need to be solved, such as the fact that it cannot yet be produced on an industrial scale and is prohibited in agricultural systems in the European Union. These situations constitute another obstacle to cultured meat (Bonny et al., 2015; Chriki & Hocquette, 2020).

1.1.3 Algae

Another alternative protein source is Spirulina, which is the most consumed algae. It contains high protein content, up to 63% of dry biomass, and can be produced on a large scale (Becker, 2007). Its high protein content and essential amino acid composition are the main features that make algae protein stand out when compared to other plant-based proteins such as soy or chickpea. It has high antioxidant activity, high levels of vitamin B12, iron, β-carotene and γ-linolenic acid (GLA) (Altmann & Rosenau, 2022; Espinosa-Ramírez et al., 2023; Hirata et al., 2000).

Microalgae biomass has been consumed as a green food supplement or food source for hundreds of years, particularly in China, Japan, and the Republic of Korea, due to its high nutrient content (Çelekli et al., 2024).

Another advantage is that they can be grown efficiently in fresh or sea water without antibiotics and pesticides, and have a lower environmental footprint in terms of energy consumption, land requirements and water use efficiency than conventional crops (Habib, 2008; Ullmann & Grimm, 2021).

However, the incorporation of microalgae into conventional food products is a challenge due to their high production costs and the fact that they produce an unpleasant taste, an unattractive dark green color, a powder-like consistency and a fishy smell (Becker, 2007).

1.1.4 Edible Insects

Edible insects, which are a natural source of protein, are consumed in 128 countries around the world and in comprises more than 2200 species, mainly beetles, caterpillars, crickets, and grasshoppers. Insects are high in protein, and most insects contain significant amounts of minerals, amino acids and vitamins (Olivadese & Dindo, 2023; Omuse et al., 2024; Zielińska et al., 2018).

Insect-based products have environmental advantages over conventional meat products, such as lower greenhouse gas and ammonia emissions, less land area required, more efficient feed conversion, and the potential to be grown with organic by-products. The insects consume less water compared to conventional livestock farming due to their cold-blooded nature, which also allows them to obtain most of their moisture from food, thus reducing the water footprint of the feed. Insects are twice as efficient at converting food into consumable tissue as chickens and pigs, and five times more efficiently than beef cattle. With fast reproduction rates and high fecundity, insects have a much broader nutritional range than conventional farmed animals (Durst & FAO, 2010; Van Huis, 2015; Van Huis & Oonincx, 2017).

Despite reluctance in Western countries, interest is growing, especially in regions such as Austria, Belgium, the Netherlands and France, where insects are being integrated into the food industry as a new food. Consumer acceptance of insect consumption is limited due to neophobia, cultural and religious perceptions, and history (Ros-Baró et al., 2022).

1.1.5 Mycoprotein

The mycoprotein derived from filamentous fungi is another meat substitute. It has a high nutritional content and functions as an antioxidant, prebiotic and blood sugar and cholesterol regulator. It is also crucial for the formation of muscle protein (Majumder et al., 2024; Ruxton & McMillan, 2010).

Due to its relatively high protein value, high fiber and reduced saturated fatty acid content, mycoprotein fits into a healthy diet. According to experimental studies, mycoprotein, which creates a feeling of fullness, contains all the essential amino acids (M. I. Ahmad et al., 2022).

The European Commission states that fungal protein is a rich source of several different mineral components, such as potassium, zinc, phosphorus, calcium, iron, and contains several water-soluble B vitamins. It also contains zinc, phosphorus, calcium, iron, potassium, and other minerals (Saeed et al., 2023).

The ecological impact of mycoprotein is lower than that of meat and accounts for less than half the greenhouse effect of animal protein when measured by weight. Mycoprotein is one of the most effective substitutes in terms of water, land and energy used in the production of meat substitutes. (Saeed et al., 2023).

Mycoprotein can be produced from rom a variety of different fungi, such as *Monascus purpureus*, *Aspergillus oryzae, Paradendryphiella salina, Neurospora intermedia, Rhizopus oryzae* and *Fusarium venenatum* (Majumder et al., 2024).

Mycoprotein is not permitted as a standalone food; however, it is currently approved for use as a food supplement, albeit in smaller quantities than full food portions (EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) et al., 2019).

1.2 *YARROWIA LIPOLYTICA*

Y. lipolytica is the most studied unicellular fungus right after *Saccharomyces cerevisiae* and as "non-conventional" yeast species classified in the kingdom Fungi, phylum Ascomycota, class Saccharomycetes, order Saccharomycetales, family Dipodascaceae, and genus *Yarrowia*. The former name of *Y. lipolytica* was *Candida lipolytica* (Barth & Gaillardin, 1997; Jach & Malm, 2022).

Y. lipolytica is a species of oleaginous yeast with a distinct physiology that enables the synthesis and accumulation of high amounts of storage lipids (Szczepańska et al., 2022).

Y. lipolytica, which has been isolated from a wide variety of places, from foodstuffs such as beer, cheese, and sausage to the human mouth (Sørensen et al., 2023; Zieniuk & Fabiszewska, 2019). In 2004, the first reconstructed *Y. lipolytica* genome was fully sequenced (Larroude et al., 2018).

Y. lipolytica is a yeast species that exhibits dimorphism and shows different growth forms depending on the environment. Under varying growth conditions, they may grow as rounded multipolar budding cells, remain attached as pseudohyphae, or develop into mycelia with septate hyphae (Madzak, 2021).

Y. lipolytica is highly versatile in its ability to utilize both hydrophilic and hydrophobic substrates, free fatty acids, vegetable oils and animal fats to produce valuable biological products. These include extracellular enzymes, organic acids such as citric/isocitric acids, pyruvate, succinate and alpha-ketoglutarate, as well as heterologous proteins such as laccase and epoxide hydrolyase. *Y. lipolytica* is good at degrading hydrophobic substrates; it can produce a wide range of useful metabolic products such as proteins, peptides, amino acids, trace minerals, vitamins and carbohydrates or, in particular, high levels of single-celled fat (SCO). It contains saturated high value-added lipids and monounsaturated fatty acids (MUFAs), similar to those found in cocoa butter. β-carotene, β-farnesene, limonene, valencene, squalene, 2,3-oxidosqualene, aromatic amino acids, and resveratrol can be produced from genetically modified *Y. lipolytica*. Enzymes such as lipases, β-mannases, laccases, amylases and proteases can be produced on an industrial scale (Darvishi Harzevili, 2014; Jach & Malm, 2022; Sáez-Sáez et al., 2020; Soong et al., 2019; Van Der Hoek et al., 2022).

It has been reported that *Y. lipolytica* can grow in pH conditions varying between 2.5 and 7.5 (Egermeier et al., 2017). In industrial production, its tolerance to low pH is an important advantage as it prevents bacterial contamination and reduces the need to neutralize pH, *Y. lipolytica* shows robustness and stability to environmental fluctuations during the fermentation process and to changes in dissolved oxygen concentration, pH, osmolarity, nutrient source, and temperature (Park & Ledesma-Amaro, 2023). Moreover, it has high tolerance to a variety of organic compounds and elevated salt concentrations (Miller & Alper, 2019).

Unlike the well-known yeast host *S. cerevisiae*, *Y. lipolytica* is a Crabtree negative yeast, indicating that that it does not create ethanol when substrate is in excess (Christen & Sauer, 2011).

Y. lipolytica owns the unique capability to redirect substantial metabolic activity via acetyl-CoA and malonyl-CoA to the creation of a wide variety of heterologous products (Markham & Alper, 2018).

1.3 CARBON SOURCES

Y. lipolytica is an interesting industrial yeast because it can utilize a broad range of carbon substrates, such as glucose, acetate, glycerol, mannitol, alkanes, lipids and oleic acid (Darvishi Harzevili, 2014; Worland et al., 2020).

Glucose, the most abundant carbon source in nature, is mostly applied in industrial-scale bioprocesses. *Y. lipolytica* uses a combination of the glycolytic cycle, mitochondrial pyruvate dehydrogenase (PDH), and ATP to convert glucose into cytosolic acetyl-CoA (Kamineni et al., 2021). But while many organisms prefer glucose as a carbon source, *Y. lipolytica* prefers glycerol over glucose (Erian et al., 2022; Mirończuk et al., 2016; Workman et al., 2013).

As biodiesel production units continue to expand, an increasing supply of high-quality glycerol byproduct is becoming available.. (Imandi et al., 2007). Glycerol has the advantages of low cost, abundant availability and reduced competition with food and feed industries (Rywińska et al., 2013). Additionally *Y. lipolytica* has tolerance to inhibitory compounds found in unrefined crude glycerol of biodiesel production (Papanikolaou et al., 2017; Spagnuolo et al., 2018).

1.3.1 Mannitol

Mannitol also known by the name mannite or manna sugar is a six carbon sugar alcohol (Ibrahim, 2016). Molecular formula is $C_6H_{14}O_6$ and molecular weight is 182.17 g/mol with a sweetness level that is 50–70% that of sucrose (M. Chen et al., 2020).

Brown seaweed biomass consists of up to 31.53% Mannitol and is found in nature in many bacteria, fungi, algae, lichens and plants (Jordan et al., 2016; Sung-Soo Jang, 2012). Mannitol has a great potential for biomanufacturing due to the abundance of seaweed in the oceans, which cover over 70% of the Earth's surface (Xin et al., 2023).

D-mannitol and other sugar alcohols are used by *Y. lipolytica* as carbon source (Darvishi Harzevili, 2014). *Y. lipolytica* utilizes mannitol by mannitol transporters and dehydrogenases, which oxidize mannitol into fructose; subsequently, the phosphotransferase system (PTS)

phosphorylates fructose, leading to the formation of fructose-6-phosphate, a key intermediate in glycolysis (Xin et al., 2023; C. Zhu et al., 2022).

1.3.2 Acetate

Acetic acid is a two-carbon monocarboxylic acid and molecular weight is 60 g/mol. Molecular formula is CH_3COOH. Acetic acid, with a pKa value of 4.75, is mostly ionized as acetate (CH3COO-) at physiological pH (Kim et al., 2021).

Acetic acid takes place in synthesis of volatile fatty acids (VFAs). Volatile fatty acids (VFAs) are organic acids can be produced through microbial fermentation, which utilizes renewable carbon sources and offers a more environmentally friendly approach. While commercial sugar-based processes yield higher productivity with fewer side products, they are costlier due to the expense of the raw materials. To address this, researchers are exploring the use of abundant lignocellulosic biomass and waste sludge as alternative carbon sources for VFA production (Bhatia & Yang, 2017).

Furthermore, acetate can be generated from a number of low-cost sources, including the gasification of organic matter, industrial waste gases, and syngas (CO/H_2), which is readily obtained through anaerobic digestion facilitated by acetogens using the Wood-Ljungdahl pathway (Huang et al., 2023; Spagnuolo et al., 2018).

Y. lipolytica can convert acetate into value-added products due to the strong tolerance of its natural acetate uptake pathway to solvents and acids. (Gao et al., 2020; Huang et al., 2023). Utilizing bioprocess control techniques, the use of acetate as a substitute substrate has developed considerably (J. Xu et al., 2017).

When acetate is used solely as a carbon source, growth requires its activation into acetyl-CoA, catalyzed by acetyl-CoA synthetase (ACS) in the cytoplasm. However, this is only possible through *Y. lipolytica* metabolic engineering strategies to improve acetate utilization, and these strategies include increased acetate tolerance, increased uptake, and improved co-utilization (Spagnuolo et al., 2018).

1.4 METABOLIC ENGINEERING

The goal of metabolic engineering is to apply molecular and genetic approaches to optimize natural metabolic pathways and regulatory networks or to combine heterologous metabolic

pathways to create a cell factory that produces cost-effective molecules on an industrial scale (Q. Zhu & Jackson, 2015)**.**

Acetyl-CoA serves as a crucial metabolic precursor for numerous biosynthetic pathways, particularly gluconeogenesis, fatty acid synthesis, and sterol synthesis, which are pivotal processes in cellular metabolism. Furthermore, it is a crucial substrate for energy production in the tricarboxylic acid (TCA) cycle depending on the cellular nutrient status, multiple mechanisms can contribute to the production of acetyl-CoA. This molecule acts as a central player, connecting various metabolic processes, which is essential for the synthesis of fatty acids and sterols from simple substrates (Y. Chen et al., 2012; Kratzer & Schüller, 1997).

Mitochondrial pyruvate dehydrogenase complex (PDC) plays a minor role in acetyl-CoA production even when pyruvate is present. Instead, acetate is formed through the combined action of pyruvate decarboxylase and acetaldehyde dehydrogenase. Formation of acetyl-Coa from acetate is a two-step process which can proceed in two ways: one involving ACS and proceeding via an acetyladenylate (AcAMP) intermediate, and the other utilizing acetate kinase (Ack) and phosphotransacetylase (Pta), forming an acetyl phosphate intermediate. In both, acetate is first converted to a high-energy intermediate and then transferred to CoA to form acetyl-CoA, facilitating its incorporation into metabolic pathways. ACS belongs to the AMP-forming enzyme family and direct activation of acetate by (ACS) is therefore an ATP-consuming reaction (Jogl & Tong, 2004; Kratzer & Schüller, 1997).

Initially, acetate is taken up into the cytosol and activated to acetyl-CoA by acetyl-CoA synthetase at the cost of two ATP equivalent molecules. The resulting cytosolic AcCoA has several metabolic targets as shown in Figure 1. It can be directly incorporated into lipids during *de novo* new fatty acid biosynthesis or transported to the mitochondria via the carnitine shuttle for energy production the tricarboxylic acid (TCA) cycle. The major difference between acetate and glucose metabolism is that acetate activates the glyoxylate shunt and gluconeogenesis pathways. The glyoxylate shunt involves the transport of isocitrate from the mitochondria to the cytosol, the breakdown of isocitrate into glyoxylate and succinate catalyzed by isocitrate lyase, and the combination of glyoxylate with AcCoA to produce malate through the action of malate synthase (Liu et al., 2016)

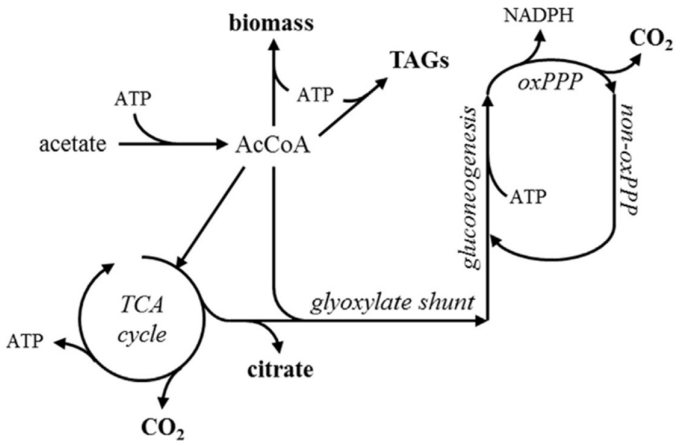

Figure 1. Overview of the major metabolic pathways involved in using acetate as the sole carbon source

A study in metabolic engineering by Lin et al. 2006 showed that increasing the ability of yeast to assimilate acetate will reduce the harmful effects of acetate, recycle wasted carbon, and increase carbon flux towards desired pathways. For this, Acetyl-CoA synthetase was overexpressed to enhance the assimilation and activation of Acetate to Acetyl-CoA. Thus, indicating a significant reduction in acetate during glucose metabolism. This showed that it also greatly increased the assimilation of acetate when used as the sole carbon source (Lin et al., 2006).

1.5 ADAPTIVE LABORATORY EVOLUTION

Adaptive Laboratory Evolution (ALE), also known as Evolutionary Engineering, is a complementary species improvement strategy. It takes advantage of the flexibility of microbial genomes by designing and implementing selective breeding regimes. These regimes confer specific selective advantages to mutants with (industrially) relevant traits. The selective advantage of these mutants over other cells in the population may stem from higher specific growth rates, lower death rates, or increased retention in culture. Evolutionary engineering has been widely used to improve traits like stress tolerance, substrate consumption kinetics, and the rates of production of breakdown products in both wild-type and engineered yeast species (Mans et al., 2018).

ALE is primarily utilized in microorganisms due to their ability to sustain rapid division in large quantities. Microorganisms have typical mutation rates and genome sizes that facilitate thorough exploration of the adaptive landscape, resulting in abundant genetic diversity and the

natural enrichment of beneficial mutations. ALE enhances high resistance traits by gradually increasing environmental pressure and regularly screening during the mating process. It involves designing suitable selection pressures based on the desired phenotype, making it broadly applicable and practical for microorganisms. Unlike conventional mutagenesis, ALE does not result in significantly more harmful mutations than beneficial ones. This is because environmental pressures introduced in ALE experiments eliminate harmful mutations, and genetic dynamics in the new population are primarily driven by beneficial mutations. Furthermore, strains evolved through ALE consistently exhibit high performance (C. Chen et al., 2024)

ALE enhances microbial growth rates and overall fitness. Through selective breeding, it promotes the dominance of strains with improved phenotypes. Competitive fitness assays monitor fitness differences between ancestral and adapted strains. ALE experiments typically span 100 to 2000 generations where stable. Stable phenotypes accumulate over time. While fitness increase may slow down, ALE remains efficient for achieving growth improvements. It is invaluable for biotechnological applications (Dragosits & Mattanovich, 2013).

1.6 YUMMOWIA PROJECT

The aim of the project is to turn *Y. lipolytica* yeast into a delicious and nutritious meat alternative product with similar or even better sensory properties than traditional meat. Unlike traditional meat production, it will be animal-free and sustainable, as well as aligned with the UN Sustainable Development Goals, thus reducing the impact on the environment. By applying synthetic biology and metabolic engineering techniques, the flavor, texture and nutritional values of *Y. lipolytica* will be modified to suit the new food application. In addition, production on an industrial scale and at low cost is aimed. A nutritious and healthy meat-like food option for everyone, including those on a vegetarian diet, as an alternative that eliminates the negative impact associated with traditional meat production and consumption. (*Yeti Foods - Bio Innovation Institute*, 2024)

1.6.1 Major Challenges

In parallel with the goals of the Yummowia project, it is aimed to turn *Y. lipolytica* into a new "genetically modified organism for food use" defined as a "GMO that may be used as food or as a source material for the production of food" ("Regulation (EC) No 1829/2003 of the European Parliament and of the Council on Genetically Modified Food and Feed.," 2003).

According to European Union legislation, genetically modified food (GMF) or feed must be evaluated case-by-case by the European Food Safety Authority (EFSA) and pass stringent regulations before being placed on the market. GM food have mandatory labeling to enable an informed and free choice to the consumers and farmers, and their traceability on the market must be guaranteed (*REGULATION (EC) No 1830/2003 OF THE EUROPEAN PARLIAMENT*, 2003).

In addition to the difficulty in legislation, especially in the European Union, another challenge in commercializing this new food product is consumer acceptance. Although GMO foods are subject to a higher level of scrutiny, the "unnaturalness" of genetic modifications also raises public concerns (Malarkey, 2003; Verhoog, 2003).

Higher public resistance to GMF is due to consumers' fear that has been enhanced by negative information from the media. Moreover, the results of a study from Laros & Steenkamp (2004) found that consumers that are more concerned about nature have more fear of GMF than consumers who trust technology in food production (Laros & Steenkamp, 2004). There are varying levels of consumer acceptance of genetic technology from country to country. For example, European consumers have the most negative attitudes, while American, Canadian and Japanese consumers are more positive (Bonneau & Laarveld, 1999; Cantley, 2012; Novoselova et al., 2007).

Consumer preferences and opinions significantly affect the development of new technologies such as genetic engineering and the implementation of sustainable production methods. One of the most important key points for consumers to accept food biotechnology is effective science-based communication. In order to gain the trust of the consumer, a simple and understandable language should be used with consumers, and the purpose and benefits of the new product should be explained. (Schmidt, 2000)

2 THE AIMS OF THE THESIS

This thesis aims to optimize the production of alternative meat by exploring diverse, eco-friendly carbon sources such as mannitol and acetate for *Y. lipolytica*. It involves enhancing *Y. lipolytica's* cell growth, growth rate, and doubling time through adaptive laboratory techniques and gene overexpression, comparing them with traditional carbon sources like glucose and glycerol. Furthermore, the research delves into understanding yeast growth and morphology with different carbon source mediums in flasks and bioreactors, and dynamics during fermentation processes.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

In this section, methods and materials have been described. Statistical analysis was performed using Excel of Microsoft Office 365. All chemicals were sourced from Sigma-Aldrich and VWR.

3.1.1 Strains and media

Yarrowia lipolytica strains and ScACS1 gene sequence utilized for Acetyl-CoA overexpression in this study are provided in Appendix A. Construction and genomic integration were facilitated using the EasyCloneYali toolbox (Holkenbrink, 2018). Detailed information on the primers, fragments, and plasmids employed can be found in Appendix A.

The media recipes and the trace metals solution recipe can be found in Appendix B. Thiamine was the sole vitamin used in all experiments, and Antifoam 204 (Sigma Aldrich) was utilized for the bioreactor experiments.

Y. lipolytica strains were cultured at 30°C and 225 rpm in standard yeast extract (YPD) liquid medium. Solid YPD medium supplemented with nourseothricin (Nat) was used for selecting *Y. lipolytica* transformants. *E. coli* strains carrying plasmids were cultured in 2xYT medium supplemented with 100 µg/mL ampicillin at 37°C.

3.1.2 Overexpression of the ACS Pathway

Plasmids were extracted from *E. coli* by using the Macherey-Nagel miniprep kit and their coding sequences were verified by Sanger sequencing provided by Eurofins Scientific SE. Linearization of plasmids was performed using Thermo Scientific™ FastDigest NotI enzyme (ThermoFisher) as per the manufacturer's protocol. Linearized vectors were transformed into *Y. lipolytica* strains following the EasyCloneYALI toolbox's transformation protocol (Dahlin et al., 2021). Transformants were verified by PCR.

3.1.3 Construction of *Y. lipolytica* Strains

Y. lipolytica strains (see Appendix A) were constructed using the EasyCloneYALI toolbox (Holkenbrink, 2018), enabling marker-free Cas9-mediated integration of expression vectors

into the genome. Transformation followed the EasyCloneYali toolbox protocol (Dahlin et al., 2021). Transformants were selected on YPD plates supplemented with hygromycin (300 mg/L). Correct integration of repair templates was confirmed by colony PCR.

3.1.4 Plasmid Curing

Plasmids were extracted from transformed strains by inoculating cells from verified colonies into 2 ml of YPD media and culturing for 1 day at 30°C with shaking at 250 RPM. Liquid cultures were diluted with sterile H_2O and plated on YPD plates, followed by incubation for 24 hours at 30°C. A selection of 4 transformants was resuspended in 200 µl of sterile H_2O, and 5 µl drops of cell suspension were incubated at 30°C for 24-48 hours on YPD and YPD with Hygromycin plates. Colonies growing on YPD plates but not on YPD with Hygromycin plates were reinoculated in 1 ml of YPD liquid media and cultured 24 hours long at 30°C and 225 RPM in the inoculation shaker at. Once confirmed, new strains were aliquoted into cryovials containing 50% (v/v) glycerol and placed into a -80°C freezer for long-term storage.

3.1.5 Cultivation

3.1.5.1 Flask Cultivation

Shake flask experiments were conducted using minimal media, as detailed in the Appendix B. Precultures were prepared from frozen stocks by inoculating them into 2.5 mL of YPD medium in Falcon tubes (10 mL) and incubated at 30°C with shaking (Excella E25 Shaker) at 225 rpm for 24 hours. Overnight cultures were then inoculated into 50 mL of Minimal media in 250 mL round-bottom shake flasks unless stated otherwise. Samples were collected at regular intervals for optical density measurement (OD600), pH monitoring, and microscopy imaging (Olympus BX61 microscope). The cultures were allowed to incubate until reaching the stationary phase at 28°C with shaking at 200 rpm. Upon reaching the desired phase, the experiments were terminated.

3.1.5.2 Bioreactor Fermentation

Using cells stored on agar plates, an initial preculture was inoculated into minimal media (see Appendix B) with an inoculation loop, using a 250 mL Erlenmeyer flask containing 25 mL of media, and incubated at 28°C with shaking at 200 rpm for 24 hours. Cells from the overnight preculture were then transferred into a bioreactor. Bioreactor-scale fermentations were

conducted in a 1-liter stirred-tank bioreactor (Applikon). Stirring was initially set to 800 rpm and 0.5 vvm of pressurized air was used to maintain aerobic conditions. When oxygen consumption increased and the DO decreased to <20%, the vvm was increased up to 2.0 and rpm to 1750 to maintain aerobic conditions. The pH was maintained at 5.5 using automated addition of either 5M NaOH (glycerol culture) or ammonia (25% and 10% for mannitol and glucose, respectively). Samples were collected in the fed-batch phase using three 10 mL Falcon tubes. The first tube was used to flush the sampling line and used for microscopy imaging, and the other two tubes were used for dry and wet cell weight analysis as well as HPLC analysis.

3.1.5.3 Dry and wet Cell Weight

For bioreactor experiments, empty Falcon tubes with their respective caps were weighed individually, and the weights were recorded. After sampling, the Falcon tubes containing the samples were weighed again to determine the weight of the culture sample. The samples in the Falcon tubes were then centrifuged at 3000 g for 5 minutes. Following centrifugation, 5 mL of the supernatant was carefully withdrawn for storage and HPLC analysis, and the remaining supernatant was removed. The tubes were then filled with water and centrifuged again to wash the cells. After removing the water by another round of centrifugation, the tubes were weighed to determine the weight of the remaining pellet ('wet cell weight'). The tubes containing the samples were then placed in an 80°C incubator until completely dried after 5-7 days. After drying, they were reweighed to determine the dry cell weight.

3.1.5.4 High-performance liquid chromatography (HPLC)

The aim of HPLC sampling is to better understand the time sensitivity of hyphae formation and to analyze the culture media at the onset of hyphae formation.

The UltiMate 3000 High-Performance Liquid Chromatography (HPLC) system (Thermo Scientific), equipped with an Agilent Hiplex H column (250 x 4.6 mm), was utilized for the quantification and detection of organic acids and the carbon source. The method employed an isocratic flow rate of 0.2 ml/min for 40 minutes, using 0.1 M sulfuric acid as the mobile phase. Organic acids were measured with a Diode Array Detector (DAD) at 210 nm, and identification was performed based on the retention times of reference compounds. The column temperature was maintained at 50°C. The components of the mobile phase used for HPLC analysis are listed in Appendix C.

3.1.5.5 Optical Density

Optical density (OD) was measured at 600 nm using a spectrophotometer (VWR mySPEC) using cuvettes and the cell culture method. Initially, the culture was diluted 2-fold at the beginning of the experiment. As the cell numbers increased, parallel dilutions were made to ensure measurements fell within the range of the spectrophotometer (~0.1-0.3).

3.1.5.6 Parameters Estimation

All parameters are calculated by first adding the data to Microsoft Excel and plotting the chart.

The biomass specific growth rate (μ) quantifies the rate at which the biomass concentration increases per unit of time during exponential growth. Calculate the growth rate by determining the slope of the ln(biomass) vs. time plot during the exponential growth phase. The slope of the linear portion of the plot represents the growth rate. It is expressed in units of $time^{-1}$ (Stanbury et al., 2003).

Doubling Time (DT) represents the time required for the population to double in size during exponential growth. Determine the doubling time using the formula;

$$DT = \ln(2) / \mu$$

where μ is the biomass specific growth rate obtained from the ln(biomass) vs. time plot.

3.2 RESULTS

3.2.1 Experimental overview

The thesis focuses around *Y. lipolytica*'s ability to utilize different carbon sources to grow, as well as investigating if the utilization can be improved either through rational genetic engineering or ALE. Experiments were conducted both in shake flasks (section 3.2.2) and bioreactors (section 3.2.3). This resulted in the construction of several strains (table 1). The carbon sources investigated were; glucose, glycerol, acetate, and mannitol.

Table 1. Strain overview

Strain ID	Name	Description
ST10000	Wildtype strain	*Y. lipolytica* wildtype strain (equipped with Cas9)
ST10129	ACS expressing strain	*Y. lipolytica* heterologously expressing *ScACS*
ST10119	Mannitol strain #3	*Y. lipolytica* strain evolved for mannitol utilization (transfer #3)
ST10131	Mannitol strain #5	*Y. lipolytica* strain evolved for mannitol utilization (transfer #5)

3.2.2 Flask Experiments

3.2.2.1 Glucose and Glycerol as only Carbon Source

Y. lipolytica exhibits a broad capacity for growth on diverse carbon sources, with glucose being the most abundant carbon source in nature (Workman et al., 2013) and glycerol the preferred carbon source (Erian et al., 2022). To get a benchmark of the performance of the wildtype strain (ST10000, equipped with Cas9) for acetate and mannitol, this strain was first quantitatively tested on glucose and glycerol.

To quantify the growth rate of the wildtype stain (ST10000), precultures of this strain were grown in minimal media containing the respective carbon source and then inoculated into a 250 ml round bottom flask containing 50 ml of minimal medium with 40 g/L glucose or 35 g/L Glycerol as only carbon source (see appendix B). The development of the optical density was monitored over time (Figure 2 A&B).

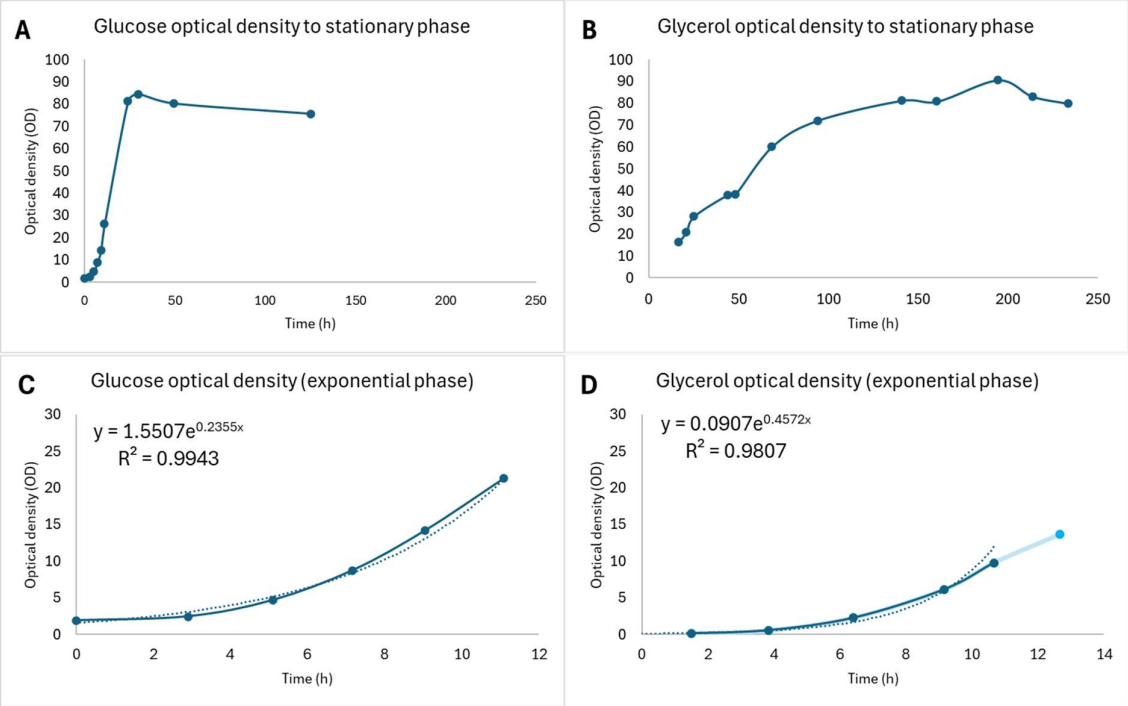

Figure 2. Growth on glucose and glycerol. Wildtype strain (ST10000) grown in minimal media with either 40 g/L glucose (A and C) or 35 g/L glycerol (B and D) as the carbon source. Growth data shown either into stationary phase (A and B) or exponential phase only (C and D). An exponential trend line was fitted to exponential phase growth data with the corresponding formula specified in the plot. Data shown comes from representative culture from duplicate shake flasks.

For determination of the growth rates, the exponential part of the curve of the glucose culture was selected between optical densities of 0-25 (Figure 2 C). The calculated growth rate for the two duplicate cultures over this time was 0.25 ± 0.01 (mean ± standard deviation) h^{-1}. For the cultures grown on glycerol, the growth rate appeared to decrease after an optical density of 10 was reached (Figure 2D) and therefore only the values op to an optical density of 10 were used in determination of the growth rate, which was determined as 0.49 ± 0.03 h^{-1}. The doubling time for glucose was 2.78 h, while for glycerol it was 1.41 h.

3.2.2.2 Acetate as only Carbon Source

Acetate poses toxicity to microbial growth and has a lower energy content compared to traditional carbon substrates such as glucose, sucrose and glycerol. Toxic effects are observed at levels below 5 g/L (Kim et al., 2021), which is significantly lower than the >40 g/L target titer desired for other minimal media formulations (see Appendix B) in the Yarrowia project.

To address this challenge, two distinct strategies were employed in this study. Firstly, the Yeti Foods reference strain was cultivated in minimal media containing varying acetate

concentrations. With this approach, it might be able to select for spontaneous mutants that are able to tolerate and grow on the supplied acetic acid. Secondly, the overexpression of Acetyl-CoA synthetase was pursued to enhance the acetate utilization capability, thereby facilitating microbial growth and metabolism.

For the first approach, the reference *Y. lipolytica* (ST10000, see Appendix A) stored at -80°C was transferred to a petri dish and incubated in an incubation cabinet at 30°C for 24 hours. Following this, it was inoculated with 2.5 ml YPD at 225 rpm and 30°C for an additional 24 hours. A resulting aliquot of 500 microliters was then transferred into 250 ml round-bottom flasks containing 50 ml minimal media supplemented with 2, 5, and 10 g/L acetic acid, with each concentration set up as duplicates. Samples were regularly collected for OD600 measurement, and experiments were terminated upon reaching the stationary phase.

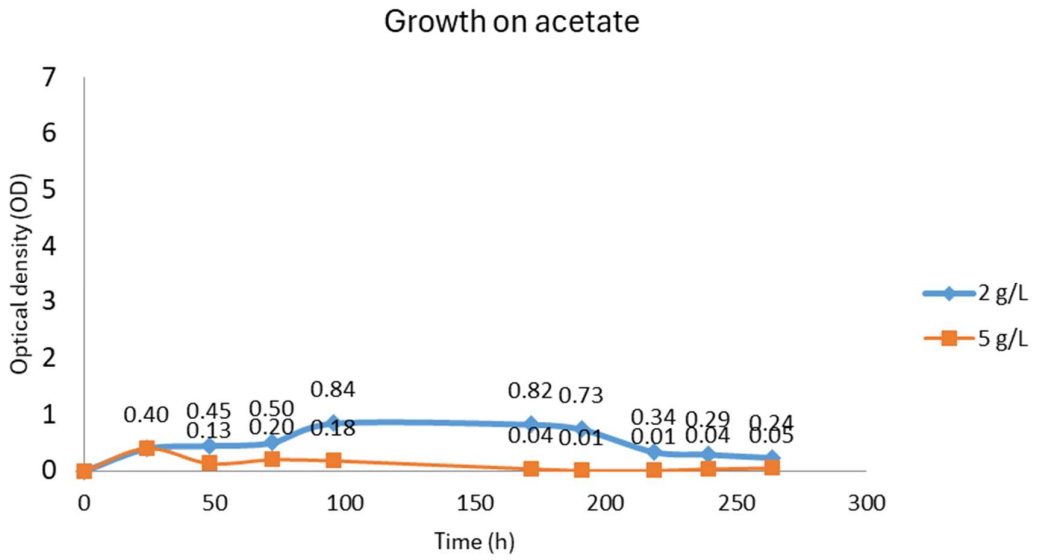

Figure 3. Growth on acetate: The optical densities of Wildtype Strain ST10000 grown in minimal media with 2 g/L and 5 g/L acetate as the carbon source. Data shown comes from representative culture from duplicate shake flasks.

Wildtype Strain ST10000 reached its highest OD value of 0.81 at 171 hours in minimal media with 2 g/L acetate, ST10000, whereas in minimal media with 5 g/L acetate, it reached its highest OD value of 0.39 at 24 hours.

In the experiment, it appeared some growth happened in the cultures containing 2 g/L acetate, indicated by the higher optical density after 100h of growing compared to the 5 g/L acetate cultures. To test if these cultures contained mutants with an improved ability to grow on acetic acid, the experiments were repeated with the the same concentrations of acetic acid. However,

the inoculum was now taken from the flask first experiments' containing 2 g/L acetate at transferred into 250 ml round-bottom flasks containing 50 ml minimal media supplemented with 2, 5, and 10 g/L acetic acid, with each concentration set up as duplicates. The results of these experiments are presented in Figure 4.

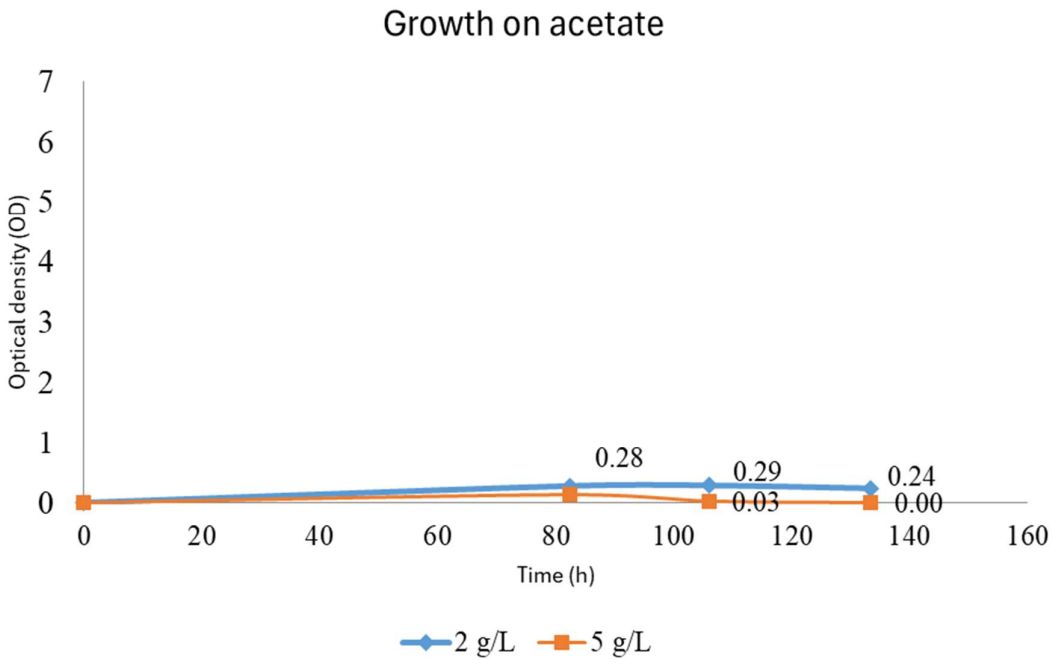

Figure 4. Growth on acetate: Optical densities of Transfer #1 transferred from minimal media with 2 g/L acetate shake flask cultures grown on minimal media with 2 g/L and 5 g/L acetate as the carbon source. Data shown comes from representative culture from duplicate shake flasks.

High acetate concentrations can indeed impede microbial growth and metabolism. A study conducted by Chen et al. demonstrated that the acetate utilization capability of *Y. lipolytica* was improved through successive overexpression of the key enzyme, acetyl-CoA synthetase (ACS) (L. Chen et al., 2021).

Therefore, in the second part of the acetate related experiments, Acetyl-CoA synthase overexpression was introduced into strain ST10000 (Yeti Foods reference *Y. lipolytica*). The resulting strain was named ST10129 and was compared to ST10000 in minimal medium containing 2 and 5 g/L of acetic acid (Figure 5).

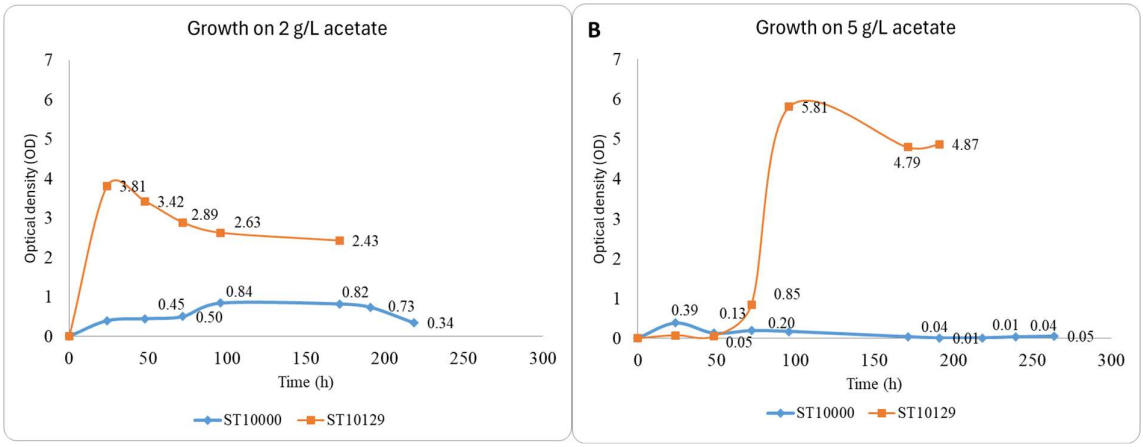

Figure 5. growth on acetate : Optical densities of Wildtype strain (ST10000) and ACS expressing strain (ST10129) in minimal media with 2 g/L and 5g/L acetate.

Compared to ST10000, ST10129 reached higher optical densities, indicating that the overexpression of ACS helped with the ability to grow on acetic acid. Although only few datapoints were collected, the growth rates of ST10129 were estimated to be 0.20 and 0.10 h^{-1} on 2 and 5 g/L respectively, whereas the growth rate of the reference strain was estimated to be <0.02 h^{-1} in both conditions. This corresponds with a doubling time of 3.5 and 7.2 hours for ST10129 in medium containing 2 and 5 g/L of acetic acid and a doubling time of >40h for ST10000 in both conditions.

As a result, the recombinant ACS-expressing strain (ST10129) demonstrated enhanced growth in minimal media containing 2 g/L acetate, reaching a maximum OD of 3.8 (Figure 5A), compared to the maximum OD of 0.84 observed for ST10000.

The experiments with the ST10129 strain were based on the research by Narisetty et al. titled "Development of a Hypertolerant Strain of *Yarrowia lipolytica* Accumulating Succinic Acid Using High Levels of Acetate"(Narisetty et al., 2022). In their study, a different engineered strain, *Y. lipolytica* PSA02004PP-ACS, was utilized with the same acetic acid concentrations (2, 5, and 10 g/L). Data from their study related to the PSA02004PP-ACS strain and the ST10129 data from this study are presented in Figures 6A and 6B.

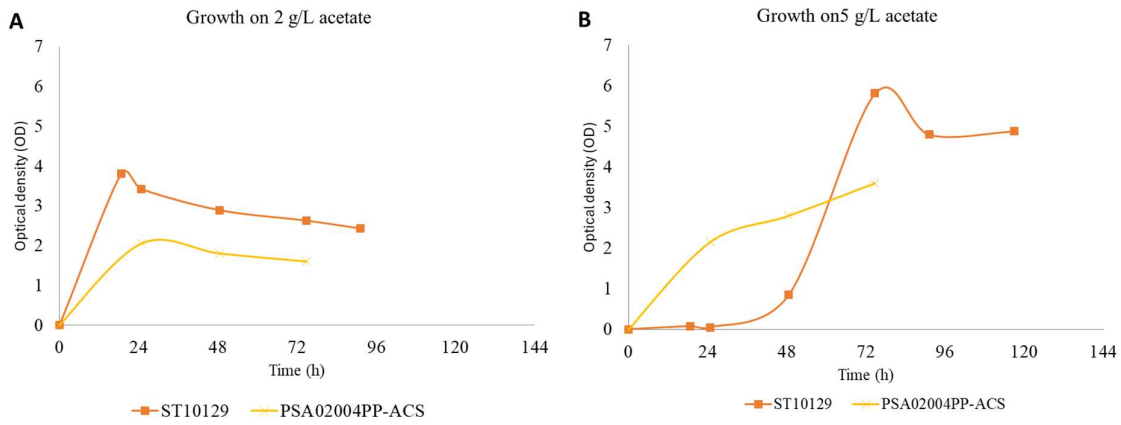

Figure 6. The time-dependent changes in cell growth were compared between experiments using the wild-type strain, an ACS overexpression-derived strain (ST10129), and the strain PSA02004PP-ACS, as utilized by Narisetty et al. The experiments involved using 2 g/L and 5 g/L acetate as the sole carbon sources. Data from experiments using the ST10129 strain represent duplicate flasks in the experiment.

Although the ST10129 strain, obtained through Acetyl-CoA overexpression, exhibited higher cell growth compared to the PSA02004PP-ACS strain used by Narisetty et al. at Acetate concentrations of 2 g/L and 5 g/L in minimal medium.

The strains ST10129 and PSA02004PP-ACS reached their respective maximum OD values of 3.8 and 2.05 at 19 hours and 24 hours when cultured in minimal media containing 2 g/L acetate. When cultured in minimal media containing 5 g/L acetate, ST10129 reached its highest value of 5.8 at 74 hours, while PSA02004PP-ACS strain reached its highest OD value of 3.6 at 72 hours.

3.2.2.3 Mannitol as only Carbon Source

Mannitol is a sugar alcohol found in high abundance in various algae, such as some brown seaweeds (kelps). Using mannitol as a carbon source could offer a path toward moving away from the reliance on land-based agriculture (Xin et al., 2023).

To investigate and improve the ability of the wildtype strain, the wildtype stain (ST10000) to grow on mannitol as the sole carbon source, an ALE method was implemented in this project. To determine the baseline, the wildtype strain (ST10000) was grown in shake flasks with low (9.4 and 18.75 g/L) concentrations of mannitol as the sole carbon source to determine the ability of the strain to grow on mannitol (Figure 7).

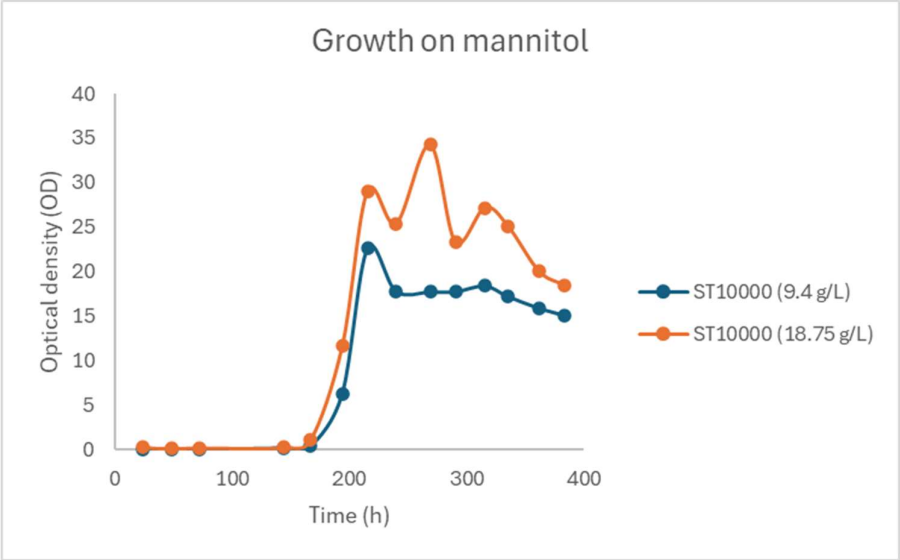

Figure 7. The optical densities of ST10000 strains were measured in two conditions: in minimal media containing 18.75 g/L mannitol and in minimal media containing 9.4 g/L mannitol.

Although no growth was observed for the first 6 days, a steep increase in optical density was observed after 150 hours.

To investigate if this growth performance could be improved, the culture was transferred to subsequent flasks containing increasingly higher mannitol concentrations (Figure 8). With each transfer, the culture was also inoculated into a new flask with the same mannitol concentration (data not shown).

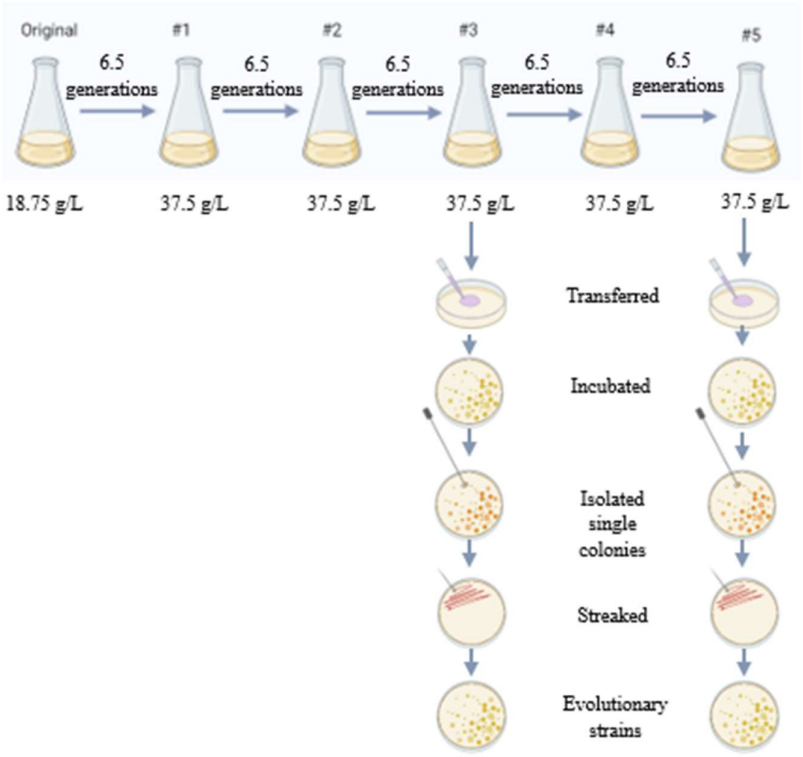

Figure 8. Generating strains using the ALE method. Figure adapted from Chen et al. by recreating it using Biorender's platform (C. Chen et al., 2024). Aliquots of 0.5 mL were taken from the flask originally labeled as "original" and transferred to the next flask. Cultures were inoculated onto agar plates containing mannitol, then incubated. Subsequently, single colonies were isolated, streaked, and grown to obtain the final colony.

The ALE experiment was conducted using 250 ml round-bottom flasks containing minimal medium with 18.75 g/L and 37.5 g/L of mannitol. The experiment began with the wildtype ST10000 inoculated in a flask labeled as "original" containing 18.75 g/L mannitol, from which a 500 microliter aliquot was transferred to the next flask with 37.5 g/L mannitol and minimal medium (transfer #1).

Regular samples were collected during the experiments for optical density measurements, and the results obtained from these samples for each experiment are separately shown in Figure 9. Growth rates calculated from these graphs are also individually presented in Figure 10.

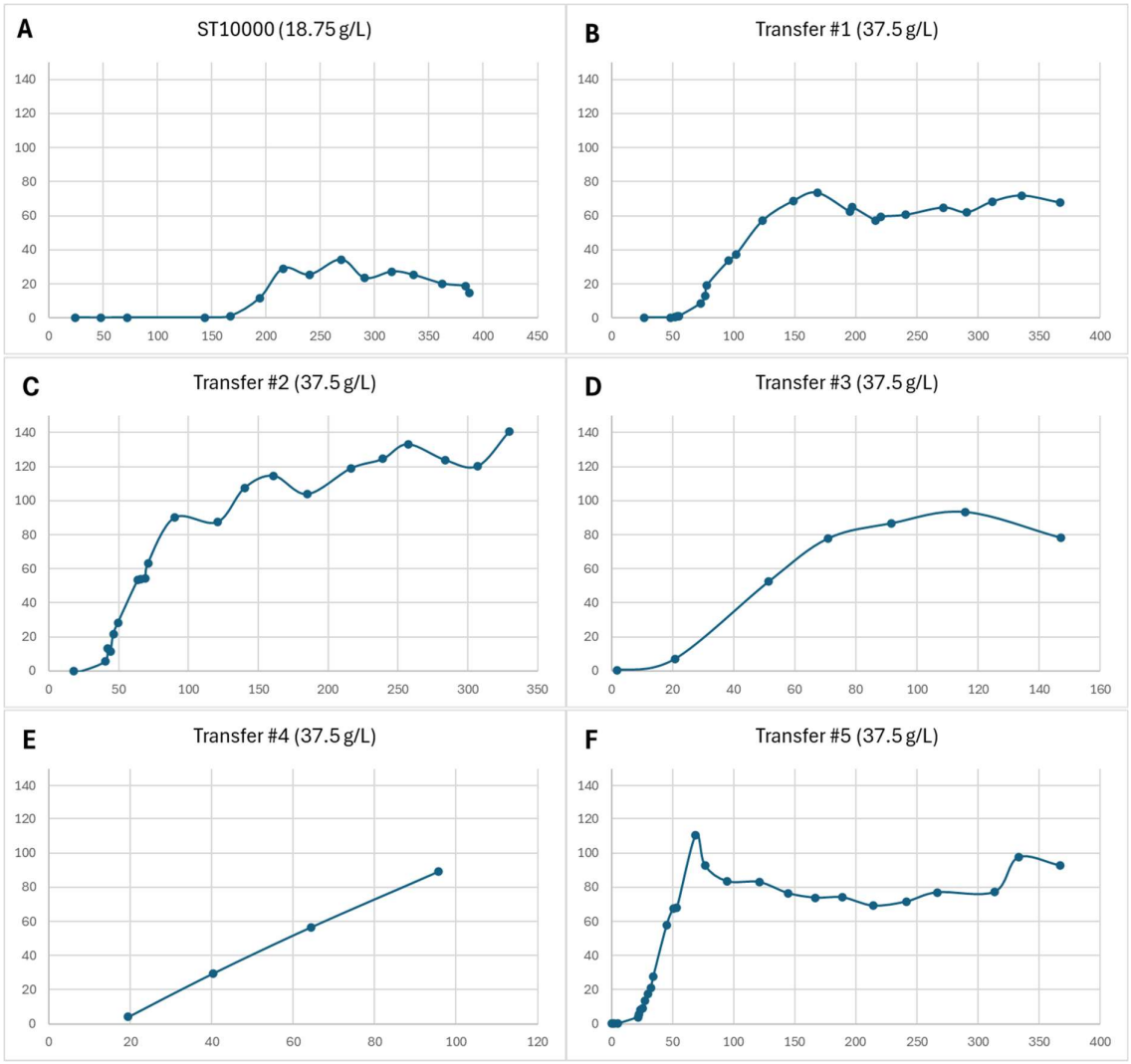

Figure 9. In shaker flask experiments, the profiles of mannitol assimilation and cell growth (OD600) over time were examined. According to the ALE method, initially, ST10000 was cultured in minimal media containing 18.75 g/L mannitol. In the subsequent flask, the mannitol concentration was doubled to 37.5 g/L, and this concentration was maintained in the following flasks, with the experiment concluded at transfer #5.

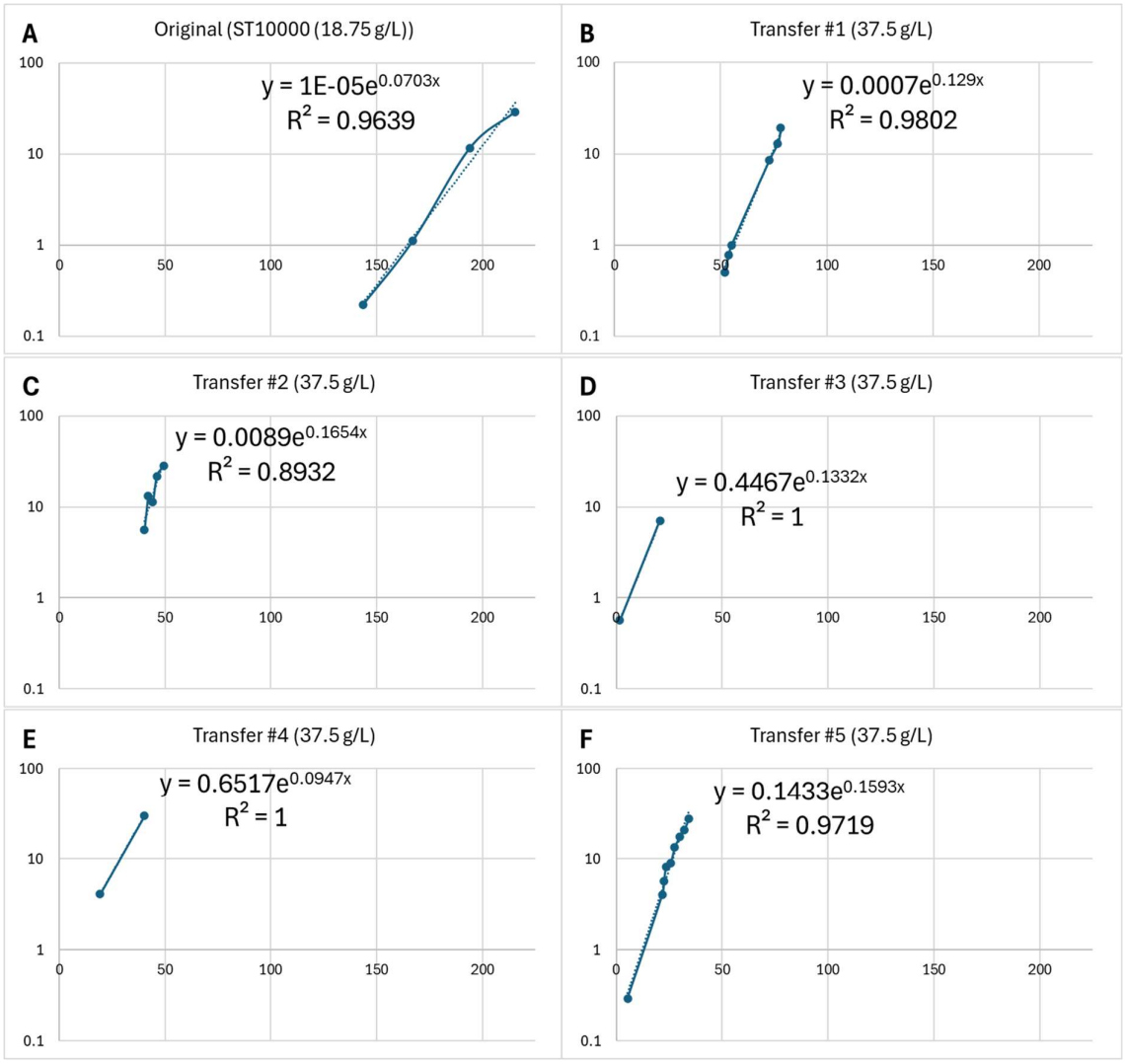

Figure 10. In shaker flask experiments, the profiles of mannitol assimilation and growth rate over time were investigated. Following the ALE method, ST10000 was initially cultured in minimal media containing 18.75 g/L mannitol (Fig 10A). Subsequently, in the successive flask, the mannitol concentration was doubled to 37.5 g/L in flask #1(Fig 10B), and this concentration was consistently maintained in the subsequent flasks until the experiment was terminated at transfer #5 (Fig 10F).

The results of the ALE experiment show changes in growth rate and doubling time across multiple transfers (see Table 2). The original strain (ST10000) exhibited a growth rate of 0.07 h^{-1} with a corresponding doubling time of approximately 9 hours. Subsequent transfers resulted in notable improvements in growth rate, with Transfer #2 showing the highest growth rate of 0.17 h^{-1} and the shortest doubling time of 4 hours.

Table 2. Strain evolution on mannitol

Strain ID	Growth Rate (μ) (h^{-1})	Doubling Time (h)
Original	0.07	9.86
Transfer #1	0.13	5.37
Transfer #2	0.17	4.20
Transfer #3 (ST10119)	0.13	5.20
Transfer #4	0.09	7.32
Transfer #5 (ST10131)	0.16	5.35

Individual cell growths, as shown individually in Figure 9, have been adapted to the same time axis to enable comparison of the evolution of wildtype ST10000 strain on mannitol in the same graph (Figure 11).

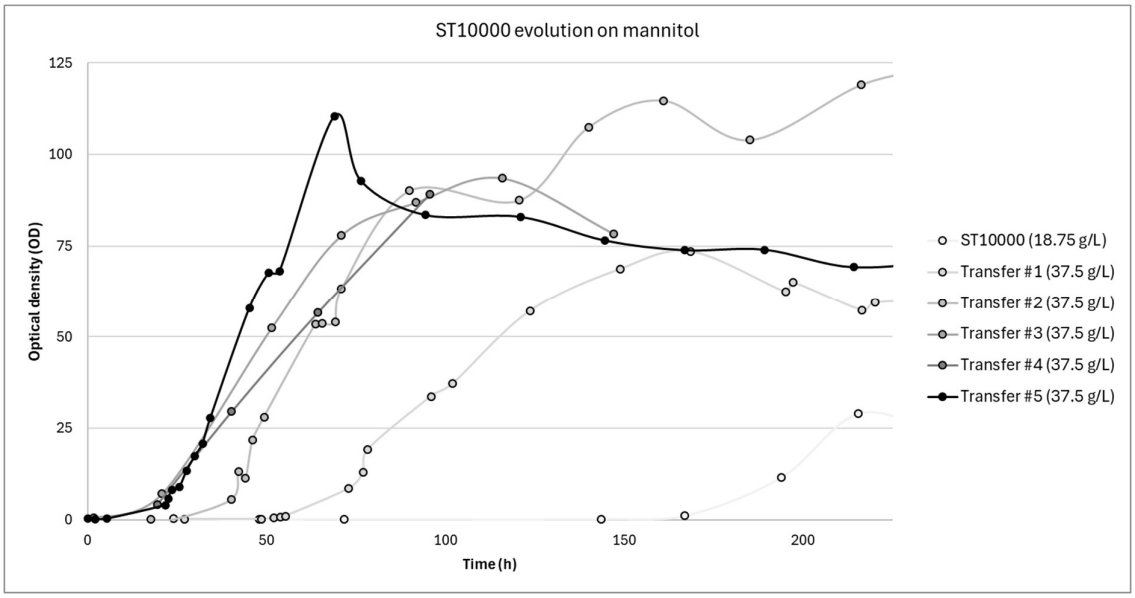

Figure 11: The comparison of strains from the ALE experiment includes ST10000, Transfer #1, Transfer #2, Transfer #3, Transfer #4, and Transfer #5 for optical density (OD) and time. The data represent duplicate flasks for ST10000, Transfer #1, Transfer #2, and Transfer #5.

In the first three transfers, the time before the strain started growing decreased from ~150 hours to within the first 24 hours for transfer #3. Besides the shorter time required for growth initiation, estimations of the growth rate from the wildtype stain (ST10000) in 18.75 g/L mannitol (0.07 h^{-1}) and transfer #5 (0.16 h^{-1}) indicated that the specific growth rate also increased.

The single-cell method (see Figure 8) was employed to prevent potential mutant mixtures in future experiments and to obtain evolutionary strains. Samples from Transfer #3 and Transfer #5 obtained from the ALE experiments were initially transferred onto Mannitol agar plates and incubated. Subsequently, four large single colonies were isolated from each petri dish, aiming to select the best-performing colony. These colonies were then streaked onto fresh petri dishes, and after incubation, four colonies from each strain were inoculated into preculture tubes containing Mannitol and minimal media. An aliquot from the fastest-growing colony was transferred to a cryotube containing glycerol and labeled as ST10119 and ST10131, respectively, and stored at -80°C.

The irreversibility test experiments were conducted on the strains ST10119 (#3) and ST10131 (#5), alongside the reference strain ST10000, to ensure the stability and consistency of the ALE process. Initially, the strains were streaked and plated on agar plates containing Mannitol, followed by incubation. Subsequently, the three strains were precultured with 2.5 mL in minimal media with mannitol as the carbon source. These cultures were then transferred as 0.5 mL aliquots to flasks containing 50 mL of minimal medium containing 37.3 g/L mannitol. The cultures were allowed to grow at 225 RPM and 28°C, with OD600 measurements taken at regular intervals to track growth dynamics (Figure 12).

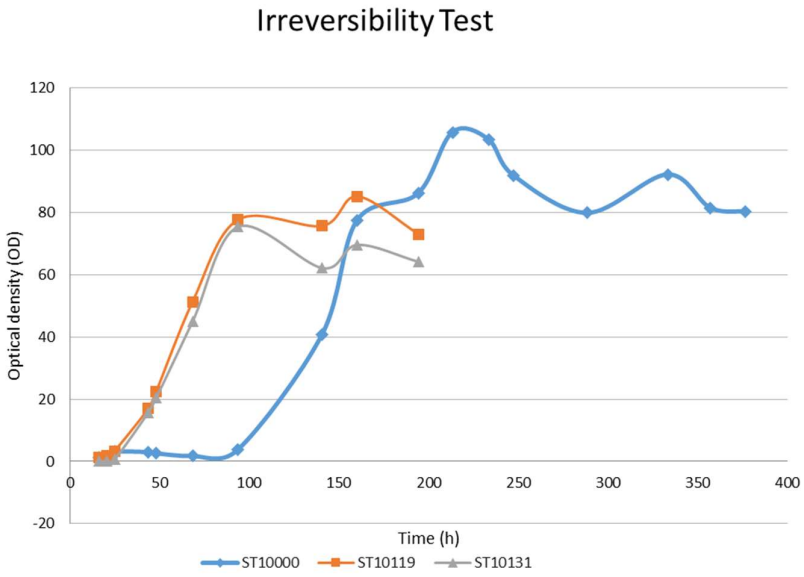

Figure 12 Irriversibility Test. In comparing the optical densities of strains Wildtype strain (ST10000), ST10119, and ST10131. Data shown comes from representative culture from duplicate shake flasks.

During the experiment, it was observed that ST10119 and ST10131 reached their maximum ODs of 85 and 75, respectively, at 160 hours and 94 hours, while ST10000 reached its maximum OD of 103 at 233 hours. Additionally, the ODs of ST10119 and ST10131 increased rapidly from the beginning, whereas ST10000 began to rise approximately 90 hours later.

3.2.3 Bioreactor Experiments

To evaluate the performance of the evolved ST10131 strain on mannitol compared to the wildtype ST10000 strain, fermentation experiments were conducted using mannitol as the sole carbon source for ST10131 and glucose and glycerol as the sole carbon sources for the wildtype.

The decision to employ a fed-batch system stems from the necessity for Yeti Foods to achieve highly efficient protein and biomass production. In such production processes, achieving high titers and productivity is paramount. A fed-batch system offers several advantages in this regard. By providing nutrients gradually throughout the cultivation process, a fed-batch system enables the maintenance of optimal growth conditions and prevents substrate inhibition. This approach facilitates achieving of higher biomass concentrations (titers) and productivity rates compared to traditional batch cultures.

For Yeti Foods to attain success, cost-effectiveness is crucial. By implementing a fed-batch system, the company aims to maximize the yield of desired products while minimizing operational costs. The ability to achieve high biomass concentrations and productivity rates per reactor volume per hour in a fed-batch system aligns with Yeti Foods' objective of optimizing resource utilization and enhancing overall process efficiency. Therefore, evaluating the performance of the evolved strain in a fed-batch setting provides valuable insights into its potential for cost-effective large-scale production, ensuring the competitiveness and sustainability of Yeti Foods in the market.

In order to reduce the time required for adaptation and thus shorten the production time, strains were first pre-grown in shake flasks on the corresponding carbon source before being inoculated into batch cultures containing 500 mL of medium. No liquid samples were taken during the batch phase, but the on-line data was logged to provide insights into culture performance.

For each carbon source related experiment, 2 sets of 1-liter Applikon reactors were utilized. The night before the experiments, the reactors were prepared using a mixture (refer to Appendix B) consisting of $(NH_4)_2SO_4$, Trace Metals, $MgSO_4$, KH_2PO_4, and H_2O, which was then autoclaved for sterilization. For the batch phase, additional components such as Preculture, Thiamin, Carbon source, Antifoam (10x), and H_2O were added. During the fed-batch phase, components including Carbon source, $(NH_4)_2SO_4$, Antifoam, and H_2O were added.

The experiments conducted in the fermentation laboratory using commonly shared bioreactors, which are recorded by Yeti Foods as R1, R2, R3 and so forth, focus on R5 and R6 for glucose, R9 and R10 for glycerol, and R11 and R12 for mannitol.

The on-line data for each experiment was logged using Lucullus Software. This data, which includes individual Dissolved Oxygen (%), stirring speed, and base addition time, was utilized to generate Figure 13, illustrating their variations over time.

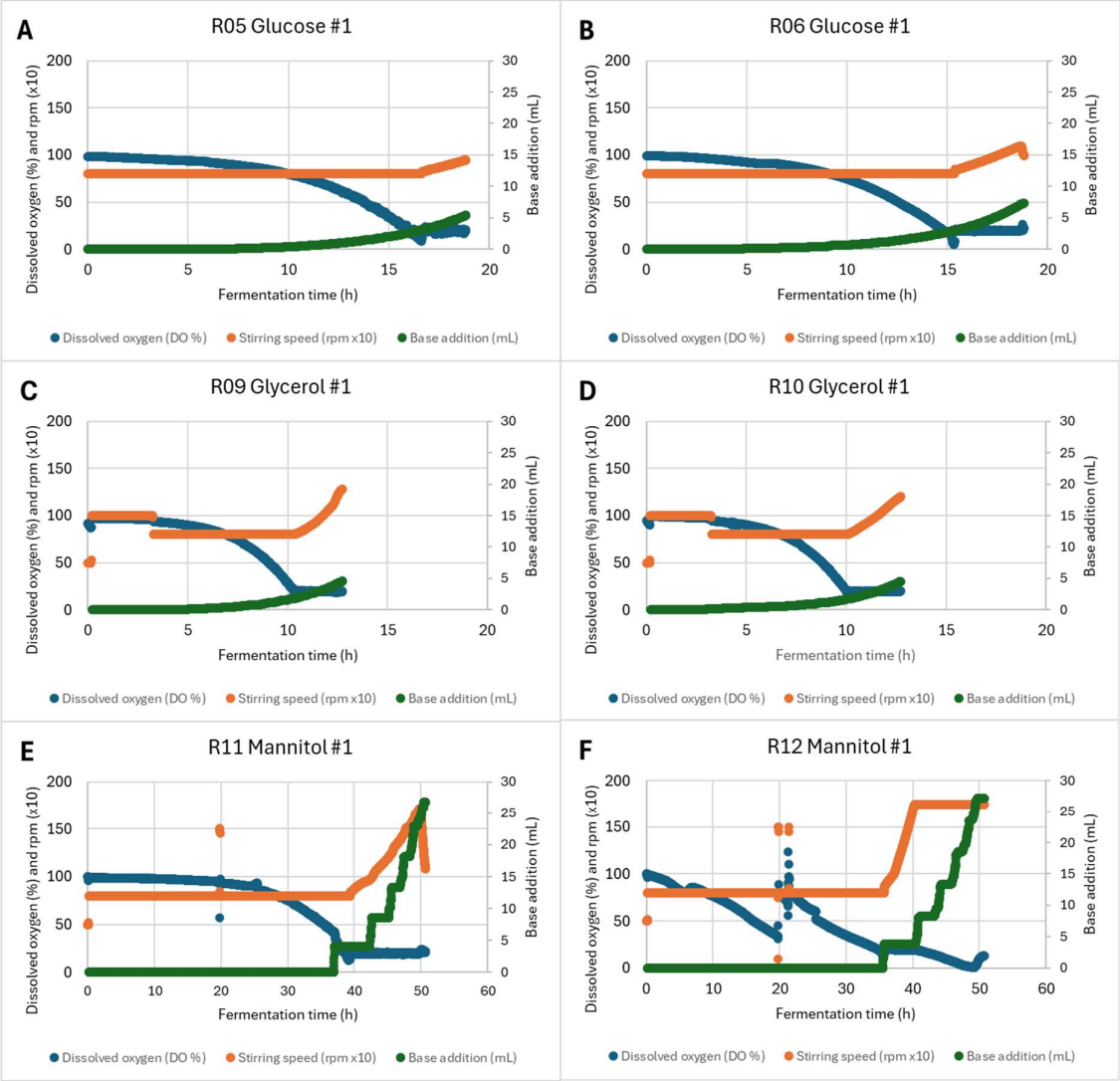

Figure 13. To ensure the reliability of the experimental results, duplicate experiments were conducted using two bioreactors for each condition. Figure A and B display Dissolved Oxygen (DO %), Stirring Speed (rpm x10), and Base Addition (mL) data for R5 and R6, focusing on glucose. Figures C and D represent glycerol experiments, while Figures E and F illustrate mannitol experiments.

From the batch data, it appeared that the evolved culture ST10131 was able to grow on mannitol directly after inoculation, as within the first 3 hours a significant decrease in the dissolved oxygen concentration could already be observed. However, it was still significantly slower than the glucose and glycerol fed cultures, as was evident from the time required to finish the substrate, ~50h for the mannitol culture vs. <20h for glucose and glycerol.

Even though the batch time was substantially longer for the mannitol culture, industrial fed-batch cultures are typically limited by the oxygen transfer rates during the later stages

(Meadows et al., 2016). Therefore, in all three cultures a fed batch with concentrated substrate was started and biomass formation was analyzed using dry weight measurements (Figure 14).

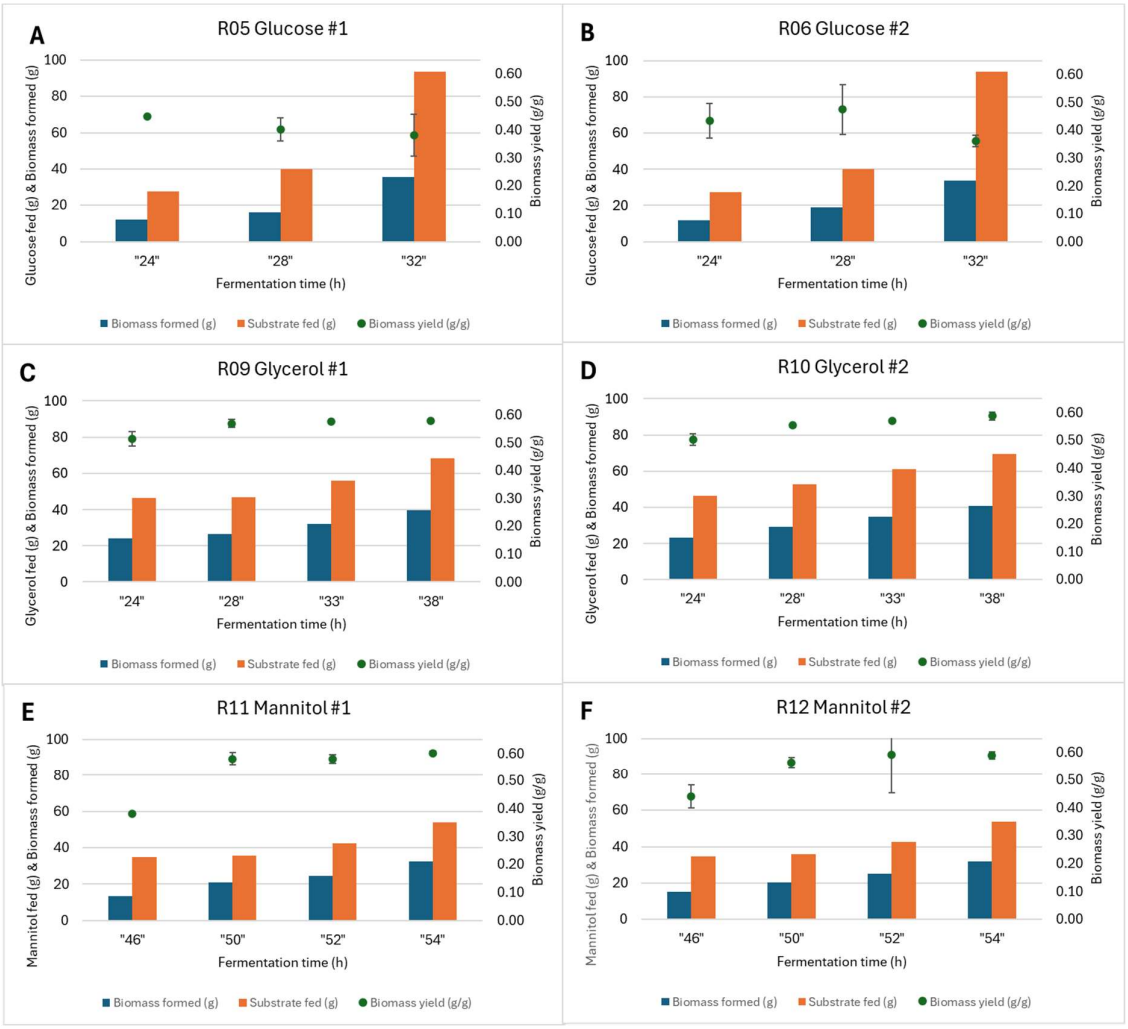

Figure 14. Bioreactor experiments results of biomass formed (g), substrate fed (g), and biomass yield (%) for three different carbon sources: glucose, glycerol, and mannitol. Data shown comes from representative culture from duplicate bioreactors.

Glucose as the carbon source yielded a maximum biomass formation of 35.78 33.85 and g at 32 hours, with a substrate fed of 93.8 g, resulting in a biomass yield of 0.38 g/g and.36 g/g. Glycerol reached its peak biomass formation of 39.62 g and 40.84 g at 38 hours, with a substrate fed of 68.4 g and 69.5 g, resulting in a biomass yield of 0.58 g/g and 0.59 g/g. Mannitol achieved its maximum biomass formation of 32.44 g and 31.79 g at 54 hours, with a substrate fed of 53.9 g, resulting in a biomass yield of 0.60 g/g and 59 g/g.

The results of the sampling completed at the end of the fermentation experiments in bioreactors, where glucose, glycerol, and mannitol were used as the sole carbon sources, are presented in Figure 15. This figure allows for the comparison of biomass formed, substrate fed, and biomass yield, enabling a comprehensive analysis of the performance of each carbon source.

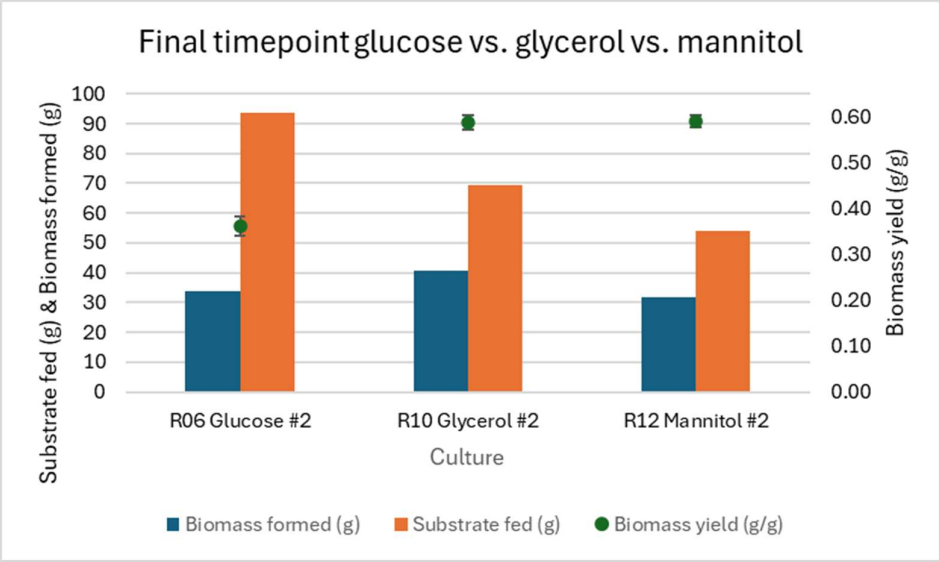

Figure 15. The bioreactor experiments, where glucose, glycerol, and mannitol were used as the sole carbon sources, were compared based on biomass formed (g), substrate fed (g), and biomass yield (%).

In a comparative analysis of biomass formation, substrate fed, and biomass yield among glucose, glycerol, and mannitol, glycerol stands out, producing the highest biomass at 40.84 g, surpassing glucose (33.85 g) and mannitol (31.79 g). Both mannitol and glycerol achieved a 59% yield, whereas glucose yielded 36%. The substrate fed for glucose was 93.8 g, glycerol was 69.5 g, and mannitol was 53.9 g.

3.3 Discussion

The experiments for this research were conducted in a shared laboratory at the BioInnovation Institute in Denmark over a 3-month period, utilizing commonly available devices and accessories. Consequently, restrictions on time and equipment usage were imposed. To ensure consistency, all experiments were performed in duplicate and repetitive which led to increased time, space and equipment requirements.

The initial flask experiments were conducted using Erlenmeyer flasks. *Y. lipolytica* with these flasks were much too foamy to calculate growth rates, optical densities for the first time points were close to 0 as all cells were in the foam (see appendix D). To fix this issue, round-bottom flasks were procured, and all subsequent experiments were restarted using these. While this resolved the foam problem in flask experiments, anti-foam agents were still necessary for bioreactor experiments (see appendix D). Xu et al. offer a potential solution to this issue (S. Xu et al., 2024). In their research, they created foamless variants of *Y. lipolytica*, which could help in future researches prevent volume loss in the bioreactor and eliminate the use of anti-foam agents.

To establish a performance benchmark for the wildtype strain (ST10000, equipped with Cas9) on acetate and mannitol, the strain was initially tested quantitatively on glucose and glycerol. The reasons for selecting these carbon sources are that glucose is the most abundant in nature, and glycerol is the preferred carbon source for *Y. lipolytica*. In the experiment, it was observed that the growth rate on glycerol was higher than on glucose.

The experiments were conducted using acetic acid as the carbon source, with low concentrations due to its toxic and inhibitory effects. Growth was observed at concentrations of 2 g/L and 5 g/L, but not at 10 g/L, prompting a repetition of the experiment. However, this time, the transfer process was conducted from a flask containing low concentration acetic acid (2 g/L) in an attempt to mitigate potential effects from different carbon sources. Nonetheless, no growth was observed at the 10 g/L concentration. This indicates the toxic and inhibitory effects of high acetic acid concentrations on the wildtype strain.

To enhance the utilization of acetic acid, Acetyl-CoA overexpression was performed. This aimed to increase the conversion of acetic acid to Acetyl-CoA in *Y. lipolytica*, allowing it to use acetic acid for necessary metabolic activities and growth. Compared to the wildtype strain,

growth increased at 2 g/L and 5 g/L acetic acid concentrations. However, there was still no growth observed at 10 g/L acetic acid concentration (Figure 5).

The ACS-expressing strain ST10129 was compared with another ACS-expressing strain, PSA02004PP-ACS, used in a previous study by Narisetty et al. Both studies utilized the same acetic acid concentrations. ST10129 strain exhibited higher growth at 2 g/L and 5 g/L acetic acid concentrations compared to PSA02004PP-ACS, but no growth was observed at 10 g/L acetic acid concentration. The probable reason for this difference is the use of different strains. These findings are presented in Figure 6.

The oceans covering 70% of the Earth's surface harbor massive amounts of seaweed. Additionally, one of these seaweed species, brown seaweed, constitutes mannitol up to 30% of its dry weight. Despite its significance, unfortunately, there is a lack of articles on the use of mannitol as a feedstock by *Y. lipolytica* due to the lack of reference articles, experiments were conducted with low mannitol concentrations of 9.4 g/L and 18.75 g/L. As a result, the concentration of 18.75 g/L showed higher growth characteristics (figure 7). Therefore, experiments continued with this concentration.

In the experiments conducted with mannitol concentrations of 9.4 g/L and 18.75 g/L, no significant growth was observed for approximately 6 days, but a sharp increase occurred after 150 hours (Figure 7). This situation is not feasible for fermentation. To improve growth performance, an Adaptive Laboratory Evolution (ALE) study was conducted. The wildtype strain was transferred five times to the next flask, and growth characteristics were examined. According to the results, the mutant strain obtained from the last transfer (transfer #5) exhibited better growth performance compared to the wildtype strain, with a growth rate of 0.16 h^{-1} compared to 0.07 h^{-1} and a doubling time of 5.35 hours compared to 9.86 hours (Table 2). Additionally, the transfer #5 strain showed a shorter time to start growth compared to other strains. Therefore, the ST10131 strain (The results were obtained approximately at the end of 40 generations.), isolated as a single culture, was used in bioreactor experiments where mannitol was used as the only carbon source.

Reversibility test experiments were conducted to ensure the stability and consistency of the Adaptive Laboratory Evolution (ALE) process using mannitol as the sole carbon source. ST10119 and ST10131 strains, along with the wildtype ST10000 strain, were subjected to growth on mannitol again. All strains exhibited growth patterns similar to the results of the ALE experiment. ST10119 and ST10131 strains showed immediate growth, while the wildtype

strain once again entered a much-delayed growth phase. This result confirms the accuracy of the ALE process (Figure 12).

The bioreactor experiments compared the performance of the evolved ST10131 strain on mannitol with the wildtype ST10000 strain, using only glucose and glycerol as carbon sources. As *Y. lipolytica* naturally produces acids, no additional acid was added to the experiments.

According to the batch data obtained from the bioreactor, it was evident that the evolved ST10131 culture exhibited growth on mannitol shortly after inoculation, with a noticeable decrease in dissolved oxygen concentration within the initial 3 hours. Nevertheless, its growth rate remained considerably slower compared to cultures fed with glucose and glycerol, as indicated by the time needed to deplete the substrate (Figure13).

Fed-batch cultures in the industry are typically limited by oxygen transfer rates during the later stages. Consequently, the fed batch in our experiments with concentrated substrate was initiated in all three cultures. Biomass formation was analyzed using dry weight measurements. Based on the results, mannitol demonstrated a slightly lower maximum biomass formation compared to glucose and glycerol. However, the biomass yield of mannitol was comparable to or slightly higher than that of glucose and glycerol, indicating efficient biomass production from mannitol. It is noteworthy that mannitol required a longer fermentation time to reach its peak biomass formation compared to glucose and glycerol, suggesting a slower growth rate or utilization rate of mannitol by the microorganism (see Figure 14).

Based on the data obtained from the bioreactor experiments (Figure 15), glycerol, as the sole carbon source, demonstrated superior performance compared to both glucose and mannitol. It achieved the highest biomass formation. Although mannitol showed a slightly lower maximum biomass formation compared to glucose and glycerol, its biomass yield was comparable to or slightly higher than that of glucose and glycerol, indicating efficient biomass production from mannitol. However, it is noteworthy that mannitol required a longer fermentation time to reach its peak biomass formation compared to glucose and glycerol, suggesting a slower growth rate or utilization rate of mannitol by the microorganism.

Another issue that needs to be addressed is the low solubility of mannitol in water, posing challenges in experimental setups, particularly with the 15% solubility limit. When conducting experiments using a 1-liter volume bioreactor, there is a loss in volume due to the dilution of mannitol.

Future experiments could include;

- In contrast to the traditionally favored slightly acidic conditions (pH 5.6–7) for oleaginous yeast cultivation, Gao et al. demonstrated that alkaline conditions (pH 7–9) could effectively alleviate the lethal effect of high-content acetate on *Y. lipolytica*. Under alkaline conditions (pH 8), the inhibitory effects reduced, allowing for high cell-density and lipid yield. (Gao et al., 2020)
- In Venter et al.'s study, the addition of 10 g/L acetate to a medium containing 30 g/L sunflower oil resulted in a significant increase in citric acid production by engineered *Y. lipolytica*. Further research could evaluate the impact of acetate in media containing different carbon sources and across various *Y. lipolytica* strains. (Venter et al., 2004)
- Novak and Pflügl's research, the use of acetate in combination with other carbon sources can help alleviate the toxicity and inhibitory effects of acetate on microorganisms. Using this approach, it becomes possible to increase the yield of the target product or reduce the formation of undesirable by-products. This research holds promise for future research efforts. (Novak & Pflügl, 2018)
- Acetate exhibits much lower utilization efficiency compared to glucose due to its limited ability to generate NAD(P)H during conversion to acetyl-CoA in *Y. lipolytica*. As cell growth and product synthesis heavily depend on NAD(P)H. Huang et al. demonstrated in their study a microbial electrosynthesis (MES) system that facilitates the direct conversion of inward electrons to NAD(P)H with an engineered strain. For this, they strengthened the conversion efficiency of acetate to acetyl-CoA by heterogeneously expressing ackA-pta genes. This strain achieved a significant increase in fatty alcohol production from acetate through pathway engineering (Huang et al., 2023).
- In addition to mannitol, acetate, glucose, and glycerol it would be interesting to test the strains made via ALE in this study for a potential improved ability to grow on the polyols such as sorbitol, xylitol, erythritol, sucrose, fructose, lactose, maltose, and ethanol.

Due to time constraints the HPLC results and pH measurements detailed in Appendix C could not be thoroughly researched. Nevertheless, they are expected to contribute to further experiments supporting the understanding of pH and acid content-related morphology. This is

particularly significant for *Y. lipolytica*, a yeast known for its capability to transition between yeast cells and hyphae in response to environmental stressors (Nicaud, 2012) .

As research on *Y. lipolytica* and similar species advance, our understanding of alternative meat options will expand. Concepts such as separating food production from land-based agriculture, ensuring food security, addressing food production during catastrophic events such as super volcano eruptions, meteor strikes or nuclear winters, or investigating food production on other planets are not currently in our focus. However, since they may become important issues in the future, they need to be thought about and studied now.

The research titled, "What food will we be eating on our journey to Mars?" discussing the potential of mycoprotein as astronaut food during space travel was authored by Lewandowski and Stryjska. Mycoprotein eliminates the need for transporting farm animals but requires cultivation in a water-based medium and heating to remove excess RNA. However, advancements in 3D printing methods may eliminate the need for a separate heating step, making mycoproteins a promising candidate for future astronaut menus. (Lewandowski & Stryjska, 2022).

4 SUMMARY

The increasing population and the consequent rise in animal-based meat consumption are expected. However, the potential environmental damages from increased animal production and the negative impacts on human health due to consumption have been examined. Since animal production is subject to external factors such as weather conditions, storms, floods, droughts, and zootechnical diseases, alternative solutions have been investigated. These alternatives include plant-based meat, cultured meat, algae, edible insects, and mycoprotein.

Yarrowia lipolytica is selected for its well-studied nature, being the second most extensively researched yeast strain after *Saccharomyces cerevisiae*. Its well-characterized genetics, metabolic pathways, and industrial bioprocessing capabilities make it a valuable model organism for studying various aspects of cellular biology and biotechnology.

Generally, traditional carbon sources, glucose or glycerol, and alternative sources such as mannitol obtainable from brown algae and acetate, a byproduct of industrial production, have been examined in detail.

All experiments were conducted in flasks and bioreactors to assess the performance and scalability of the microbial fermentation process under different conditions and volumes.

Due to the toxicity of acetate in cell growth, metabolic engineering has been performed by *Y. lipolytica* to convert acetate into acetyl-CoA. Although the engineered strain performed better on acetate at lower concentrations compared to the wildtype strain (ST10000) and previous studies, no significant results were obtained at higher concentrations. Therefore, it is unsuitable for fermentation applications as a sole carbon source.

Due to its limited applications and usage, mannitol is used in fields such as pharmaceuticals, chewing gum, candies, and some psychological disorders. Although there have been studies focused on its production, there has been no research that we can compare or reference in our experiments regarding its use as a carbon source.

Mannitol has been explored for fermentation processes aimed at minimizing batch time through adaptive laboratory evolution, with the goal of rapidly increasing cell quantity in our experiments. While the improved strain didn't exhibit the same rapid growth as a glucose-grown culture, a high yield was achieved in a fed-batch culture using mannitol. This serves as

an initial proof-of-concept for utilizing this carbon source as a feedstock for Yeti Foods products.

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7 APPENDIX

A Primers, Biobricks, Plasmids, Strains and Genes

Figure S1: Primers

Primer ID	Sequence:	Description
PR-10058	GGCTAAACCCTATTCTCTGGCGGG	Sequence TU2 plasmids
PR-10059	TACGAACCGCCTACCGACCAAAT	Sequence TU2 plasmids
PR-00008	CTACTCTGGCGTCGATGAGGGAggcggcaaccgagcgttctg	Amplification of BB00011
PR-00009	CGGTTGTAGTGAGGGCGGATTGagaatcaggggataacgcaggaaagaac	Amplification of BB00011
PR-10050	CAAGCTGGTCTCgGGCATTAGTTGCGTAAGCGTTGCACGT	Construct new integration backbone
PR-10051	GAGGATGGTCTCgACTAcccaagaatgcatctgagtggcct	Construct new integration backbone

Figure S2: Biobrics

Biobrics ID	Primer 1	Primer 2
BB00078 (ScACS1-A Acetyl-CoA synthase, part A)	-	-
BB00079 (ScACS1-B Acetyl-CoA synthase, part B)	-	-
BB10013 (TU1_empty fragment)		
BB00011 (Twist compatible Backbone)	PR-00008	PR-00009
BB10007 (IntD1_BsaI_Backbone)	PR-10050	PR-10051

Figure S3: Plasmids

Name	Description	Biobricks	Backbone	Source
pYu10008	Linear gRNA for integration of gene 1 with Hygromycin resistance	-	-	EasyClone Yali
pYu10045	Transcriptional unit for GFP expression	-	-	Yeti Foods
pYu11190	Transcriptional unit for ScACS1 overexpression	BB00078 (ScACS1-A Acetyl-CoA synthase, part A), BB00079 (ScACS1-B Acetyl-CoA synthase, part B)	pYu10045 (TU2_GFP_BBaJ23111)	This work
pYu11364	Integrative vector for ScACS1	pYu11190 (TU2_ScACS1), pYu10032 (TU1_empty fragment)	BB10007 (IntD1_BsaI_Backbone)	This work
pYu10032	TU1_empty fragment	BB10013 (TU1_empty fragment)	BB00011 (Twist compatible Backbone)	This work

Figure S4: Strains

Strain ID	Parent Strain	Repair Template	Plasmid gRNA
ST10000 (Yali Cas9) -Wild Type Strain	-	-	-
ST10119	T10000 (Yali Cas9) with-Mannitol ALE #3	-	
ST10131	ST10000 (Yali Cas9) withp Mannitol ALE #5		
ST10129 (IntD1::ScACS1) wild type ACS expressing strain	ST10000 (Yali Cas9)	pYu11364 (IntD1_TU2_ScACS 1)	pYu10008 (YLgRNA_IntD_1_Hph)

Figure S5: Genes

Name	Nucleotide sequence
ScACS1	ATGTCTCCCTCTGCCGTCCAGTCTTCTAAGCTCGAGGAGCAGTCTTCTGAGATCGACAAGCTCA AGGCTAAGATGTCTCAGTCTGCCGCTACTGCCCAGCAGAAGAAGGAGCACGAGTACGAGCACC TCACCTCTGTTAAGATTGTCCCCCAGCGACCCATCTCTGATCGACTGCAGCCCGCTATCGCCACC CACTACTCCCCCCACCTTGACGGTCTGCAGGACTACCAGCGACTGCACAAGGAGTCCATTGAGG ACCCCGCCAAGTTCTTCGGTTCTAAGGCCACCCAGTTTCTCAACTGGTCCAAGCCCTTCGACAA GGTTTTCATCCCCGACCCCAAGACCGGACGACCCTCTTTCCAGAACAACGCCTGGTTCCTTAAC GGTCAGCTGAACGCTTGCTACAACTGTGTCGATCGACACGCTCTGAAGACCCCCAACAAGAAG GCCATCATCTTCGAGGGTGACGAGCCCGGTCAGGGTTACTCCATTACCTACAAGGAGCTTCTCG AGGAGGTCTGCCAGGTCGCTCAGGTCCTCACTTACTCTATGGGTGTCCGAAAGGGTGACACTGT TGCTGTCTACATGCCCATGGTGCCCGAGGCTATTATTACCCTGCTCGCTATCTCCCGAATTGGTGC CATCCACTCCGTCGTCTTTGCCGGTTTTTCTTCCAACTCCCTCCGAGATCGAATCAACGACGGAG ACTCCAAGGTTGTTATCACCACCGATGAGTCCAACCGAGGTGGTAAGGTCATTGAAACTAAGCG AATTGTCGACGACGCTCTCCGAGAGACTCCCGGAGTCCGACATGTGCTTGTCTACCGAAAGACT AACAACCCTTCTGTCGCTTTCCACGCCCCCCGAGATCTTGACTGGGCTACTGAGAAGAAGAAGT ACAAGACCTACTACCCCTGTACCCCCGTCGACTCTGAAGATCCTCTGTTCCTCCTCTACACCTCC GGCTCTACCGGAGCTCCCAAGGGTGTCCAGCACTCTACCGCCGGTTACCTCCTTGGCGCTCTGC TCACCATGCGATACACCTTCGACACTCACCAGGAGGATGTCTTCTTCACTGCTGGTGATATCGGT TGGATTACCGGACATACCTACGTCGTTTACGGACCTCTCCTTTACGGTTGCGCTACCCTGGTTTTC GAGGGTACTCCCGCCTACCCTAACTACTCCCGATACTGGGACATCATTGATGAGCATAAGGTTAC CCAGTTCTACGTCGCCCCCACCGCCCTGCGACTCCTGAAGCGAGCCGGTGACTCCTACATTGAG AACCATTCTCTGAAGTCCCTCCGATGTCTGGGTTCCGTCGGCGAGCCCATCGCCGCCGAGGTTTG GGAGTGGTACTCTGAGAAGATCGGTAAGAACGAGATCCCCATCGTTGACACCTACTGGCAGACC GAGTCTGGCTCTCACCTCGTCACCCCCCTCGCCGGCGGTGTCACCCCTATGAAGCCCGGTTCCG CCTCTTTCCCCTTTTTCGGTATTGACGCTGTTGTCCTCGACCCTAACACCGGTGAGGAGCTGAAC ACTTCTCATGCCGAGGGTGTTCTCGCTGTTAAGGCTGCCTGGCCCTCCTTTGCCCGAACCATTTG GAAGAACCACGACCGATACCTCGACACCTACCTTAACCCCTACCCCGGCTACTACTTCACCGGT GACGGAGCCGCTAAGGACAAGGACGGTTACATTTGGATCCTTGGCCGAGTTGATGACGTTGTTA ACGTCTCCGGTCACCGACTCTCCACCGCCGAGATCGAGGCTGCCATCATTGAGGATCCCATTGTC GCTGAGTGCGCCGTTGTCGGTTTCAACGATGATCTCACCGGCCAGGCTGTGGCCGCTTTCGTGG TCCTCAAGAACAAGTCCTCTTGGTCCACCGCCACTGACGACGAGCTGCAGGACATCAAGAAGC ACCTGGTTTTTACCGTCCGAAAGGATATCGGCCCTTTCGCCGCTCCCAAGCTTATTATCCTCGTTG ACGACCTGCCTAAGACCCGATCTGGAAAGATTATGCGACGAATCCTTCGAAAGATCCTCGCCGG AGAGTCCGATCAGCTCGGTGACGTCTCTACCCTGTCTAACCCCGGCATCGTCCGACATCTCATCG ATTCCGTCAAGCTCTAA

B Media Recipes

YPD: 1% Yeast Extract, 2% Peptone, 2% Glucose

500X Trace metals: 4.5 g/L CaCl2·7H2O, 4.5 g/L ZnSO4 ·7H2O, 4 g/L FeSO4·7H2O, 1 g/L H3BO3, 1 g/L MnCl2·4H2O, 0.4 g/L Na2MoO4·2H2O, 0.3 g/L CoCl2·6H2O, 0.1 g/L CuCl2·5H2O, 0.1 g/L KI, 15 g/L EDTA

2xYT: 16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl

Figure S6: Composition of the media used in Flask experiments

Media	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	
	Minimal medium, Glucose (40 g/L)	Minimal medium, Glycerol (35 g/L)	Minimal medium, Acetate (2 g/L)	Minimal medium, Acetate (5 g/L)	Minimal medium, Acetate (10 g/L)	Minimal medium, Mannitol (9.3 g/L)	Minimal medium, Mannitol (18.6 g/L)	Minimal medium, Mannitol (37.3 g/L)	Minimal medium, Mannitol (74.6 g/L)	
Carbon source volume	40	35	2	5	10	30	60	120	240	ml
(NH4)2SO4	17	17	17	17	17	17	17	17	17	ml
Thiamin	1	1	1	1	1	1	1	1	1	ml
Trace metals	2	2	2	2	2	2	2	2	2	ml
MgSO4	1	1	1	1	1	1	1	1	1	ml
KH2PO4	50	50	50	50	50	50	50	50	50	ml
H2O	389	394	427	424	419	399	369	309	189	ml

Figure S7: Composition of the media used in Bioreactor experiments

	Reactor mixture (stock solutions)			To add (stock solutions)			Feed (stock solutions)		
Carbon Source	**Compound**	**Stock (g/L)**	**Volumes (mL)**	**Compound**	**Stock (g/L)**	**Volumes (mL)**	**Compound**	**Stock (g/L)**	**Volumes (mL)**
Glycerol	$(NH_4)_2SO_4$	300	10	Thiamin	1000	11	Glycerol	800	136.75
	Trace metals	500	21	Glycerol	500	17.5	$(NH_4)_2SO_4$	300	62.5
	$MgSO_4$	241	16.5	Preculture	_	5	Antifoam (10x)	_	5
	KH_2PO_4	120	67	Antifoam (10x)	_	5	H_2O	_	53.55
	H_2O	_	285.5	H_2O	_	61.5			
Mannitol	$(NH_4)_2SO_4$	300	10	Thiamin	1000	11	Mannitol	155.3	245
	Trace metals	500	21	Mannitol	155.3	60	Antifoam (10x)	_	5
	MgSO4	241	16.5	Preculture	_	5			
	KH_2PO_4	120	67	Antifoam (10x)	_	5			
	H_2O	_	285.5	H_2O	_	19			
Glucose	$(NH_4)_2SO_4$	300	10	Thiamine	1	11	Glucose	500	245
	Trace metals	500	21	Glucose	500	20	Antifoam (10x)	_	5
	$MgSO_4$	241	16.5	Preculture	_	5			
	KH_2PO_4	120	67	Antifoam (5x)	_	2.5			
	H_2O	_	285.5	H_2O	_	61.5			

C Experiment #5 Outcomes

HPLC: The mobile phase consisted of Oxoglutaric Acid (2.00 mg/2ml), Acetic Acid (2.63 mg/2ml), Malic Acid (2.44 mg/2ml), Succinic Acid (2.95 mg/2ml), Fumaric Acid (2.97 mg/2ml), and Citric Acid (2.10 mg/2ml).

Figure S8: HPLC results belongs to experiment #5

		Response					Concnetration mg/L				
Sample Nr	Sampling Time (h)	Citric acid	Ketoglutaric acid	Succinic acid	Malic acid	Fumaric acid	Citric acid	Ketoglutaric acid	Succinic acid	Malic acid	Fumaric acid
1	0	20	367	0.1	0.6	0.6	42.42	177	<LOQ	<LOQ	<LOQ
2	2	4	286	1	0.7	0.2	<LOQ	138	<LOQ	<LOQ	<LOQ
3	5	4	271	0.5	0.5	0.4	<LOQ	131	<LOQ	<LOQ	<LOQ
4	22	4	258	8	1	1	<LOQ	124	38	<LOQ	<LOQ
5	24	5	232	12	1	1	<LOQ	112	55	<LOQ	<LOQ
6	26	6	259	17	0.6	0.6	<LOQ	125	76	<LOQ	<LOQ
7	28	4	241	22	1	1	<LOQ	116	100	<LOQ	<LOQ
8	30	4	250	32	2	2	<LOQ	121	142	<LOQ	<LOQ
9	32	93	215	42	0.1	2	192	104	187	<LOQ	<LOQ
10	34	68	198	26	0.3	0.3	140	95	116	<LOQ	<LOQ
11	45	3	134	1	0.3	0.3	<LOQ	64	<LOQ	<LOQ	<LOQ
12	51	68	104	1	2.5	3	140	50.61	<LOQ	<LOQ	<LOQ
13	69	8	25	2	6.5	6.	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
14	77	6	1	6	1	0.8	<LOQ	<LOQ	28	<LOQ	<LOQ
15	95	63	11	15	1	1	130	<LOQ	68	<LOQ	<LOQ
16	121	65	0.7	2	3.	3	134	<LOQ	<LOQ	<LOQ	<LOQ
17	145	61	0.8	24	3	3	126	<LOQ	109	<LOQ	<LOQ
18	167	74	1	18	4	4	153	<LOQ	80	<LOQ	<LOQ
19	189	81	0.9	12	7	3	168	<LOQ	55	<LOQ	<LOQ
20	214	98	0.4	25	6	6	203	<LOQ	111	<LOQ	<LOQ
21	333	3	0.9	0.6	5	5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Figure S9: Microscopy images belong to experiment #5

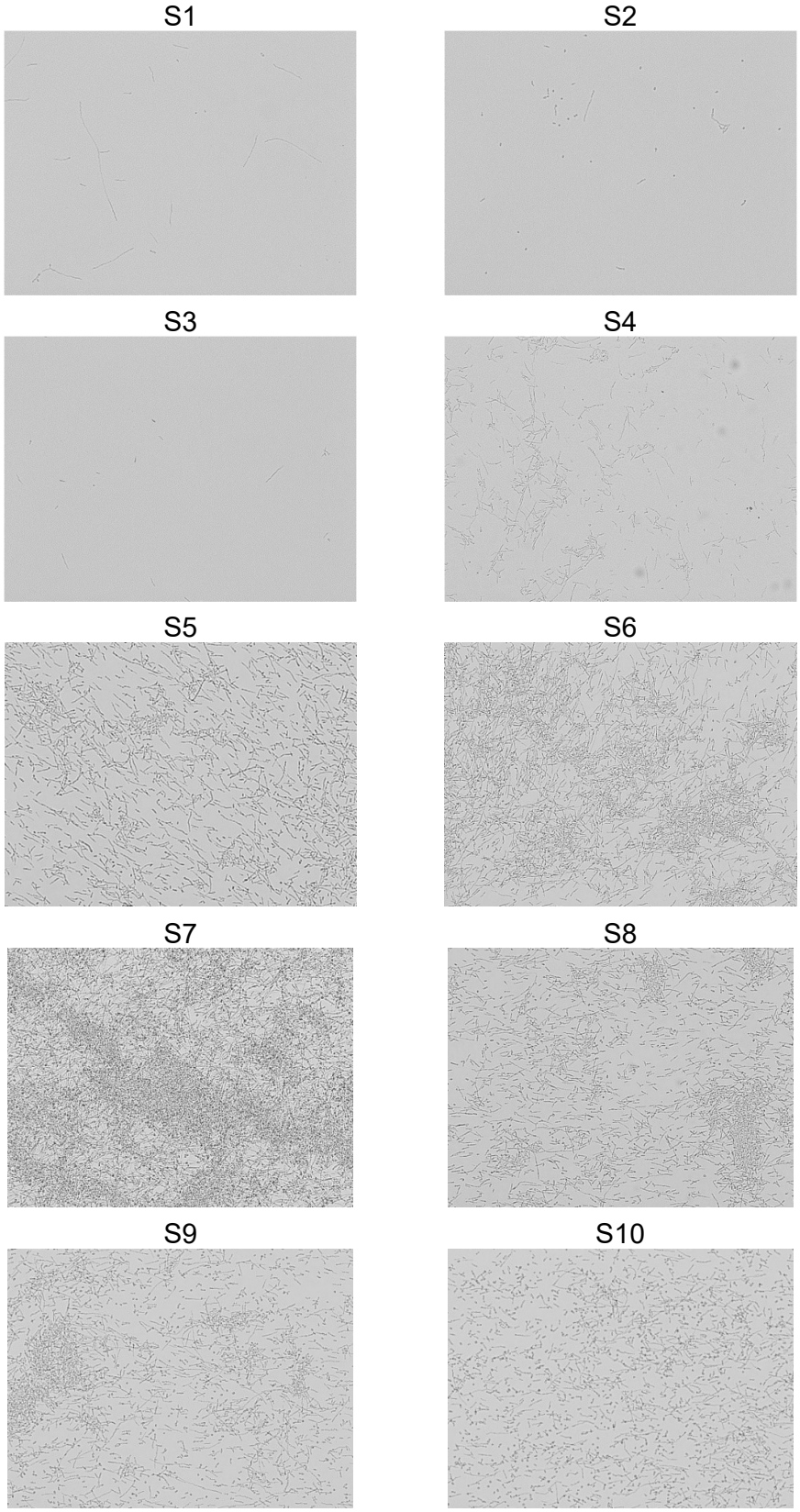


Figure S9: Microscopy images belong to experiment #5 (continue)

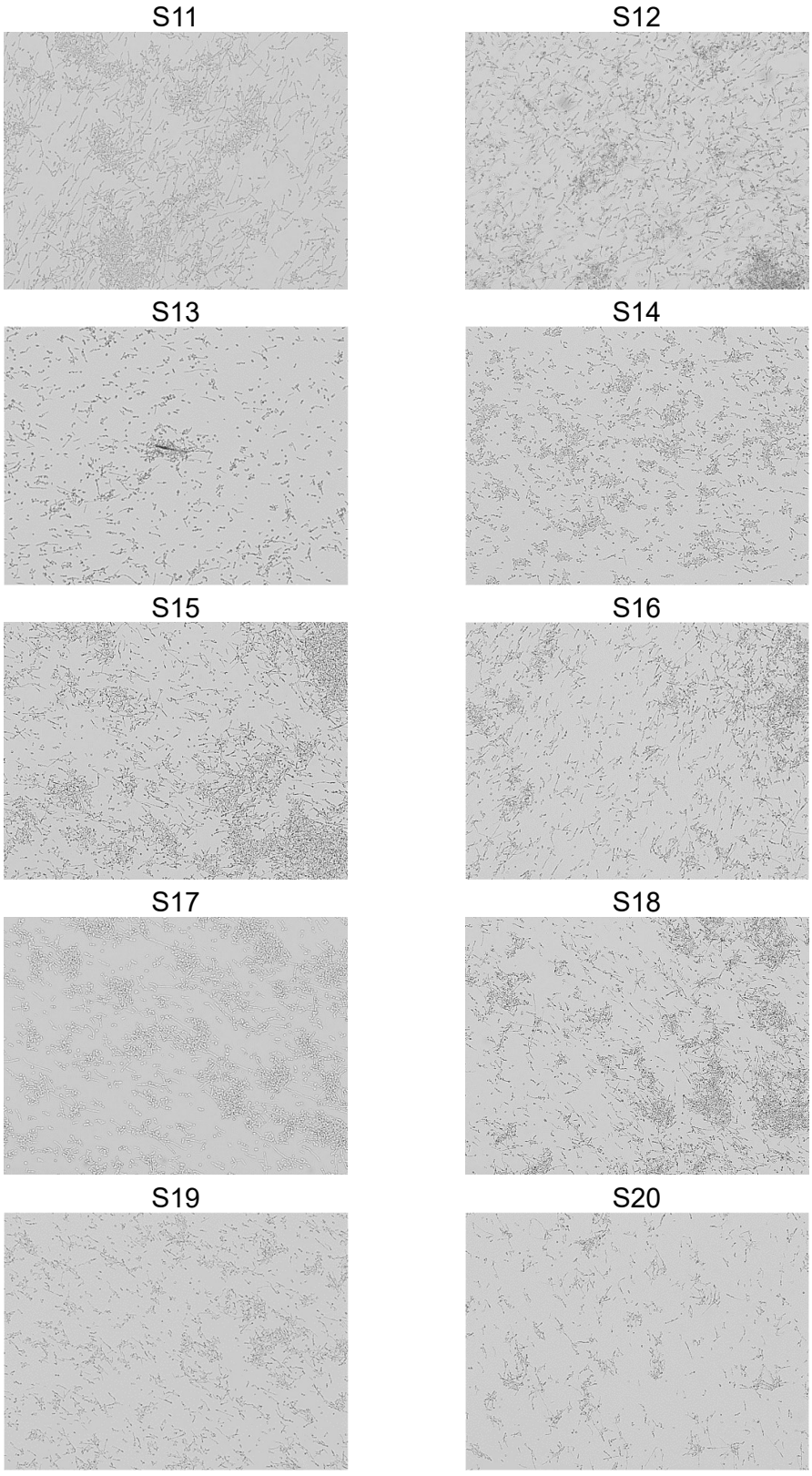


Figure S9: Microscopy images belong to experiment #5 (continue)

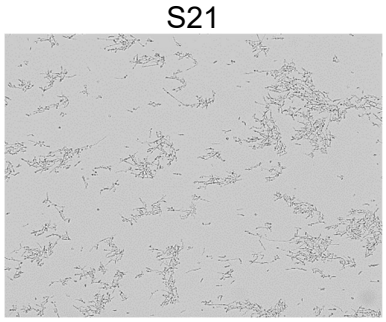
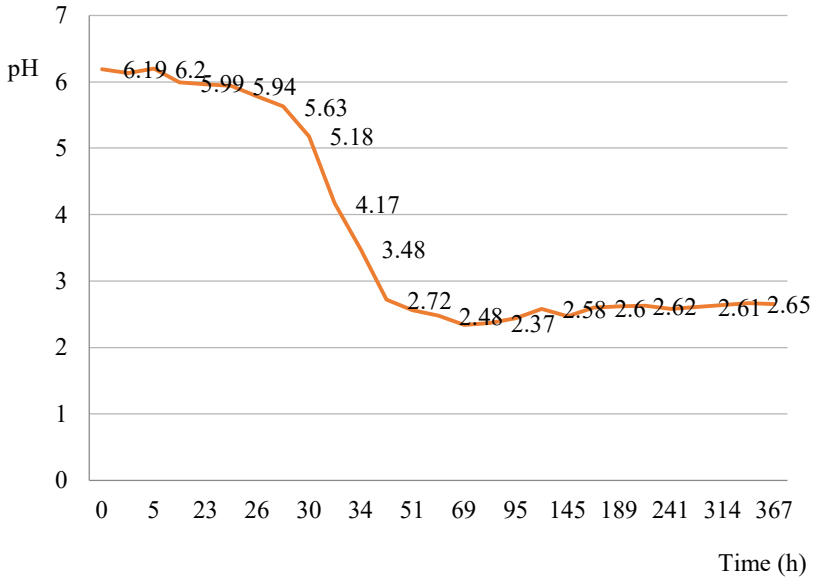


Figure S10: pH results images belong to experiment #5



D Foaming

Figure S11: Foaming Problem (in the Erlenmeyer Flask)

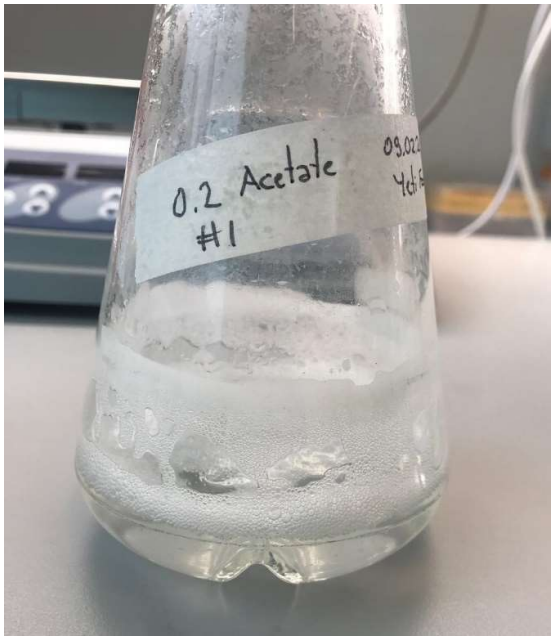


Figure S12: Foaming Problem (in the Bioreactor)

E Optical Density Data of The Experiments

Figure S13: Glucose shake flask data

Hours	Glucose replicate 1	Glucose replicate 2
0	1.67	1.88
3	2.34	2.44
5	4.69	4.67
7	9.04	8.74
9	14.28	14.20
11	26.20	21.30
24	81.20	77.40
30	84.40	85.60
50	80.20	79.40
125	75.60	72.60

*Values in yellow were assumed to be in the exponential phase and used for growth rate calculation

Figure S14: Glycerol shake flask data experiment 1 (exponential)

Hours	Glycerol replicate 1	Glycerol replicate 2
0		
2	0.15	0.08
4	0.57	0.58
6	2.28	2.37
9	6.16	6.37
11	9.78	10.04
13	13.65	14.65

Figure S15: Glycerol shake flask data experiment 2 (stationary)

Hours	Glycerol replicate 1
0	
17	16.45
21	20.99
25	28.20
44	38.05
48	38.40
68	60.15
94	72.10
141	81.25
160	80.80
194	90.60
214	83.10
233	79.85
260	77.45
289	83.50
333	81.75
357	83.25
377	88.85
402	85.05

Figure S16: Mannitol shake flask data evolution

Hours	ST10000 (9.4 g/L)	ST10000 (18.75 g/L)	Hours	Transfer #1 (18.75 g/L)	Transfer #1 (37.5 g/L)	Hours	Transfer #2 (37.5 g/L)	Transfer #2 (37.5 g/L)
0			0			0		
24	0.00	0.23	27	-0.04	0.14	18	0.05	0.05
48	0.04	0.12	49	0.39	0.19	23	0.02	-0.04
72	0.02	0.19	52	0.86	0.50	40	15.35	5.58
143	0.12	0.22	54	1.24	0.79	42	22.08	13.13
167	0.37	1.11	55	1.69	1.00	44	15.65	11.35
194	6.24	11.61	73	11.75	8.56	46	26.75	21.80
215	22.60	29.02	77	18.50	12.98	49	31.00	28.05
240	17.79	25.36	78	21.25	19.20	64	66.05	53.55
269	17.73	34.25	96	38.94	33.69	65	60.35	53.75
291	17.75	23.33	102	40.60	37.13	69	66.05	54.15
316	18.40	27.18	124	30.30	57.15	71	77.85	63.20
336	17.23	25.13	149	32.25	68.75	90	102.80	90.05
362	15.90	20.05	169	23.65	73.50	121	90.85	87.45
383	15.10	18.50	195	21.65	62.45	140	82.60	107.45
387	10.35	14.65	197	26.50	65.00	161	81.75	114.70
			217	20.55	57.30	185	79.80	103.90
			220	15.45	59.40	216	73.30	118.95
			241	16.60	60.50	239		124.50
			272	18.75	64.75	257		133.20
			291	17.95	62.00	284		124.00
			312	20.10	68.30	307		120.30
			336	20.35	71.85	330		140.40
			367	12.75	67.70			

Hours	Transfer #3 (37.5 g/L)	Hours	ansfer #4 (37.5 g/	Hours	ransfer #5.1 (37.5 g/L	Transfer #5.2 (37.5 g/L)
0		0		0	0.24	0.26
2	0.56	19	4.10	2	0.12	0.23
21	7.09	40	29.50	5	0.20	0.29
52	52.55	64	56.65	22	3.47	4.03
71	77.90	96	89.05	23	4.22	5.65
92	86.85			24	7.10	8.23
116	93.50			26	7.94	9.02
147	78.35			28	10.89	13.37
				30	13.35	17.45
				32	17.13	20.88
				34	24.28	27.83
				45	47.55	57.95
				51	59.60	67.65
				54	68.15	68.10
				69	93.40	110.40
				77	106.55	92.70
				95	100.10	83.45
				121	91.30	82.95
				145	75.20	76.50
				167	71.05	73.85
				189	85.75	74.00
				214	78.75	69.20
				241	80.30	71.55
				267	86.25	76.95
				314	88.60	77.25
				333	104.35	97.40
				367	98.70	92.70

* Data in yellow used for growth rate estimations

Figure S17: Irreversibility Test with ST10119, ST10131 and ST10000

Hours	ST10000	Hours	ST10119	ST10131
17	1.25	17	1.32	0.04
21	1.52	21	1.93	0.08
25	3.16	25	3.37	0.62
44	3.09	44	16.93	15.64
48	2.71	48	22.34	20.30
68	1.85	68	51.21	44.85
94	3.82	94	77.63	75.30
141	40.80	141	75.65	62.05
160	77.40	160	85.08	69.50
194	86.05	194	72.95	64.05
214	105.70			
233	103.43			
248	91.83			
289	79.80			
333	92.13			
357	81.45			
377	80.13			

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22/05/2024