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Master Thesis

Bioethanol Production in Kveik Yeast: Testing Usefulness of Farmhouse Brewing Yeast for Industrial use.

Master in applied experimental biotechnology

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Abbreviations

AF – Alcoholic Fermentation

ATP - Adenosine Triphosphate

BBD-RSM - Box-Behnken Design-Response Surface Methodology

BCAA - Branched-Chain Amino Acids

CDW - Cell Dry Weight

CFSTR - Continuous-Flow Stirred Tank Reactor

DoE - Design of Experiments

EA - Ethyl Acetate

EtOH - Ethanol

HPLC - High-Performance Liquid Chromatography

KYM - Kveik Yeast Medium

NADP - Nicotinamide Adenine Dinucleotide Phosphate

OD - Optical Density

POF - Phenolic Off-Flavors

PFW – Potato fruit water

S. cerevisiae - Saccharomyces cerevisiae

Sbt- Syringe bottle

SSF - Simultaneous Saccharification and Fermentation

YPG - Yeast Extract Peptone Glucose

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Abstract

Kveik yeast, a strain of *S. cerevisiae* originating from Norwegian brewing, exhibits thermotolerance, contributing to rapid fermentation within 24-48 hours, and can withstand stressful conditions. This thesis investigated various aspects of four selected kveik yeast strains, including their potential for bioethanol production, by analysing their thermotolerance, sugar conversion efficiency, and fermentation efficiency. The study employs batch and continuous fermentation methods for ethanol concentration, glucose conversion, productivity, yield, and overall efficiency. The research focuses on kveik yeast and examines critical parameters such as temperature, glucose concentration, inoculum size, and fermentation time. It uses predictive modelling techniques such as the design of experiments (DoE) and contour plot methodologies to optimize ethanol production using kveik Ebbegarden (Y9).

The batch fermentation model facilitated fermentation using the syringe piston expansion method. Following the Design of Experiments (DoE) analysis guided by the contour plot, ethanol production reached 48.04 g/l with an initial glucose concentration of 80 g/l at 40°C. This was achieved after 24-48 hours of fermentation using suspended cells. Similarly, the continuous fermentation model employed alginate bead immobilization within a Continuous Flow Stirred Tank Reactor (CFSTR). Under these conditions, ethanol production was 21.95 g/l with an initial glucose concentration of 56 g/l at a dilution rate of 0.34 /h. This fermentation process lasted 3-4 hours.

The findings revealed that temperature and initial glucose concentration significantly impact ethanol production. Additionally, the study shows differences in sugar conversion, ethanol concentration, yield, and productivity between batch fermentation with suspended cells and continuous fermentation with immobilized cells. Although the ethanol production and yield of immobilized cells are lower than those of suspended cells, continuous fermentation with immobilized cells achieves a significantly higher ethanol production rate per hour. The study concludes that kveik yeast is suitable for bioethanol production, especially in under warmer conditions, due to its high-temperature tolerance and fast fermentation rate. Furthermore, Immobilized kveik yeast can increase efficiency and economic benefits in industrial applications by allowing for the reuse of yeast beads for over ten cycles, resulting in shorter fermentation times of 3-4 hours.

1. Introduction

1.1 Significance of *Saccharomyces cerevisiae* in Bioethanol Production

Saccharomyces cerevisiae, a budding yeast, has been used for thousands of years to ferment food and beverage; it possesses a remarkable capacity for converting sugars into alcohol and carbon dioxide through alcoholic fermentation. It exhibits versatility by fermenting under both aerobic and anaerobic conditions. In the presence of oxygen, *S. cerevisiae* undergoes respiration, converting glucose into energy. Similarly, under anaerobic conditions, it engages in alcoholic fermentation, yielding ethanol and carbon dioxide. (Dondrup et al., 2023), (Dashko et al., 2014)

S. cerevisiae, also known as both brewer's and baker's yeast, has been widely researched for its ability to produce ethanol. This organism has a high glucose-to-ethanol conversion rate and can tolerate high levels of ethanol, making it an ideal choice for large-scale fermentation processes. Ethanol production by *S. cerevisiae* has significant economic and environmental implications, as it is used as a fuel additive and a renewable energy source (Parapouli et al., 2020). Apart from its role in ethanol production, *S. cerevisiae* produces other fermented foods and drinks, such as bread, cheese, and yogurt. Its ability to convert sugars to alcohol and carbon dioxide is exploited in producing biofuels, pharmaceuticals, and other industrial products (Maicas, 2020). *S. cerevisiae* is a very versatile and important organism with a rich history of use in human civilization. Under anaerobic conditions, it can produce ethanol at a high rate. These properties make it a valuable tool for various industrial applications. (*Cerevisiae - an Overview | ScienceDirect Topics*, n.d.), (Parapouli et al., 2020). Apart from its fermentative abilities, *S. cerevisiae* has a remarkable ability to withstand high sugar concentrations, making it suitable for different industrial applications. Additionally, it can produce a wide range of aromatic and volatile compounds, which makes it even more desirable in beer production.

The efficient ethanol production in beverage fermentations relies on yeast cell growth, closely related to alcohol production. Fermentative yeasts, such as *S. cerevisiae*, use sugars anaerobically as electron donors (glucose) and acceptors (pyruvate), which serve as carbon sources for biomass growth. However, anaerobic fermentation is less energetically favourable than respiratory metabolism. *S. cerevisiae* utilizes both respiration and fermentation pathways

for ATP production. Respiration yields more ATP in the presence of oxygen, while fermentation provides a lower yield without it. The Crabtree effect, observed in *S. cerevisiae*, involves the organism producing ethanol and other two-carbon compounds like pyruvate, even in the presence of oxygen. Rather than utilizing its respiratory machinery to metabolize saccharides and facilitate biomass growth, *S. cerevisiae* generates and accumulates ethanol, which can have toxic effects (Pronk et al., 1996). The Crabtree effect is essential for both Crabtree-negative and Crabtree-positive yeasts. Crabtree-positive yeasts likely evolved in sugar-rich environments alongside flowering plants. This evolution was driven by genetic changes such as whole-genome duplication and regulatory rewiring (Pfeiffer & Morley, 2014). The Crabtree effect is characterized by the rapid conversion of sugars to ethanol, even aerobically, termed "pre-fermentation" ethanol production. This process continues until sugar depletion, known as the long-term Crabtree effect, while the short-term effect results in immediate aerobic alcoholic fermentation upon excess sugar addition (Hagman et al., 2014).

1.2 Kveik Yeast

The use of traditional yeasts for beer production on Norwegian farms has become more prevalent in recent times. These yeasts are known as gjær, gjest, barm, and kveik in Norway, with the term kveik gaining global recognition. Historical records suggest that until the late 1800s, almost every farm had its unique yeast culture, mainly used in beer making.

As of 2022, there are currently 24 recognized Kveik cultures that are commercially available. These cultures have been named either after the places where they were discovered or after their owners, including Voss, Hornidal, Ebbegarden, Stranda, Årset, Midtbust, and Oslo. It is important to note that traditional kveik cultures often blend various yeast strains, sometimes including bacteria, most commonly *S. cerevisiae species* (Klimczak & Cioch-Skoneczny, 2022). Together, they play a crucial role in converting fermentable sugars into ethanol and generating a diverse array of flavorful compounds that are distinct to each beverage (Cubillos et al., 2019).

1.2.1 Kveik Yeast in Traditional Norwegian Brewing:

Kveik yeasts are known for their fast fermentation rates and high tolerance for higher fermentation temperatures. Most strains have an optimal range of 30-37 °C and can withstand various stress factors, which makes them a popular choice among rural Norwegian brewers (Klimczak & Cioch-Skoneczny, 2022), Biörnstad, 1972; Isbell, 2023). The term "kveik" refers to homegrown yeast, once widespread in Norway but now rare, with remaining pockets like Voss and potentially Sunnmøre (*Kveik*, n.d.). These yeasts produce non-phenolic off flavor (POF-) beers with short fermentation times, akin to other domesticated beer yeasts. These yeast are reused through serial re-pitching (Preiss et al., 2018).

1.2.2 Kveik Yeast Subgroups in Western Norway:

In Western Norway, home to diverse traditional beers such as stjørdalsøl, konnjøl, and maltøl, Kveik yeasts are categorized into two subgroups, each with distinct characteristics. One subgroup hail from the Granvin, Stranda, Lærdal, and Voss regions, while the other corresponds to the Sykkylven, Hornindal, and Stordal regions. These regions reflect their geographical divide around the Jostedal glacier (Kawa-Rygielska et al., 2022). Kveik yeasts have been integral to Western Norway's farmhouse ale brewing for centuries, and they exhibit thermotolerance, contributing to rapid fermentation completion within 1-2 days when pitched into wort at temperatures above 30°C (Foster et al., 2022a). Some of the Kveik yeast used in this project are as follow:

- **Voss**

  The Voss kveik, originating from Sigmund Gjernes in Voss, Norway, is distinctive due to Voss's isolated location south of the Jostedal glacier. Preserved through years of dedicated efforts by Sigmund and others, this kveik has a unique taste and aroma with fruity notes, prominently featuring orange and citrus characteristics. (Garshol, 2020)
- **Ebbegarden**

  Owned by Jens Aage Øvrebust, the Ebbegarden kveik from Stordal produces a tropical fruit aroma and enhances hop bitterness. Comprising both bacteria and S. cerevisiae, it is harvested from the top of the fermentation vessel after 120 hours. Its flavor and aroma are characterized by tropical fruit notes, with intensity varying based on fermentation temperatures (Garshol, 2020), (Aasen, 2020)

- **Eirtheim**

  The Eitrheim kveik passed down through generations in Tokheim, Norway, has fruity notes of ripe pear, plum/prune, and honey. The intensity varies with fermentation temperatures, being medium at higher temperatures and less intense at lower temperatures (Kveik Yeastery, n.d.).

- **Stalljen**

  Stig Jarle Seljeseth owns the Stalljen kveik, originating from the 1970s, consisting of five strains of S. cerevisiae and no bacteria. Harvested from either the top or bottom of the fermentation vessel after 24 hours, Stalljen yields flavors of apples, ripe fruit, and hints of cloves and anise. The intensity varies with fermentation temperatures (Garshol, 2020), (Aasen, 2020).

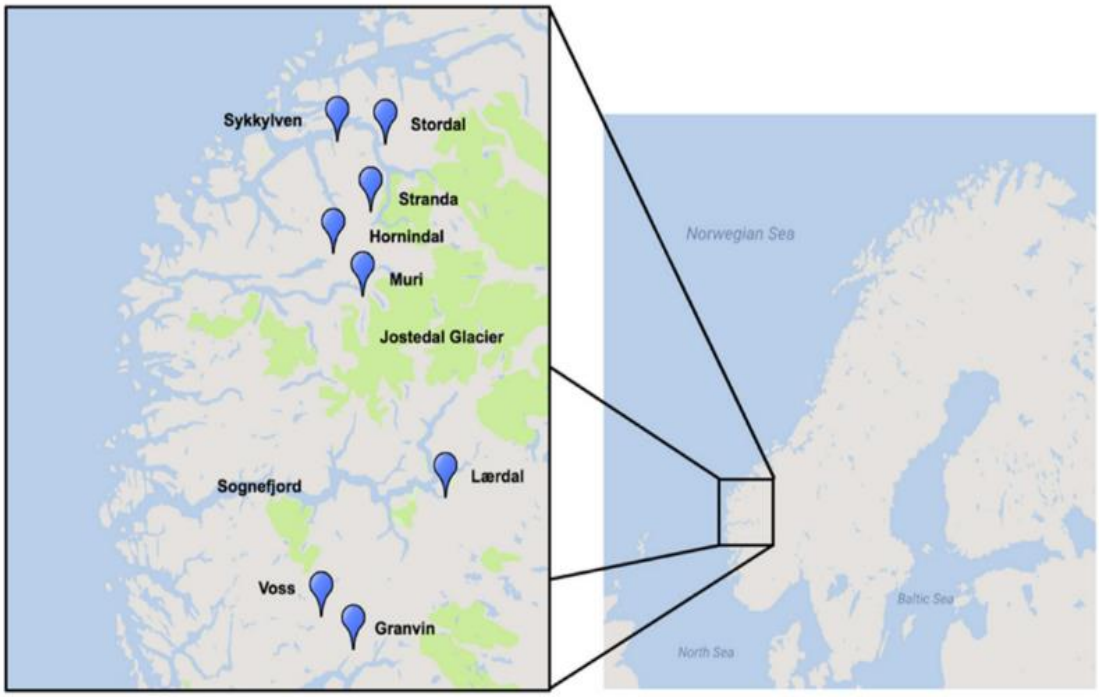

Figure 1: Geographical distribution of Kveik yeast samples sourced for this project. Maps was taken from (Preiss et al., 2018). Parks, including the Jostedalsbreen (Jostedal glacier) national part, are highlighted in green.

1.2.3 Preservation and Storage of Kveik Yeast:

Traditional Norwegian brewing techniques involved using a perforated log called a "kveikstokk" or items like cloth, bricks, juniper twigs, or blades of grass to collect yeast. The collected yeast was then coated in flour, dried, and repeated multiple times before being hung to dry or treated with hot ash to expedite the drying process (Kawa-Rygielska et al., 2021), (Habschied et al., 2022a). Kveik yeasts can be dried for extended periods, sometimes over a year, and the top and bottom parts of a wooden ring called *gjækrans* are collected after the initial fermentation to preserve yeast. This traditional storage method has been in use since the early 17th century (Klimczak & Cioch-Skoneczny, 2022).

Figure 2: A Norwegian yeast ring (gjærkrans) used to harvest and preserve kveik (Garshol, 2020). Photo: ***Lars Marius Garshol****.*

1.2.4 Unique Characteristics of Kveik Yeast:

Various Kveik yeast exhibit differing fermentation temperature ranges but share a common trait of accelerated fermentation compared to conventional beer yeasts. Notably, Kveik yeasts have a heightened capacity to accumulate the disaccharide trehalose. The trehalose functions

as a protective shield against temperature and ethanol-induced stress. This accelerated trehalose accumulation is attributed to a mutation within the trehalose Synthase complex (Dondrup et al., 2023). Genetically differing from domesticated *S.cerevisiae* yeast commonly used for generations in Norway's traditional farmhouse brewing, Kveik yeasts are usually related to a particular family and are passed down from generation to generation. They are known for being non-purified and gaining popularity due to thermotolerance, ethanol tolerance, and good flocculation properties (Habschied et al., 2022b, 2022a), (Habschied et al., 2022b).

In contrast to commercial brewers who typically ferment their ales at cooler temperatures ranging from 15 to 20°C, Kveik yeast, originating from ancient farmhouse brewing practices, can withstand notably higher temperatures, often exceeding 30°C. This capability allows Kveik yeast to ferment faster, providing greater efficiency for commercial brewers. Moreover, unlike modern commercial yeast strains that can produce unwanted flavor compounds at higher temperatures, Kveik yeast maintains a cleaner flavor profile even at elevated temperatures. The historical tradition of local farmhouse brewing supports the effectiveness of warmer fermentation practices in producing high-quality beer (Garshol, 2020).

1.3 Bioethanol Production from Yeast Fermentation

1.3.1 Overview of Global Bioethanol Production

The world's economy heavily depends on fossil fuels for energy and production. This widespread use has increased pollution and greenhouse gas levels, especially in urban areas. As the world's population grows and industries expand, energy consumption rises. Limited fossil fuel reserves have led to decreased transport fuel imports, and global oil production is predicted to decline (Sarkar et al., 2012). As the energy crisis looms and environmental problems caused by fossil fuel overuse worsen, renewable biomass biofuels are gaining more attention worldwide. Bioethanol is a popular transportation biofuel from plant sources such as wheat, sugar beets, corn, straw, and wood. Biofuels from organic waste materials and biomass offer significant ecological advantages over traditional fossil fuels. Worldwide, bioethanol is expected to have a production volume of over 130 billion litres annually (OECD, 2015). Brazil and the United States are the major producers, accounting for 90% of global production

(Limayem & Ricke, 2012), (*Feedstock for Bioethanol Production from a Technological Paradigm Perspective*, n.d.).

Although first-generation feedstocks like cereal grains, sugar cane, and sugar beets have been widely used, there are growing concerns about their impact on food sustainability. Therefore, alternative sources like second-generation lignocellulosic and third-generation algal biomass are being explored to overcome biofuel production's environmental and ethical challenges. In alcoholic fermentation, yeast plays a crucial role by converting sugars such as glucose, galactose, and fructose into ethanol and producing carbon dioxide and other by-products. These versatile metabolic processes can occur effectively under anaerobic conditions, making bioethanol a sustainable and renewable energy source with enhanced adaptability and viability (Tse et al., 2021)**.** Using single-cell microorganisms like yeast for sugar fermentation has been a common practice in biotechnology for centuries. Previously, it was used to produce alcoholic beverages like beer and wine. This method is widely used in the industry to produce fuel ethanol from renewable energy sources (Kosaric & Velikonja, 1995), (Zabed et al., 2014). *S. cerevisiae* is a yeast type commonly used for ethanol production through fermentation. It is more efficient than other options for converting sugar to alcohol. It can produce flocs during growth, making it easier to settle or suspend as required. Additionally, it has a high tolerance to ethanol (Kosaric & Velikonja, 1995).

1.3.2 Stress Factors Affecting Yeast in Alcoholic Fermentation

Alcoholic fermentation (AF) is the primary process involved in ethanol production. During alcoholic fermentation, glucose and fructose in the substrate are converted into ethanol and carbon dioxide, as shown in Equation 1. This process starts with the breakdown of sugar in the glycolytic pathway, which generates adenosine triphosphate (ATP), the cell's primary energy source. Pyruvate is the final product of glycolysis, which yeast can further convert into acetaldehyde and ethanol (Genisheva et al., 2014).

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \quad \text{— (1)}$$

This equation shows that the molar yield is 2 mol ethanol per 1 mol glucose; however, in mass term Yp/s = 0.51gEtOH/ gGlu.

Various stresses during propagation, fermentation, and storage can impact yeast cell performance and health. These stressors include oxidative reactions, osmotic pressure on the membrane, ethanol production, and thermal stress. High temperatures can stress yeast cells, leading to protein denaturation and disruption of cellular structures, which can cause cell death and depleted glycogen reserves. These fermentation issues lead to stuck or prolonged fermentation and lower alcohol production, making the yeast unsuitable for reuse (Gibson et al., 2007). During osmotic stress, the yeast cell's response varies depending on initial cell health, wort sugar composition, and fermentation stage. Studies suggest that yeast cells are more vulnerable during the exponential fermentation phase due to decreased levels of reserve carbohydrates like glycogen and trehalose. High sugar levels in the culture medium create a concentration gradient across the cell membrane, resulting in osmotic stress. This hyper-osmotic pressure can lead to solute diffusion into cells, causing water leakage, reducing cell viability, and affecting metabolism (Gibson et al., 2007; Isbell, 2023).

As the alcohol content increases, the toxicity of the yeast's environment rises, leading to reduced yeast activity, slower fermentation, and decreased alcohol yield. The rise in alcohol toxicity can further impact yeast viability and vitality, ultimately affecting fermentation efficiency and ethanol production.

1.4 Fermentation method used for Bioethanol production

1.4.1 Batch, Fed-batch, and continuous modes

Fermentative microorganisms utilize carbon sources, primarily sugar, to produce acids, alcohols, and gases. In industrial applications, fermentation produces biopharmaceuticals, food and feed supplements, biofuels, and chemical building blocks. Achieving cost-effective processes involves considering factors such as media and supplement costs, process runtime, microbial growth and viability, product titer and yield, and product quality concentrations of nutrients and byproducts in the culture medium play crucial roles. Therefore, during process development, bioprocess engineers decide on the application of a batch, fed-batch, or continuous bioprocess.(Yang & Sha, n.d. 2019)

In batch fermentation, microorganisms are introduced to a fixed medium volume in a fermenter, and as microbial growth progresses, nutrients deplete, and byproducts accumulate, causing changes in the culture environment. The growth curve typically undergoes distinct phases, including a lag phase with slow growth, an exponential growth phase with constant microbial division, and a stationary growth phase characterized by slowed growth as nutrients deplete and byproducts accumulate. Bioprocess engineers often harvest the culture at this point to prevent entering the death phase, marked by a decrease in viable cell density. Batch fermentation is a cost-effective method that offers several benefits over other fermentation approaches, with a low risk of contamination and ease of sterilization and feedstock management (Li & Sha, 2016; Yang & Sha, 2019). Additionally, it is most commonly used in long-term, small-scale, or solid-state fermentation processes (Wang et al., 2013).

The Fed-batch fermentation process is a modified version of batch fermentation commonly used in the bioprocessing industry. In this approach, microorganisms are initially inoculated and allowed to grow in a batch regime for a specified period. After this, nutrients are added incrementally to the fermenter throughout the remaining fermentation duration to sustain microbial growth (Yang & Sha, 2019). The consistent addition of nutrients contributes to increased product yields by enhancing cell density during the exponential phase. For example, it is possible to maximize ethanol yield by providing yeast cells with a continuous supply of sugar during the stationary phase (Tse et al., 2021).

Continuous fermentation involves constantly adding fresh medium and simultaneously harvesting used medium and cells, facilitating the replacement of consumed nutrients and removing toxic metabolites. Maintaining a constant culture volume ensures optimal conditions for cell growth. The addition of substrate and final product harvesting occurs continuously in this process (Díaz-Montaño, 2013)**.** Cell immobilization technologies, often employed in continuous fermentations, enable cell's continuous recovery and utilization. The dilution rate is a crucial factor influencing continuous fermentation, with different rates leading to varying product yields and qualities. Continuous processes provide significant economic advantages by eliminating the need for repeated fermentation preparation, cleaning, and sterilization between batches, saving workforce, energy, and time. Additionally, they prolong microbial exponential growth phases, reducing processing time and ensuring high final product production levels (Díaz-Montaño, 2013), (T. Li et al., 2014).

For efficient ethanol production, it is essential to have a yeast strain that can rapidly ferment and produce high concentrations of ethanol under conditions of high osmotic stress and ethanol

levels. However, during batch fermentation, growth may be hindered by inhibition from substrates or end-products. Alternative fermentation modes, such as fed-batch or continuous fermentation modes are being explored and optimized to improve yeast ethanol tolerance (Balat, 2011).

1.5 Key Factors Influencing Bioethanol Production in Fermentation

1.5.1 Temperature impact

The temperature is an important factor in fermentation since adding heat to the system increases the reaction rate (Mallouchos et al., 2003). High temperatures, on the other hand, stress microorganisms, reduce their growth, and cause the inactivation of enzymes and ribosomes. Optimal temperature regulation is essential for ensuring the best microbial activity during fermentation (Phisalaphong et al., 2006). The ideal temperature for the fermentation process of free cells of *S. cerevisiae* is approximately 30°C (Torija et al., 2003), while immobilized cells transfer heat more efficiently from the particle surface to the inside of the cell, resulting in a higher heat transfer rate. (Liu & Shen, 2008). Also, a lower ethanol yield is obtained at reduced temperatures, and a longer time is required to complete fermentation (Jones et al., 2002).

1.5.2 Influence of Glucose concentration

The initial sugar concentration is a critical parameter directly impacting the fermentation rate and microbial cells. Typically, the fermentation rate increases with high sugar concentration up to a certain level. However, an excessively high sugar concentration (higher than 12%) can surpass the uptake capacity of microbial cells, resulting in a steady fermentation rate. Higher sugar concentration in batch fermentation increases ethanol productivity and yield, resulting in longer fermentation time and higher recovery costs (Laopaiboon et al., 2007).

1.5.3 Other Factors: Inoculum size and fermentation time

Inoculum concentration does not significantly affect final ethanol concentration but does affect sugar consumption rate and ethanol productivity (Laopaiboon et al., 2007). Short fermentation times can result in inadequate microorganism growth, which can lead to inefficient fermentation. On the other hand, longer fermentation times, particularly in batch mode, can harm the microbial growth due to the high ethanol concentration in the fermented broth (Nadir et al., 2009).

1.6 Cell Immobilization: Optimizing Bioethanol Production

Cell immobilization in bioreactors aims in addition to easy reuse of biomass to reduce inhibition caused by high substrate and product concentrations during ethanol production. This approach improves ethanol production and reduces operational costs (Duarte et al., 2013). In various studies, immobilized cells on different supports have proven more effective than free cells including ease of product separation, the ability to reuse biocatalysts, high volumetric productivity, improved process control, reduced susceptibility of cells to contamination and, making them a promising option for cost-effective ethanol production (Silbir et al., 2014; Ylitervo et al., 2011; Yu et al., 2007). These immobilized cell systems influence yeast metabolism and enhance alcohol fermentation (Yang & Sha, 2019).

Choosing the right immobilization technology and carrier material is crucial for an efficient cell-immobilization system, considering factors like operating costs, material stability, product quality, legality, and safety. Gels made from Sodium alginate, and polyvinyl alcohol enhance the ethanol yield and improve reusability over an extended period (Chacón-Navarrete et al., 2021). One popular and simple technique for immobilizing cells in laboratory setups is by entrapping them within calcium alginate beads, which are known to sustain high cell viability and activity. (Liu & Shen, 2008).

Alginate beads are a cost-effective and straightforward option for cell immobilization, providing gentle conditions suitable for industrial applications. Calcium alginate beads are preferred among the various supports because they are biocompatible, affordable, readily available, and simple to prepare. However, some drawbacks such as gel degradation, mass

transfer limitations, low mechanical strength, potentially leading to cell release, and large pore size need consideration (Bangrak et al., 2011; Zhou et al., 2010).

In fermentation, using immobilized cells is common and brings several advantages over free cells. Immobilized cells increase cell density per reactor volume, optimizing space and resources. They are easier to separate from the reaction medium, making downstream processing more streamlined. Immobilized cells often demonstrate higher substrate conversion rates, and experience less inhibition by-products, leading to shorter reaction times, and improving control over cell replication(Duarte et al., 2013).

Different methods for cell immobilization include adsorption, crosslinking, encapsulation, and entrapment (Chibata et al., 1986). Entrapment involves polymerizing acrylamide monomers in an aqueous solution with suspended microorganisms, effectively addressing degradation and mass transfer limitations, allowing for high biomass loading, and enhancing ethanol productivity. This method is preferred for its simplicity, non-toxicity, cost-effectiveness, reversibility, and good mechanical properties (Chibata et al., 1986; Mohd Azhar et al., 2017). In entrapment, yeast cells are confined within a carrier in a porous matrix, facilitating interaction with the medium, including metabolism, mass transfer, and nutrient exchange. Cell containment can be achieved through direct immobilization within the carrier's formation or by releasing cells into an existing matrix. Despite challenges such as carrier destabilization at low pH, gas diffusion issues (e.g., CO2), severe mass transfer limitations, low mechanical strength, and large pore size, entrapment technology offers advantages like simplicity, non-toxicity, cost-effectiveness, biocompatibility, and high availability and good mechanical properties (Chacón-Navarrete et al., 2021).

Adsorption is a widely used and economical technique that attaches cells to a material surface through electrostatic forces. This method is known for its simplicity, cost-effectiveness, and rapid execution (Margaritis & Merchant, 1987). In encapsulation, yeast cells are enclosed within a thin semi-permeable membrane. This method limits cell growth and controls the size of nutrients and products, influencing the rate of reaction. (Ylitervo et al., 2011).

1.7 Aim of the study

Exploring alternative yeast strains for bioethanol production is a crucial area of focus due to their potential to increase fermentation efficiency and improve sustainability. A farmhouse brewing yeast strain called kveik yeast, originating from traditional Norwegian brewing practices, has emerged as a promising candidate for bioethanol production. Kveik yeast, a variant of *S cerevisiae*, is known for its fast fermentation at elevated temperatures and remarkable stress tolerance, making it ideal for bioethanol production in warm conditions. This thesis investigates the characteristics and capabilities of kveik yeast in bioethanol production.

The main aim of this study is to explore the potential of kveik yeast, known for its rapid fermentation at high temperatures and stress tolerance, in bioethanol production. Specifically, the study aims to investigate the unique characteristics of kveik yeast, including its thermotolerance, stress resistance, and fermentation efficiency. The research assesses the potential of kveik yeast by analysing its fermentation methodology, ethanol yield, and overall efficiency. Various fermentation methods, such as batch and continuous fermentation, will be examined to enhance ethanol production efficiency. The analysis will investigate key factors impacting ethanol production, including temperature, glucose concentration, inoculum size, and fermentation time, specifically focusing on kveik yeast.

This project aims to explore the following research question regarding ethanol production from kveik yeast:

- What are the optimal fermentation conditions (temperature, glucose concentration, and inoculum size) for maximizing ethanol production using kveik yeast?
- Can kveik yeast efficiently produce bioethanol in an industrial setting by utilizing high glucose concentrations at elevated temperatures?
- What is the most effective mode of fermentation for ethanol production using kveik yeast and what are the differences in efficiency between batch and continuous fermentation mode when using kveik yeast?

This study aims to investigate research questions and conduct detailed exploration and experimentation to provide valuable understanding into maximizing the utilization of kveik yeast in industrial-scale bioethanol manufacturing by examining the fermentation process, ethanol yield, productivity, and overall efficiency under various conditions.

2. Material and Methods

2.1 Materials

2.1.1 Kveik Yeast

The study selected four yeast strains obtained from Kveik Yeastery in Brumundal, Norway: Voss (Y1), Ebbegarden (Y9), Eirtheim (Y14), and Stalljen (Y22). These strains were chosen for their distinct traits, apart from unique flavor profiles, but for specific fermentation characteristics (Table 1). The material was supplied as dried granules, packaged in small sachets, designed specifically for the artisan brewery market (Fig 1). Although kveik yeast is not a single strain, but rather a mix of several *S. cerevisiae* sub-strains (Nadia, 2021), they are referred to as strains in the following:

Figure 3: Photoset sachets with dried Kveik yeast

Table 1: Kveiks used in this study, along with the place of origin, owner, and fermentation temperature (Garshol, 2020), (*Kveik Yeastery,* n.d.)

Kveik	Origin	Owner	Fermentation temperature **(°C)**
Voss (Y1)	Voss, Vestland	Sigmund Gjernes	18-42ºC
Ebbegarden (Y9)	Stordal, Møre og Romsdal	Jens Åge Øvrebust	18-38 ºC
Eitrheim (Y14)	Tokheim, Vestland	Reidar Eitrheim	18-42 ºC
Stalljen (Y22)	Hornindal, Møre og Romsdal	Stig Seljeset	8-38 ºC

2.1.2 Growth Media Preparation

Two media formulations were prepared to optimize yeast growth and fermentation conditions. Yeast Peptone Glucose (YPG) is a nutrient-rich mixture with a composition of 20 g/l glucose, 20 g/l peptone, and 10 g/l yeast extract, accommodating glucose concentration from 2% to 12% (w/v). If a solid YPG medium is required, 20 g/l agar was added to YPG media before autoclaving.

Kveik Yeast Medium (KYM), developed by the Kveik Yeastery AS, is a nutrient-rich blend with the composition of 0.25 g/l $CaSO_4$, 0.25 g/l $CaCl_2$, 6.5% (0.4 µl/l) $ZnSO_4$, 0.09 g/l yeast vitamin, 0.07g/l Branched Chain amino acid-BCAA (nitrogen source) in the form of casein hydrolysate, and 20% (w/v) sugar beet molasses as the carbon source. It provides a rich environment for yeast growth and fermentation processes. The mixture of vitamins, nitrogen, and carbon sources, especially the 20% (w/v) molasses, provides enough fermentable sugars to sustain yeast metabolism. Molasses, BCAA, and yeast vitamins were provided by Kveik Yeastery.

KYM and YPG media were autoclaved at 121 °C for 20 minutes after preparation and stored at 5 °C in a cool room before use to maintain sterility. When working with the Kveik yeast one stains were cultured each time to avoid cross-contamination.

The inoculation of Kveik yeast strains Y9, Y1, Y14, and Y22 involved taking 10 ml of YPG and KYM media and adding 4-5 dried yeast granules into KYM and YPG medium. The mixture was incubated at 37 °C for 16 hours and stored in a 4 °C cooling room before use in the experiment. Only inoculums less than 2 weeks old were used to reduce initial ethanol level.

2.1.3 Other chemicals

- 1000 ml of 0.1M $CaCl_2$, 0.2M NaCl
- 500 ml of 0.9 % NaCl
- 1000 ml of 0.1M succinate- NaOH pH 4.3
- 500 ml of 0.1M $CaCl_2$
- Potato fruit water PFW used as an alternative N-source: A by-product from starch processing obtained from HOFF AS, Brumundal, Norway.

2.2 Methodology

2.2.1 Experimental setup

The experimental study aimed to achieve the research objectives and was conducted within the laboratory facility at the Biohus of Inland Norway University of Applied Sciences in Hamar, Norway. The study adhered to a well-defined experimental design or workflow, illustrated in Figure 4-6. The experimental workflow included the overall method and process used in the whole project including batch model and continuous model validation. This visual representation outlines the systematic and structured approach to investigate the research goals.

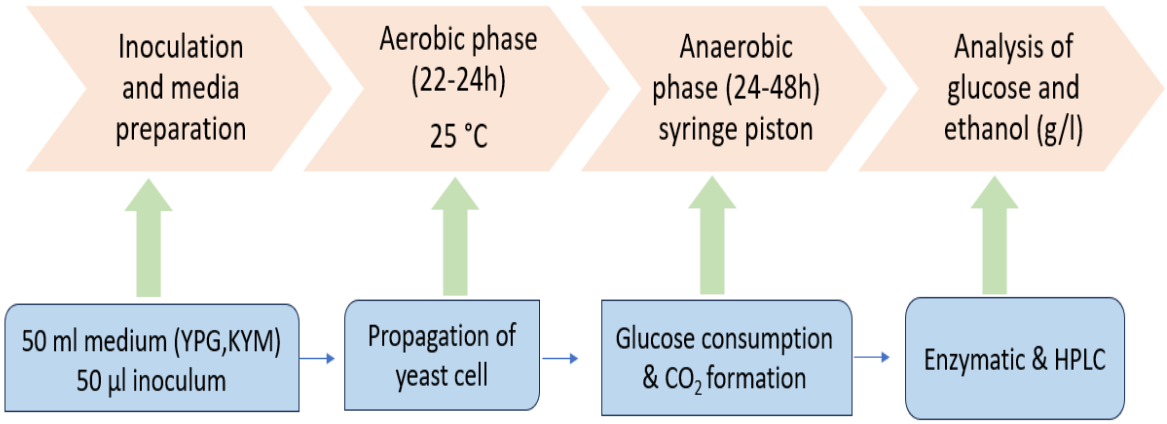

Figure 4: Workflow for the batch model fermentation including inoculum and media preparation, aerobic phase, anaerobic phase, and analysis method.

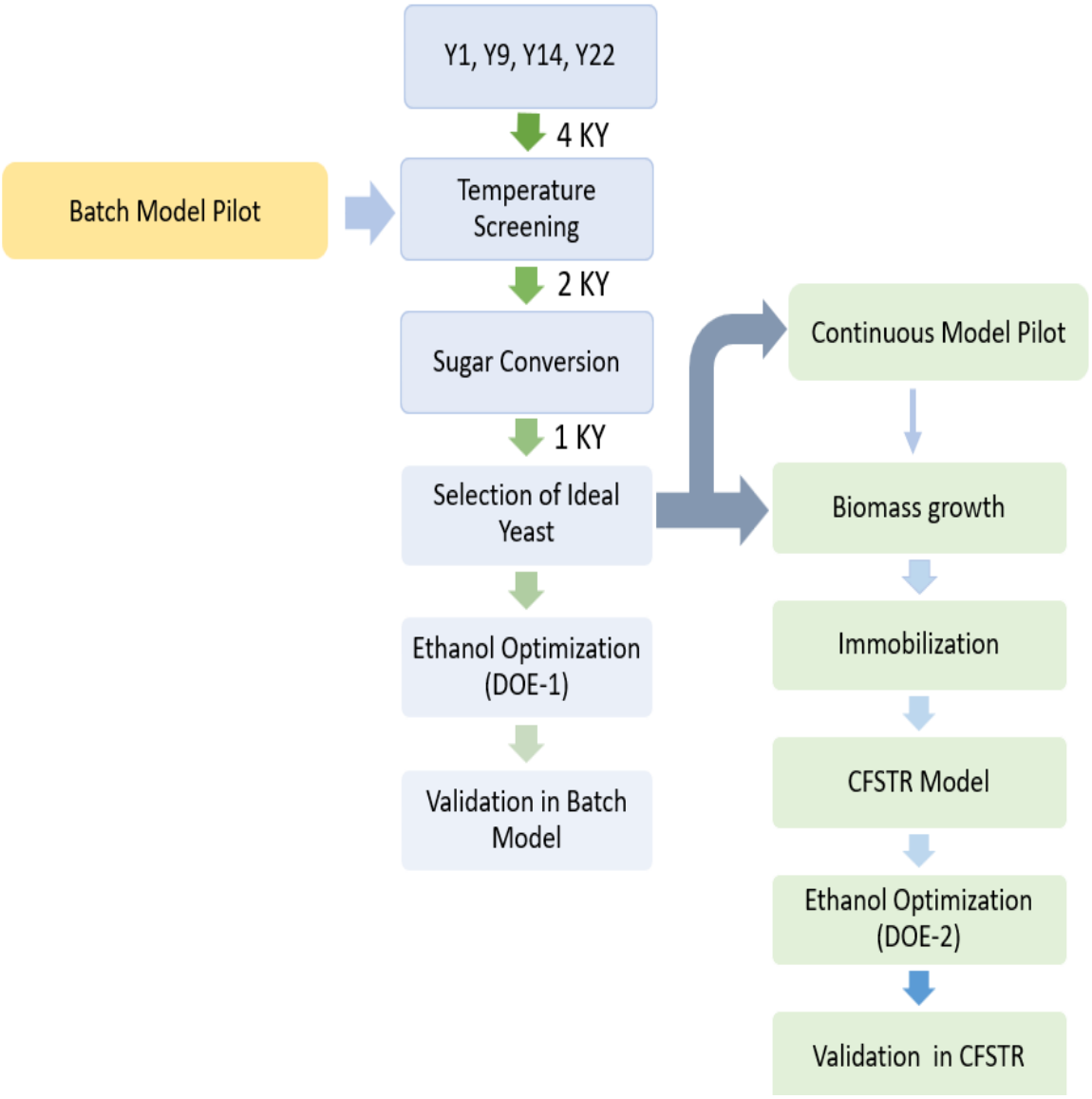

Figure 5: Experimental workflow for Fermentation study model including Batch and continuous model validation using yeast culture and immobilized yeast bead.

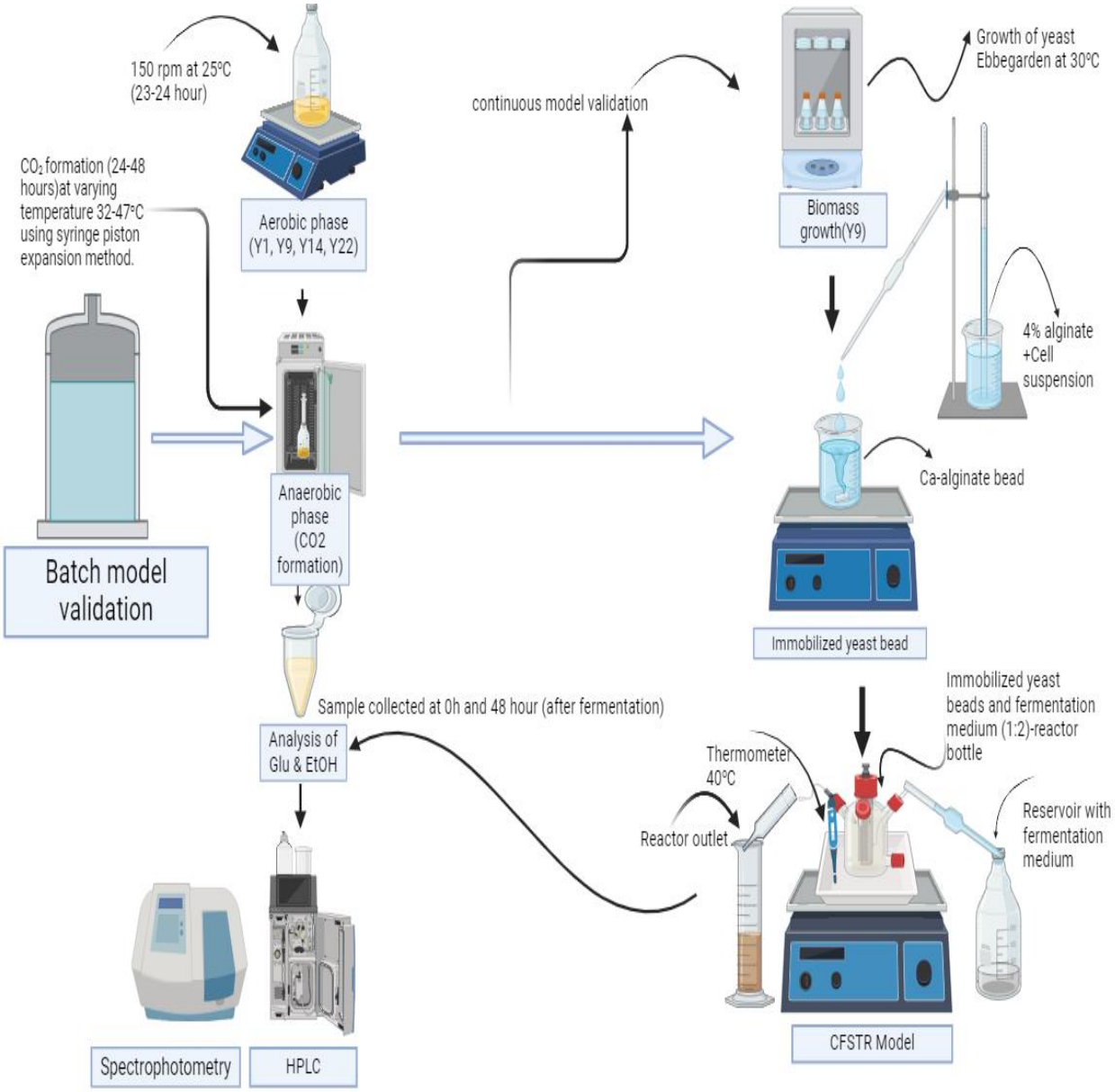

Figure 6: Diagram showing Experimental setup of batch and continuous fermentation created using Bio-Render.

2.2.2 Screening Test in Batch Fermentation Model

The first stage of experiments involved pilot and screening tests to determine a suitable setting for a study model using Kveik yeast (figure 4-6).

The inoculum (50 µl) was added to 50 ml of medium in a 250 ml bottle. The aerobic propagation phase occurred on a rotating stirring disc at 150 rpm at room temperature (25 ^{o}C) for 21-24 hours, with the bottle loosely covered with aluminium foil. The anaerobic fermentation phase utilized the syringe piston expansion method to measure gas (e.g., CO_2) evolution (fig 6). This involved using a 100-ml syringe connected to the 250-ml bottle with KYM and YPG media containing the propagated Kveik. The syringe piston was fitted through a hollowed silicone stopper and fixed at the top of the bottle. The stopper was tightly covered using parafilm to prevent air entry or gas leaks.

The syringe piston expansion method allowed an oxygen-free environment during the anaerobic phase. Gas evolution during fermentation causes the syringe piston to move upward, reaching a maximum of 100 ml. After reaching the 100 ml mark, the syringe piston was re-adjusted to 0 ml, and readings were taken continuously for 10-12 hours. The setup was left overnight, and the syringe piston was adjusted to 0 ml in the morning. Readings continued until gas evolution stopped. The anaerobic condition was maintained for around 48-50 hours. Samples were collected for optical density (OD_{660}), glucose, and ethanol tests at 0 hours (initial propagation phase) and 48 hours (after the completion of the anaerobic fermentation phase). OD_{660} was measured in a Spectronic 20D+ (Milton Roy, Houston, TX, USA) spectrophotometer. Samples were diluted with a sterile medium.

Glucose consumption was measured with Siemens Urine analysis sticks, interpreting color changes according to the manufacturer's instructions. The absence of colour change indicated normal glucose levels, while the highest observed value was 111 mmol/l, representing the upper limit of the test range.

2.2.2.1. Pilot Experiment-1: Initial Setup Evaluation

The first pilot experiment was an initial evaluation of the model setup introducing 50 µL of Ebbegarden (Y9) inoculum into a 250 mL bottle containing 50 mL of either KYM or YPG media (n=2). The aerobic propagation phase occurred at room temperature (25 °C) on an orbital shaker (150 rpm) for 23-24 hours. Subsequently, the anaerobic fermentation phase was

initiated at 37 °C using the syringe piston expansion method, maintaining an oxygen-free environment (Fig 6). Gas evolution was monitored continuously for 7-8 hours. Glucose consumption was assessed at 0h, 22 hours (after the aerobic phase), and 32 hours (after the anaerobic phase) using glucose sticks.

2.2.2.2. Pilot Experiment-2: Yeast Vitamin Supplementation

In the second pilot experiment, 50 ml of YPG media was supplemented with yeast vitamin (0.09 g/l), an ingredient of KYM, while another 50 mL of YPG media without vitamin supplementation served as a control. This setup replicated the conditions of Experiment 1, involving a 24-hour aerobic phase at 25 °C and a subsequent 24-hour anaerobic phase at 37 °C. Gas evolution was measured by the syringe piston every 1-2 hours, maintaining anaerobic conditions for around 48-50 hours (Fig 6). Glucose consumption was assessed at 0-, 23-, and 48-hours using glucose sticks.

2.2.2.3. Pilot Experiment-3: Yeast Pre-growth Temperature

The third pilot experiment explored yeast pre-growth conditions by adjusting the aerobic propagation phase temperature to 37 °C instead of 25 °C. Yeast inoculum (Y9) was added to 50 ml of YPG media (n=2). The 24-hour aerobic phase at 37 °C involved an orbital shaker at 150 rpm, while the subsequent 24-hour anaerobic fermentation phase at 37 °C utilized the syringe piston method for gas measurement. Glucose consumption was monitored using glucose sticks at 0, 23, and 48 hours.

2.2.2.4 Temperature Effect on fermentation

The experiment involved anaerobic fermentation of four Kveik yeast strains (Ebbegarden Y9, Eitrheim Y14, Stalljen Y22, and Voss Y1) at 32 °C to 47 °C. Following an initial aerobic phase with a 50 µL inoculum in 50 ml of YPG (n=1) and KYM media (n=2), at room temperature (25 °C), anaerobic fermentation was conducted at various temperatures (32 °C, 37 °C, 42 °C, 47 °C) for an additional 24 hours. Gas evolution during fermentation was

monitored using the syringe piston expansion method (Fig 6). Measurements were taken every 1-2 hours continuously for 9-10 hours, left overnight, and resumed until gas evolution stopped. Samples were collected at 0 hours (initial phase) and 48 hours (after anaerobic phase) for glucose and ethanol analysis by enzymatic method and HPLC.

2.2.2.5 Glucose Variation at 42 °C

The impact of glucose concentration on ethanol production was examined using yeast strains Ebbegarden (Y9) and Eitrheim (Y14) at a constant temperature of 42 °C. YPG medium with varying glucose concentrations (20 g/l, 40 g/l, 80 g/l, 120 g/l) underwent aerobic growth for 24 hours at room temperature, followed by an anaerobic phase for 24 hours measured CO_2 evolution through the syringe piston method. Samples at 0 and 48 hours were collected for glucose and ethanol tests using enzymatic and HPLC methods.

2.2.3 Design of Experiment (DOE1)

Table 2: Design of the experiment (DoE1) with varying factors including temperature (39 °C, 42 °C, and 45 °C), Glucose condition (80 g/l, 100 g/l, and 120 g/l), and Potato fruit water (0%,5%, and 10%) (w/v).

Exp No	Temperature (°C)	Glucose (g/l)	Potato fruit water (%)
1	39	80	0
2	45	80	0
3	39	120	0
4	45	120	0
5	39	80	10
6	45	80	10
7	39	120	10
8	45	120	10
9	42	100	5
10	42	100	5
11	42	100	5

Table 2 presents the eleven experiments conducted using Design of Experiment (DoE1) with the Kveik yeast Ebbegarden (Y9) as the chosen strain. MODDE Pro software (Sartorius Umetrics) was used to make a full factorial design, considering three factors: temperature (39 °C, 42 °C, and 45 °C), sugar concentration (80 g/l, 100 g/l, and 120 g/l), and the addition of potato fruit water at 0%, 5%, and 10% (w/v). YPG media, containing varying concentrations of glucose (80 g/l, 100 g/l, 120 g/l), yeast extract (10 g/l), and peptone (20 g/l), was autoclaved at 120 °C for 15 minutes. Heat-treated Potato fruit water was added after autoclaving.

Each experiment involved an aerobic phase with a 50 µL inoculum of Ebbegarden (Y9) in 50 ml of YPG media (n=1) at 25 °C on an orbital shaker at 150 rpm for 24 hours. After the aerobic phase, the anaerobic phase began in the incubation cabinet at varying temperatures, each lasting an additional 24 hours. To monitor anaerobic fermentation, the media bottle with YPG media and Y9 inoculum was tightly sealed with parafilm to prevent air entry. As yeast fermented, CO_2 gas moved the syringe piston upward, indicating the reading value. Readings were taken every 1-2 hours, with the setup left overnight by resetting the syringe piston at 0ml. This process continued until gas evolution stopped, maintaining anaerobic conditions for approximately 48-50 hours. Samples were collected at 0 hours (initial phase) and 48 hours (after the anaerobic phase) for glucose and ethanol tests using enzymatic methods and HPLC.

3.2.4. Model Validation Experiment

In the model validation experiment, the aim was to confirm the predictions modelled by the Design of Experiment (DOE). The experiment was carried out under the determined optimal conditions at 40 °C temperature, 78 g/l glucose concentration, and 4.6% potato fruit water (PFW). Triplicate experiments were conducted using YPG medium, and inoculum sizes of 50 µl and 100 µl were tested (n=3). Each experiment comprised a 24-hour aerobic phase, initiated by adding the Y9 inoculum to the YPG medium, followed by a 48-hour anaerobic phase with a total experimental duration of 72 hours. Glucose and ethanol were analysed by using enzymatic and HPLC methods.

2.2.4 Immobilized Yeast Ethanol Fermentation in Continuous Flow Stirred Tank Reactor (CFSTR) Model

2.2.4.1 Experimental Design

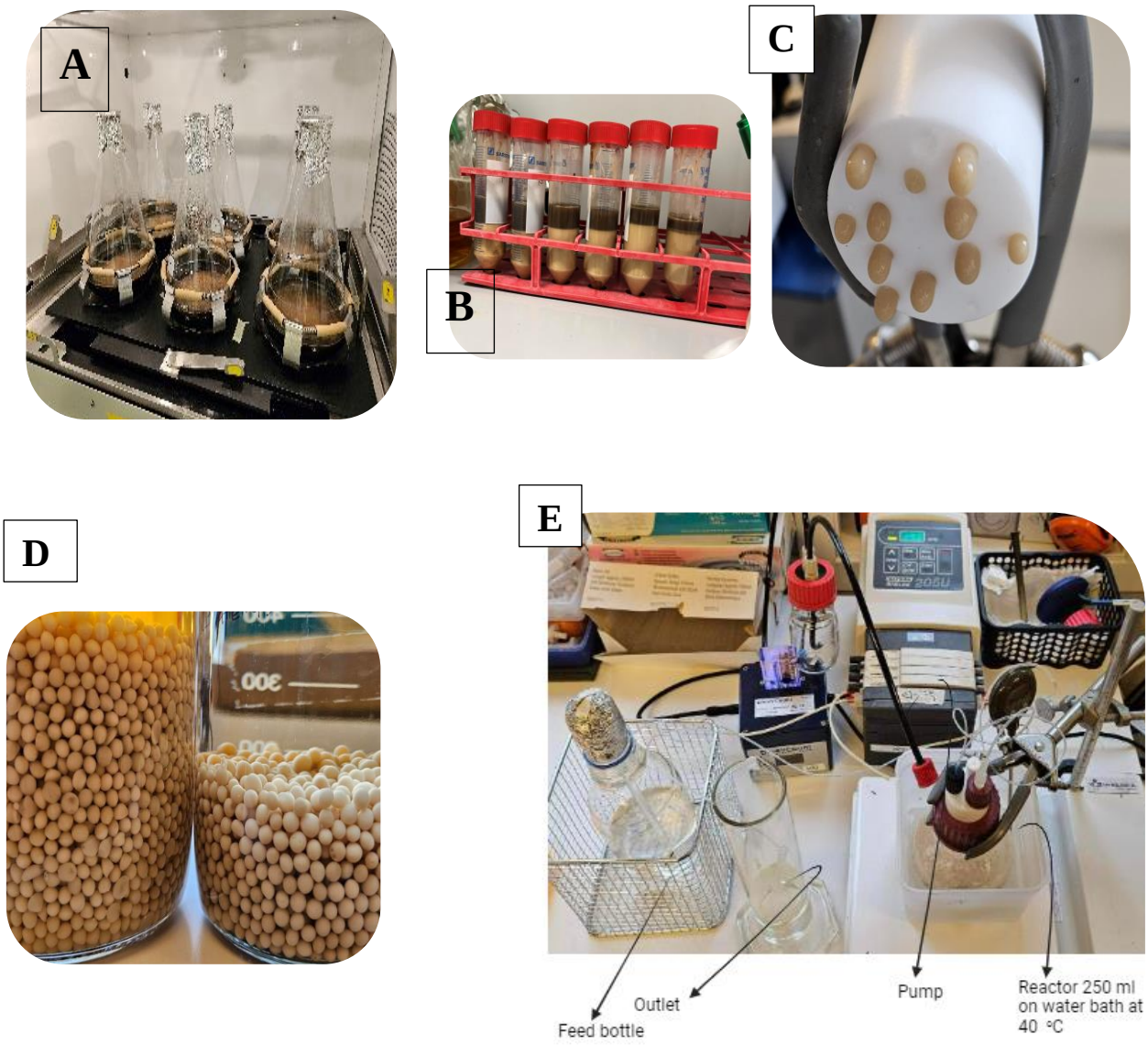

Figure 7 illustrates the process of preparing immobilized yeast alginate beads and their use in CFSTR model. Panel A shows the growth of yeast strain Ebbegarden, Panel B displays the harvested yeast biomass, Panel C shows a 4% alginate suspension prepared by +cell, Panel

D displays the yeast alginate beads with a diameter of 3-5 mm, and Panel E depicts the CFSTR setup.

The immobilized ethanol reactor involved yeast pre-growth, biomass harvesting, alginate bead production, and CFSTR setup. Yeast was pre-grown in KYM media and biomass was collected after centrifugation. Alginate beads were produced by combining yeast cell suspension with sodium alginate (4%) at a ratio of 1:1 (v/v). The resulting immobilized yeast bead was used in the CFSTR setup and dripped into a $CaCl_2$ solution facilitating controlled glucose feeding and dilution rates. This brief outline encapsulates the essential stages of the CFSTR experimental sequence.

2.2.4.2 Immobilization Process

The entrapment method was used for immobilized bead production. The production of yeast beads consisted of three steps: Fermentation medium preparation, biomass production and cell harvest, alginate preparation, and bead casting.

2.2.4.3 Fermentation Medium Preparation

The following solutions were prepared for the fermentation medium i.e., minimal N-free glucose medium.

The fermentation medium was prepared by combining 125 ml of varying concentration glucose solutions, 50 ml of 0.1M CaCl2, 125 ml of 0.1M succinate, and 5 ml of yeast vitamin (100x stock solution). These components were added to 195 ml of water to achieve a total volume of 500 ml for the fermentation medium.

2.2.4.4 Yeast Biomass Production

KYM media was prepared in six 3000ml Erlenmeyer flasks, each containing 750 ml of KYM media with 20% molasses and additional glucose at three levels in a pairwise bottle (30 g/l, 60g/l). The Ebbegarden yeast was pre-cultured in KYM inoculated at 37 ^{o}C and used for inoculation. Yeast growth was observed after 24 hours of stirring (150 rpm) at 30 ^{o}C in a New

Brunswick Innova 44 shaking incubator. The culture broth was collected and centrifuged (SORVALL Evolution) at 5000 rpm for 15 minutes (SLA 3000). The resulting yeast pellets were measured and stored in a cooled room for subsequent use in the immobilization process.

2.2.4.5 Alginate Solution Preparation

A 4% Na-alginate solution was prepared by dissolving 32.0 g of alginic acid in 800 ml of water. The Highly Viscous solution underwent intense mixing using a magnetic stirrer for 16 hours to solubilize. Subsequently, the solution was filtered for any lumps using a metal screen funnel.

2.2.4.6 Bead Production Process

To produce Ca-alginate beads, the following solutions were prepared. 1000 ml of 0.1M $CaCl_2$ and 0.2M NaCl, 500 ml of 0.9% NaCl.

The process of casting the beads involved combining 25 ml of wet yeast cells with 40 ml of a 0.9% NaCl solution. Then, 40 ml of 4% Na-alginate was mixed with 40 ml of the yeast cell suspension to create a homogeneous mixture. This mixture was then pumped through a mounted manifold with thin openings, creating a shower of droplets falling approximately 20 cm into the $CaCl_2$-NaCl solution. The alginate solidified spontaneously in contact with Ca^{2+} ions. The casting setup ensured the uniform spherical size of the beads. The solution was kept at slow circulation on a magnetic stirrer.

The resulting round-shaped beads were collected on a Buchner funnel after 1 hour of soaking and amounted to 60 ml. The beads were stored in a solution consisting of 0.1M succinate (125 ml), 0.1M $CaCl_2$ (50 ml), mixed with remaining water (325 ml) up to 500 ml to maintain their integrity and viability. All the bead production was performed by the same method.

2.2.5 Sample Collection and Analysis

During the CFSTR experiments, 2 ml samples were collected from the reactor outlet at regular intervals over 3-4 hours and analysed for glucose and ethanol. The collection intervals were 0, 20, 40, 60, 90, 150, and 210 minutes. Yeast beads were re-used for 9 cycles each casting approximately 4 hours.

2.2.6 Screening test in immobilized yeast ethanol fermentation using CFSTR model

2.2.6.1 Pilot Experiment Overview

The pilot experiments provided an initial exploration of the Continuous Stirred Tank Reactor (CFSTR) system, offering valuable observations into its functionality.

Four pilot experiments were conducted to evaluate the performance of the CFSTR system. Two experiments involved 2% (w/v) glucose concentration at dilution rates of 0.2 /h and 0.9 /h, while two experiments involved 1% (w/v) glucose concentration with 0.2 /h and 0.9 /h dilution rates.

To set up the CFSTR, 120 ml of fermentation medium was mixed with 60 ml of yeast beads in a reactor bottle total volume of 150 ml. Another bottle containing 300 ml of fermentation medium was used as the feeding bottle. The fermentation medium was continuously fed from the feeding bottle to the reactor bottle. This flow system provided a constant liquid volume (v) by a peristaltic pump. An outlet line on the same pump head served as the outflow that contained the fermentation product was collected in a cylinder as shown in Figure 6.

The pilot experiments were performed to provide the initial understanding of the CFSTR model, the impact of glucose concentration, dilution rate, and yeast bead size.

The RPM setting of peristaltic pumps was adjusted to give a flowrate corresponding to a set dilution rate. The dilution rate is described as shown in Equation 2:

Dilution rate (D = F/V (/h)= Volumetric flow rate (ml/h) constant reactor volume(ml) — (2)

2.2.7 Design of Experiment-2 (DoE2)

Table 3: Design of Experiment-2 (DoE-2) consisting of seven experiments with varying Glucose and dilution factors.

Exp No	Run Order	Glucose concentration (g/l)	Dilution rate (/h)
1	3	40	0.18
2	7	80	0.18
3	6	40	0.9
4	1	80	0.9
5	5	60	0.34
6	2	60	0.34
7	4	60	0.34

Table 3 presents the seven experiments conducted using the Design of Experiment (DoE-2) with the immobilized yeast bead (Y9). MODDE Pro software (Sartorius Umetrics) was used to make a full factorial design with two factors at two levels and a centerpoint. The factors were glucose concentration (40 g/l, 60 g/l, and 80 g/l), and dilution factors (0.18 /h, 0.34 /h, and 0.9 /h).

In the Design of the Experiment (DoE-2) with immobilized yeast bead (Y9), seven experiments were conducted using the CFSTR model at 40 °C. Each experiment included a unique combination of glucose concentration and dilution rates.

For each experiment, the CFSTR setup involved mixing 120ml of fermentation medium with 60 ml of yeast beads in the reactor bottle (1:2) with a total volume of 150 ml. Simultaneously, a feeding bottle with 300ml of fermentation medium facilitated continuous flow into the reactor bottle. Samples were collected at specific time intervals (0, 20, 40, 60, 90, 150, and 210 min) from the reactor bottle outlet.

Enzymatic and HPLC analyses were conducted to analyse glucose consumption and ethanol production in the fermented product. Table 3 summarizes the details of the seven experiments conducted under the DoE-2 framework.

2.2.8 Model Validation Experiment

A model validation experiment was conducted to confirm modelled from the DoE-2 outcome predictions. Optimal conditions, 80 g/l glucose 0.34 /h dilution rate, were applied in the CFSTR model at 40 °C in the replicate experiments (n=2). This experiment validated the model under specified optimal conditions.

2.3 Analytical Methods

HPLC and enzymatic methods were used for the glucose and ethanol analysis.

Enzymatic UV determination of glucose and ethanol was measured according to R-Biopharm manual instruction at 340nm (see Appendix).

Glucose and ethanol were calculated as follows:

Glucose concentration (g/l) = $0.864 * \Delta A_{glucose}$ — (3)

Concentration ethanol (g/l) = $0.190 * \Delta A_{ethanol}$ — (4)

2.3.1 HPLC Analysis

The HPLC was performed on the Dionex Ultimate 3000 system using an RI detector. The column was a Rezex RHM Monosaccharide H^+; H23-11233=25; 300*7.8; CV= 9ml

This analysis was run isocratically at 60 ^oC with 0.6 ml/min flow. The mobile phase was filtered, by distilled water. Detection was done with an RI detector. Column: Rezex RHM Monosaccharide H^+; H23-11233=25; 300*7.8; CV= 9ml

- Max pressure 69 bar
- Max temperature 85^{o}C

2.3.2 Dry weight determination

After completion of anaerobic fermentation, a 25ml sample was taken from the biomass-containing medium and placed in a beaker. The beaker was covered with aluminium foil and dried in a hot oven cabinet at 80 °C for 24 hours. The dried biomass in the beaker was then weighed using a precision analytical balance to determine the dry weight.

$$\text{Dry weight (g/l)} = \frac{\text{Weight of dried biomass (g)}}{\text{Total volume of sample (l)}} \quad — (5)$$

2.3.3 Fermentation parameters

Ethanol Yield (g/g) was calculated as final ethanol concentration (g/l) to the sugar consumption (Initial glucose (S_R) - Final glucose concentration (S_r). Sugar conversion (%) was calculated as a ratio of sugar consumption to the initial sugar concentration (g/l). Ethanol productivity (g/l/h) was calculated as the ratio of ethanol concentration (g/l) and fermentation time (h).

$$\text{Yield (g/g)} = \frac{g\ EtOH\ produced}{g\ glucose\ consumed} \quad — (6)$$

$$\text{Sugar conversion (\%)} = \frac{Glu(initial) - Glu\ (end)}{Glu\ (total)} \quad — (7)$$

$$\text{Productivity (g/l/h) in batch mode} = \frac{EtOH\ at\ steady\ state\ (\frac{g}{l})}{Fermentation\ time\ (h)} \quad — (8)$$

$$\text{Productivity (g/l/h) in CFSTR} = [EtOH] * D \quad — (9)$$

2.3.4 Statistical Methods

Modde program

A full factorial design method was used to investigate the main factors that influence ethanol production optimization during batch and continuous fermentation. The purpose of this statistical design approach was to obtain a model that describes the impact of each factor and its interactions on ethanol production. The full factorial design with three factors during batch fermentation and two factors during continuous fermentation was carried out in 11 and 7 runs, respectively, with three center points carried out separately. A randomized order was applied to minimize systematic errors. The optimization process considered three factors: glucose concentration, temperature, and nitrogen source (PFW) for the batch model, and dilution rate and glucose concentration for the continuous model. The factors involved in the model are shown in Tables 2 and 3. The goodness of fit and parameter significance of the model was investigated using the full factorial design method. The analysis of results and statistical calculations were performed using MODDE Pro software (Sartorius Umetrics). The DoE methodology is useful for obtaining maximum information from a minimal number of well-planned experiments by varying all the process factors simultaneously.

3. Results

3.1 Pilot Experiments:

The pilot experiments were conducted to better understand the dynamics of yeast fermentation, with a particular focus on the selected Kveik yeast strains Voss (Y1), Ebbegarden (Y9), Eitrheim (Y14), and Stalljen (Y22). In Pilot 1, yeast strain Ebbegarden (Y9) was used in YPG and KYM media. The experiment involved observing CO_2 gas formation with the syringe piston expansion method and monitoring glucose consumption at the start (0 h), after anaerobic phase (24 h), and end (48h) using glucose sticks. Pilot 2 was introduced with supplementation with yeast vitamins to assess their impact on gas yield and ethanol production, drawing comparisons with the baseline of Pilot 1. Pilot 3 explored variations in yeast pre-growth conditions, altering the aerobic phase temperature from the initial room temperature (25 °C) to an elevated 37 °C. These preliminary experiments were essential in establishing foundational aspects of the experimental protocol, determining suitable temperature and duration for the aerobic phase, and exploring potential factors influencing yeast behaviour under different conditions. The observations made during these pilot experiments provided valuable insights guiding subsequent screening and optimization phases in this study.

Optical density (OD_{660})

Table 4: The optical density (OD_{660}) at different time points: 0h (initial phase), 18h (after aerobic propagation), 26h (during the fermentation process), and 48h (after completing the anaerobic fermentation phase). P1, P2, and P3 indicate the pilot experiments.

	0 h	18h	26h	48
P1	0.15	0.51	3.27	1.41
P2	0.26	0.65	3.53	0.70
P3	0.15	2.52	4.45	4.4

Table 4 illustrates the optical density data from OD660 at different time points (0 h, 18 h, 26 h, and 48 h) for conditions P1, P2, and P3 (1:5), it was observed that P1 and P2 showed an

initial increase in growth from 0 h to 26 h (0.15 to 3.27 and 0.26 to 3.53), followed by a reduction at 48 h (1.41 and 0.70). On the other hand, P3 demonstrated consistent growth, maintaining a stable optical density of 4.4 at 48 h.

During the anaerobic fermentation phase, it is essential to convert glucose into ethanol and CO_2 rather than promote yeast growth. While P1 and P2 showed good gas formation during this phase, P3 did not produce gas. However, the higher temperature used during the aerobic phase for P3 (37 °C) may have contributed to the lack of gas production during the anaerobic phase, as the yeast may have already produced some ethanol during the aerobic phase.

3.1.1 Pilot Experiment 1

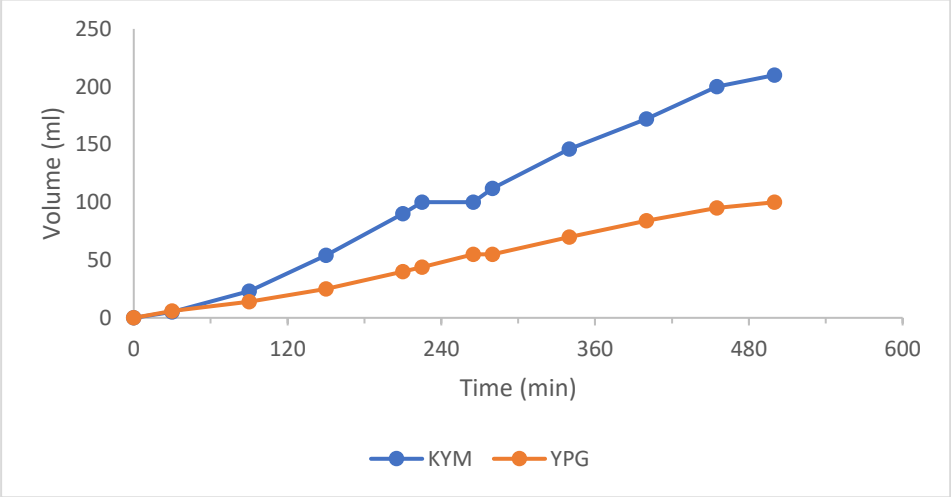

Figure 8: CO_2 formation during anaerobic phase by Kveik yeast Ebbegarden (Y9), cultured in the syringe piston model KYM and YPG media (50ml) at 37 °C (n=1).

Figure 8 illustrates the response of the Kveik yeast Ebbegarden (Y9) under cultured on YPG and KYM media. The experiment consisted of an aerobic phase at room temperature (25 °C) for 21-24 hours and an anaerobic phase at 37 °C for almost 8 hours. Ebbegarden growing in KYM medium has more than two times gas development than compared to growing in YPG medium with the end values being 210 ml CO_2 for KYM and 100 ml for YPG.

3.1.2 Pilot Experiment 2

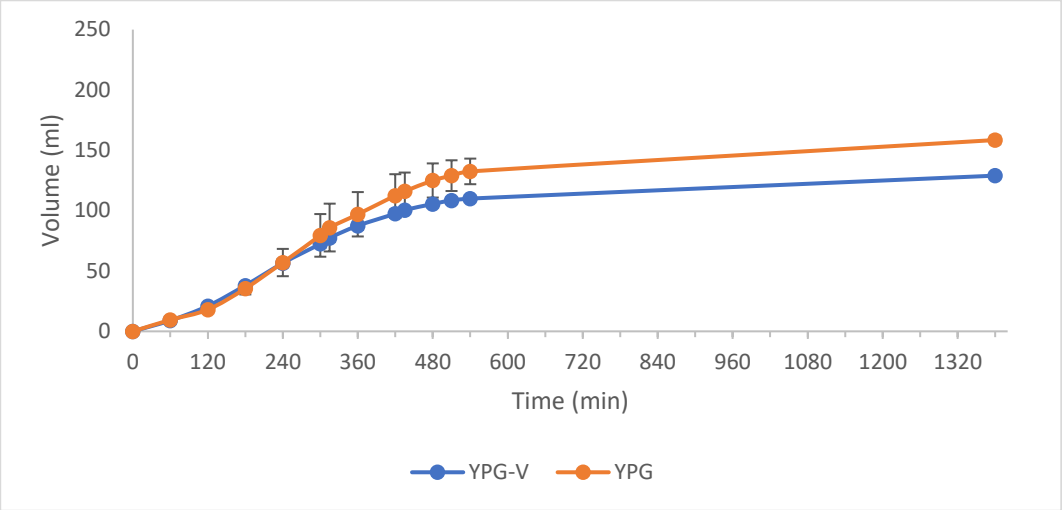

Figure 9: Comparison of CO_2 formation in an anaerobic phase between YPG medium with and without yeast vitamin supplement at 37 °C (n=2).

Figure 9 shows the result of pilot experiment 2, which aimed to investigate the impact of yeast vitamins on gas yield and ethanol production. The experiment was designed similarly to Experiment 1, with a 24-hour aerobic phase at 25 °C, followed by an additional 24-hour anaerobic phase at 37 °C. No significant difference in CO_2 formation was observed during the first 6 hours with a CO_2 volume of around 90 ml in both mediums with and without yeast vitamins, the medium without vitamins exhibited a slight increase in gas formation compared to the medium with vitamins. At the end of 48 hours, the medium without vitamins stabilized at 158.5 ml of CO_2, while the medium with vitamins remained constant at 129 ml of CO_2.

3.1.3 Pilot Experiment 3

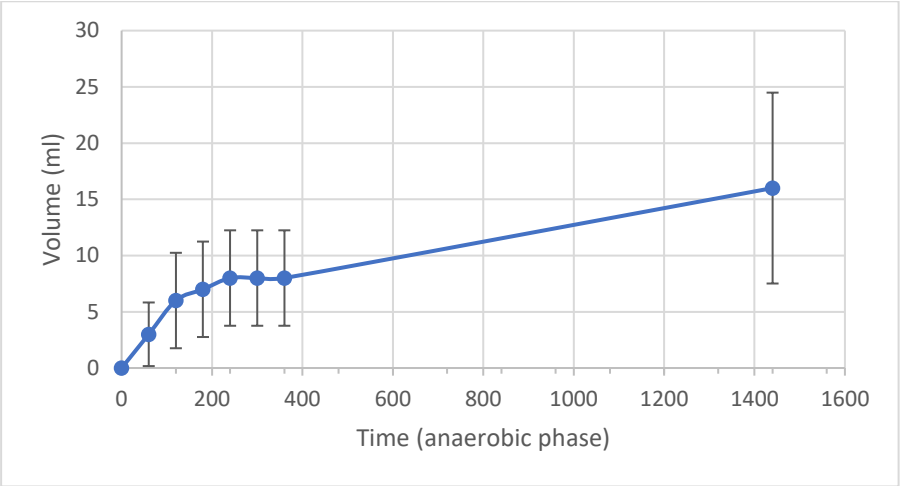

Figure 10: CO_2 formation of YPG medium at 37 °C (n=2)

Figure 10 shows the results of an experiment aimed at testing the temperature for the aerobic phase (yeast propagation) before initiating the anaerobic phase. The aerobic phase was conducted at 37 °C, followed by a 24-hour anaerobic fermentation, also at 37 °C. As indicated in the figure, with sugar being exhausted during the aerobic phase, the recorded CO_2 formation was only 16 ml after 24 hours of fermentation. The replicate variation was significant.

From the pilot experiment, the following points were observed:

- Pilot 1: Difference YPG-KYM on available C-source, higher gas yield in Kym. YPG is easier to work with for defined sugar levels.
- Vitamin supplement on apparent effect on YPG. YPG is a balanced medium, and vitamins are not a limiting factor.
- Pilot 3: Propagation/ aerobic phase run at 25 °C to avoid depleting C-source.

Syringe piston model useful for rapid screening, supplemented with the analytical assay.

3.2 Screening of temperature robustness and ethanol production

3.2.1 CO_2 formation during the anaerobic phase at varying temperatures.

In the experimental setup using a syringe model, four types of Kveik yeast were tested under four different temperature conditions, across two types of medium, resulting in a total of 32 experiments (Fig 11).

Table 5: Accumulated gas (ml) after 24 hours of anaerobic fermentation of Kveik yeast Y1, Y9, Y14, and Y22 on YPG and KYM media

Kveik yeast	32ºC		37ºC		42ºC		47ºC	
	YPG	KYM	YPG	KYM	YPG	KYM	YPG	KYM
Y1	113	339	135	321.5	165	345	143	117
Y9	134	274.5	67	310	89.5	414	176	388
Y14	74	382	115	439	123	332	146	211
Y22	98	281	128	394	178	362.5	169	186

Table 5 displays the accumulated gas production (ml) after 24 hours of anaerobic fermentation for Kveik yeast Y1, Y9, Y14, and Y22 on both YPG and KYM media at temperatures of 32 °C, 37 °C, 42 °C, and 47 °C.

At a temperature of 37 °C, Y9 produced 67 ml of CO_2 on YPG media and 310 mL on KYM media. Y14, on the other hand, produced 115 ml on YPG media and 439 mL on KYM media. Interestingly, all Kveik yeasts produced nearly equal amounts of gas on YPG media, but in KYM media, there was almost double the amount of gas formation compared to Y1 and Y22 at all temperatures. Moreover, at a temperature of 47 °C, the gas production of Y1 and Y22 consistently decreased suggesting that Y9 and Y14 are better at producing CO_2, especially at elevated temperatures and in KYM media. These results suggest that Y9 and Y14 are particularly suitable for gas formation in the KYM medium compared to the YPG medium and more precisely at elevated temperatures (37 ^oC). The graphical representation of the CO_2 curve is shown in Figure 11.

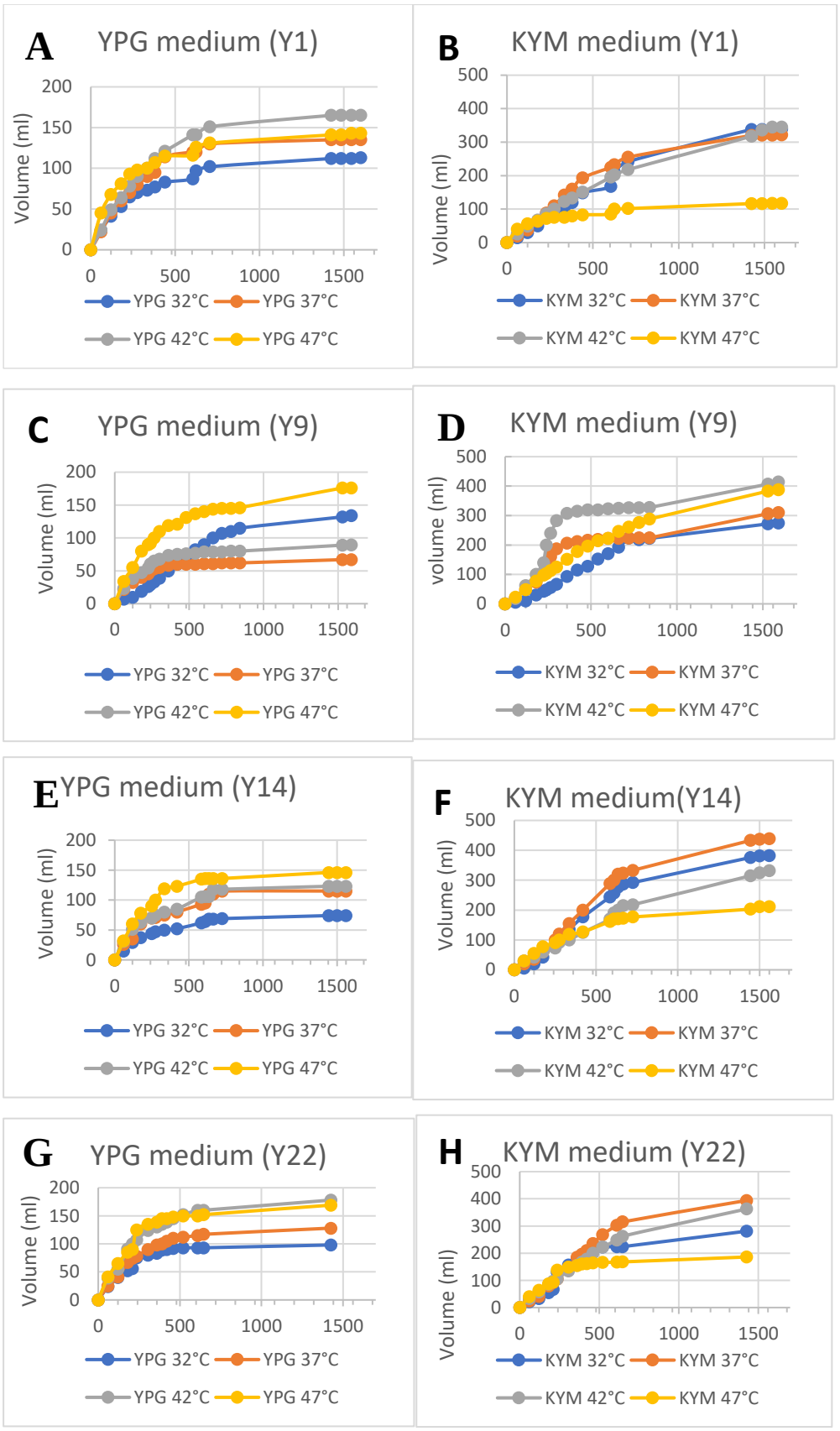

Figure 11: CO_2 gas formation during ethanol fermentation at 32 °C, 37 °C, 42 °C, and 47 °C in KYM and YPG medium (n=1).

3.2.2 Glucose consumption and ethanol production by YPG and KYM media at various temperatures.

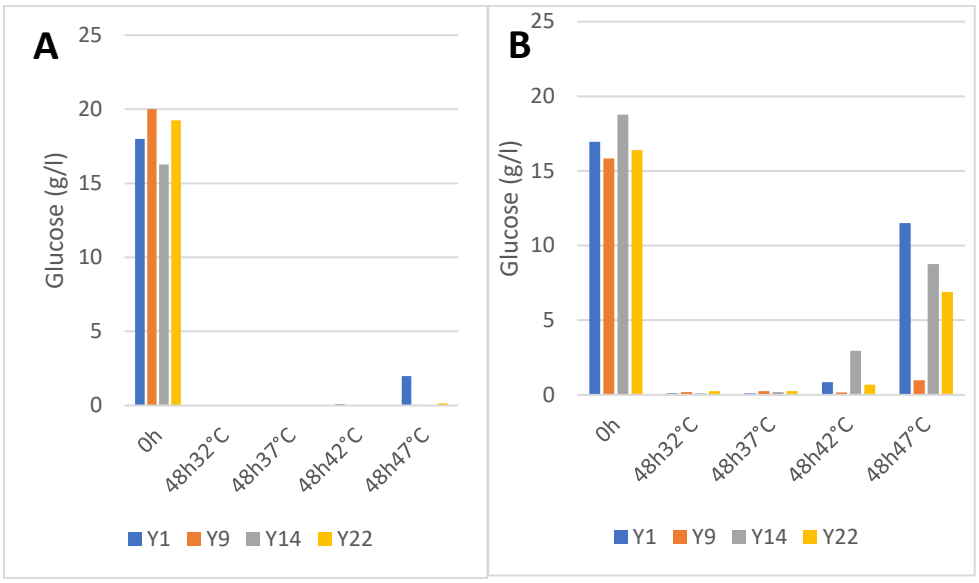

Figure 12: Glucose consumption by Kveik yeast Voss (Y1), Ebbegarden (Y9), Eitrheim (Y14) and Stalljen (Y22) at temperatures 32 ºC,37 ºC 42 ºC, 47 ºC in YPG medium and KYM medium. Bar represents glucose (n=1) at the start (0h) and after 48 hours at respective temperatures.

In this combined representation, denoted as Figure 12, panel A represents the glucose consumption of Kveik yeast (Y1), (Y9), (Y14), and (Y22) at different temperatures (32 °C, 37 °C, 42 °C, and 47 °C) in YPG medium analysed by HPLC. The bar diagram illustrates the yeast's ability to metabolize glucose, containing 16 g/l glucose for all tested temperatures.

Moving to Panel B, Figure 12 shows the glucose consumption by the same yeast in a KYM medium under varying temperatures. The bar diagram shows the glucose consumption of the yeast in KYM media, with a glucose concentration of 16 g/l molasses. In the YPG medium, almost all glucose has been exhausted and converted into ethanol. In the KYM medium, most of the glucose was consumed at 32 °C and 37 °C. However, at 42 °C, there was still approximately 2 g/l of glucose remaining after 48 hours. At 47 °C, all Kveik yeast strains, except Y9 (with 0.98 g/l remaining), have struggled to convert sugar into ethanol with rest values of 11.51 g/l for Y1, 8.77 g/l for Y14 and 6.9 g/l for Y22.

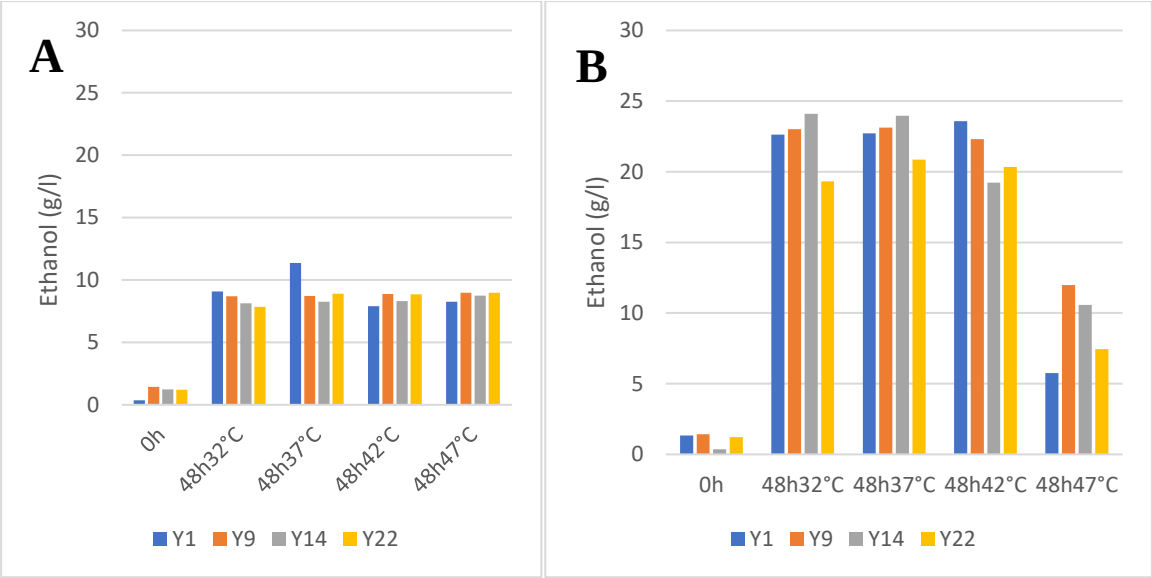

Figure 13: Ethanol production by Kveik yeast Y1, Y9, Y14 and Y22 at temperature 32 ^{0}C, 37 ^{0}C, 42 ^{0}C and 47 ^{0}C in YPG and KYM medium (n=1).

Figure 13 illustrates the ethanol production profiles of Kveik yeast strains Y1, Y9, Y14, and Y22 in both YPG and KYM media, analyzed through HPLC in Panel A and Panel B. The bar graph shows ethanol levels after 48 hours of fermentation at different temperatures (32°C, 37 °C, 42 °C, and 47 °C). In the KYM medium (Panel B) at temperatures ranging from 32°C to 42 °C, there's a paradoxical doubling of ethanol production, reaching 21-23 g/l, compared to the YPG medium where the average remains at approximately 7-11 g/l, consuming almost all glucose concentration. This occurs despite similar initial glucose concentrations of 16-17 g/l in both YPG and KYM media (Fig. 12). However, at the elevated temperature of 47°C, ethanol levels decrease to 7-11 g/l. Additionally, both KYM and YPG media show about 2 g/l of ethanol at 0 hours, produced during the pre-growth phase. Ethanol yield in YPG was 0.50 g/g, while in KYM it was 1.17 g/g, which was twofold higher than YPG indicating other glucose in Molasses that are consumed or converted.

3.3 Glucose conversion and ethanol production in selected Kveik Yeast Y9 and Y14.

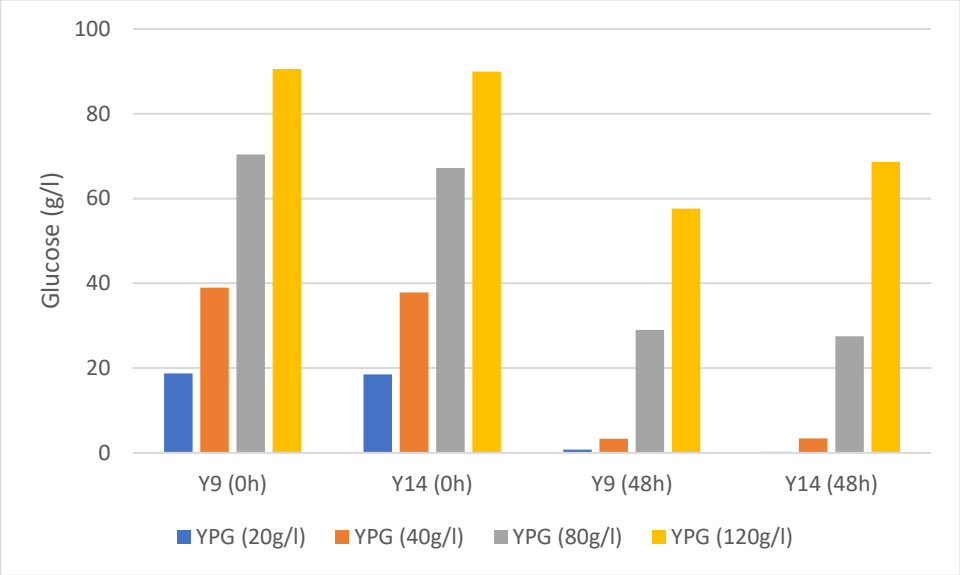

Figure 14: Glucose consumption by Kveik yeast Y9 and Y14 at glucose concentrations at 42 ^{0}C (n=1). The bar diagram represents the start and rest glucose (at t=0h and t=48h)

The Kveik strains Y9 and Y14 were investigated from their performance on temperature robustness and further glucose consumption with sugar levels that are more realistic in industrial situations.

Figure 14 shows the glucose utilization of two Kveik yeast strains, Y9 and Y14, at a temperature of 42 °C in YPG medium. The experiment involved varying glucose concentrations of 20 g/l, 40 g/l, 80 g/l, and 120 g/l over 48 hours, including aerobic and anaerobic fermentation phases. At lower concentrations (20 g/l and 40 g/l), both Y9 and Y14 efficiently consumed glucose. However, at 80 g/l and 120 g/l, glucose was only partly consumed (Table 6). During ethanol fermentation in the syringe model, the growth of Kveik yeast Y9 and Y14 showed gas evolution peaks within 24 hours of the anaerobic phase (total 48 hours) and stabilized after that.

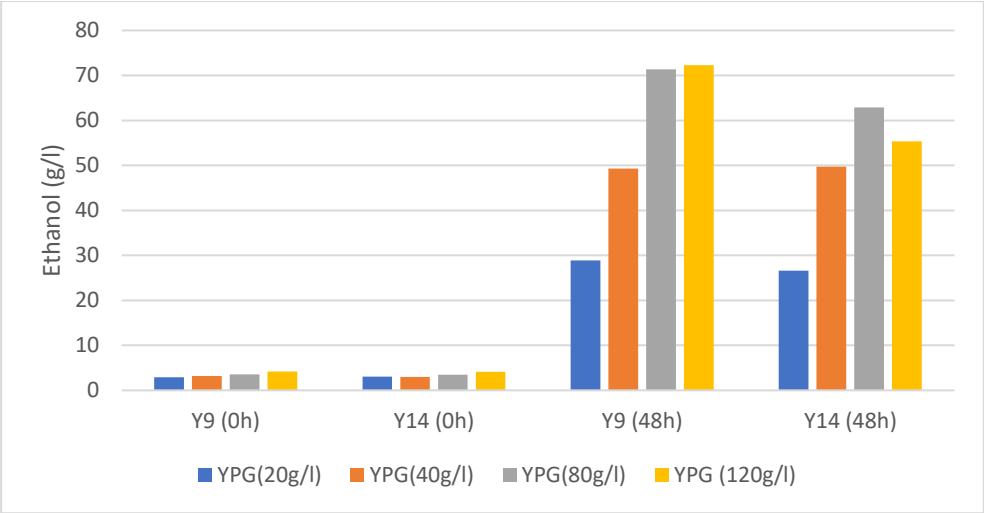

Figure 15: Ethanol production by Kveik yeast #Y9 and #Y14 at glucose concentrations 20 g/l, 40 g/l, 80 g/l and 120 g/l at 42 °C (n=1).

Figure 15 illustrates the ethanol production of two different types of Kveik yeast, Y9 and Y14. The experiment was conducted in YPG medium at a temperature of 42 °C. Higher ethanol production was observed with glucose concentrations of 80 g/l and 120g/l at 48 hours, reaching around 65 g/l whereas, YPG medium with 20 g/l glucose exhibited lower ethanol production i.e. 28.89 g/l and 26.62 g/l compared to those with higher glucose concentration.

Table 6: At different glucose concentrations in YPG (42 °C), ethanol yield (Yp/s), and glucose conversion (%) for Kveik yeast Y9 and Y14.

	20 g/l		40 g/l		80 g/l		120 g/l	
	EtOH Yield (g/g)	Glucose conversion (%)	EtOH Yield (g/g)	Glucose Conversion (%)	EtOH Yield (g/g)	Glucose Conversion (%)	EtOH Yield (g/g)	Glucose Conversion (%)
Y9	0.17	96	1.38	91.5	1.73	58.7	2.20	36.3
Y14	0.15	98.6	1.44	91	1.58	59	2.61	23.6

Table 6 represents ethanol yield (Yp/s) from consumed glucose for Kveik yeast Y9 and Y14 at various glucose concentrations. The highest ethanol yield was observed at 120 g/l glucose concentration, with 2.20 g/g values for Y9 and 2.61 g/g for Y14. Lower ethanol yields were recorded at 20 g/l, and intermediate yields were observed at 40 g/l and 80 g/l glucose concentrations. The data implies a potential overestimation of ethanol and underestimation of

glucose, especially considering that the generally accepted maximum value for ethanol yield on glucose is around 0.5 g/g. Overall kveik Y9 (Ebbegarden) demonstrates the best conversion potential for further exploration.

3.4 Optimizing Ethanol Production in Kveik Y9

A systematic Design of Experiments (DOE1) using a full factorial design of three factors was implemented to screen conditions for ethanol production by kveik Y9. The table below presents the experimental table, featuring variations in temperature, glucose concentration, and potato fruit water supplementation as a nitrogen source.

Table 7: Design of Experiment (DOE1) containing 11 sets of experiments with the first 1-8 being the corner of the design and 9-11 being the central point of graphic design to screen conditions for ethanol production using Kveik yeast Ebbegarden (Y9).

Exp No	Tempt (ºC)	Glucose (g/l)	Potato fruit water (v/v) %	Gas volume (ml)	Ethanol (g/l)	Glucose conversion (%)
1	39	80	0	785	55.4	98.4
2	45	80	0	265	14.3	12.3
3	39	120	0	784	52.5	59.9
4	45	120	0	199	12.4	11.3
5	39	80	10	753	49.6	98.0
6	45	80	10	138	9.4	11.9
7	39	120	10	757	44.0	49.5
8	45	120	10	156	10.7	6.0
9	42	100	5	609	43.5	42.3
10	42	100	5	610	43.5	42.4
11	42	100	5	611	43	42.0

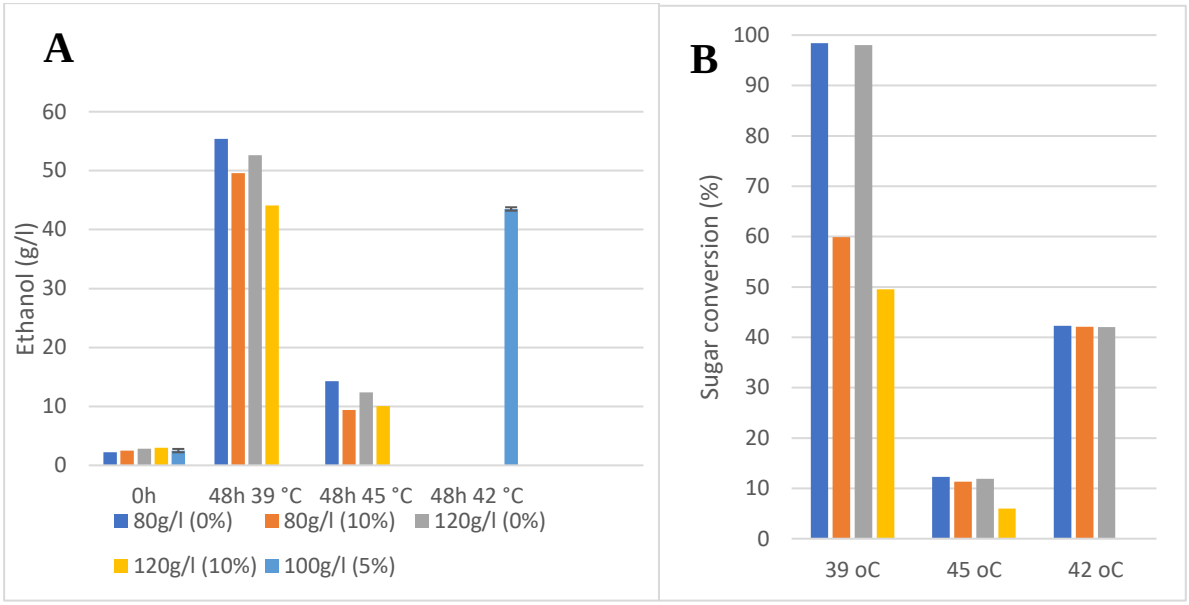

Figure 16: Panel A represents the ethanol production at 39 °C, 42 °C, and 45 °C with 80 g/l, 100 g/l 120 g/l of glucose concentration with 0%, 5%, and 10% of potato fruit water respectively as a nitrogen source. The single bar at 48h 42°C represents the central point experiment (n=3) with standard deviation =0.2. 0h indicates the start of the aerobic phase. Panel B displays the correlation between temperature and sugar conversion.

The amount of ethanol (g/l) produced from fermentation at different temperatures was analysed at the inoculation (0h) and after completion of fermentation 48 hours (Fig 16). The sample fed with 80 g/l of glucose and 0% potato fruit water and fermented at 39 °C produced the highest amount of ethanol i.e., 55.4 g/l, which was closely followed by all the fermentation samples at 39 °C. Whereas samples fermented at 45 °C fed with 80 g/l of glucose and 10% of potato fruit water produced the comparatively lowest amount of ethanol at the end of the fermentation i.e., 9.4 g/l. Overall all the samples at 39 o C and 42 °C produced a relatively 4-5 times higher amount of ethanol than those fermented at 45 °C. The production of a small amount i.e. 2 g/l of ethanol before the aerobic phase was observed at the initial phase (0 hours) from all the fermentation samples.

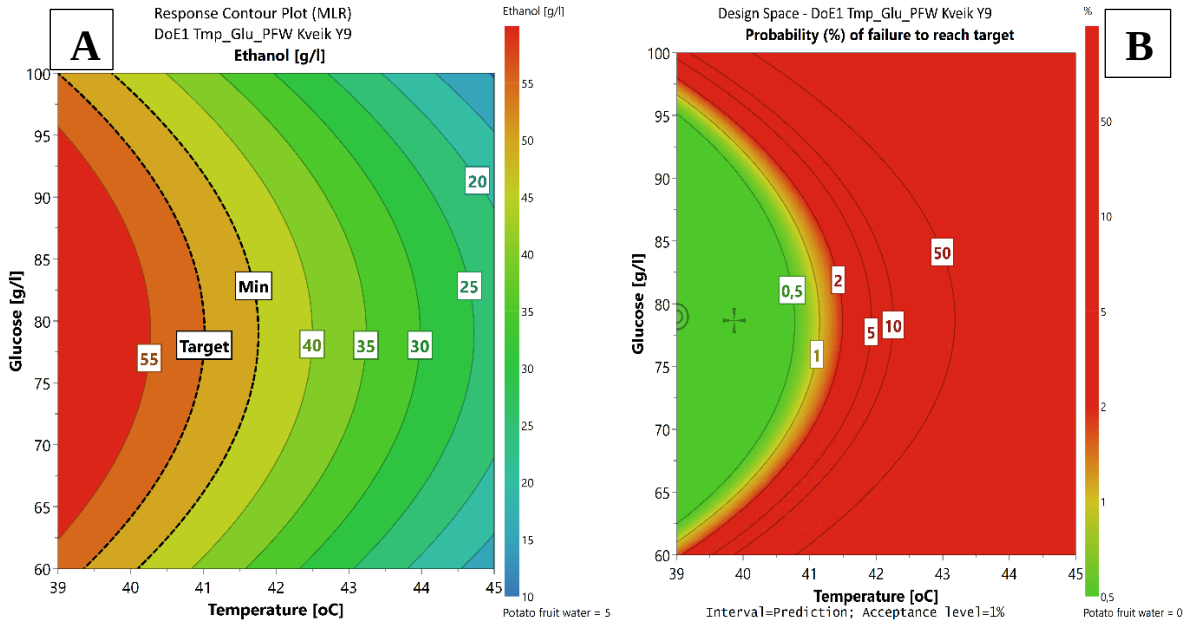

Figure 17: Panel A showing Contour plot and panel B prediction plot (probability of failure to reach target) modeled with temperature, and glucose concentration affecting ethanol production at 0 PFW. Panel A with red colour represents the heat plot with the highest concentration of ethanol, and panel B with green colour indicates the region with the highest probability of best outcomes ($p<0.01$).

After conducting 11 experiments, the ethanol yield, productivity, and glucose conversion data were modelled using the MODDE software (table 7). MODDE produced a contour plot that predicts robust setup conditions for ethanol production as 78 g/l glucose concentration, 4.6 % PFW at 40 ^{o}C.

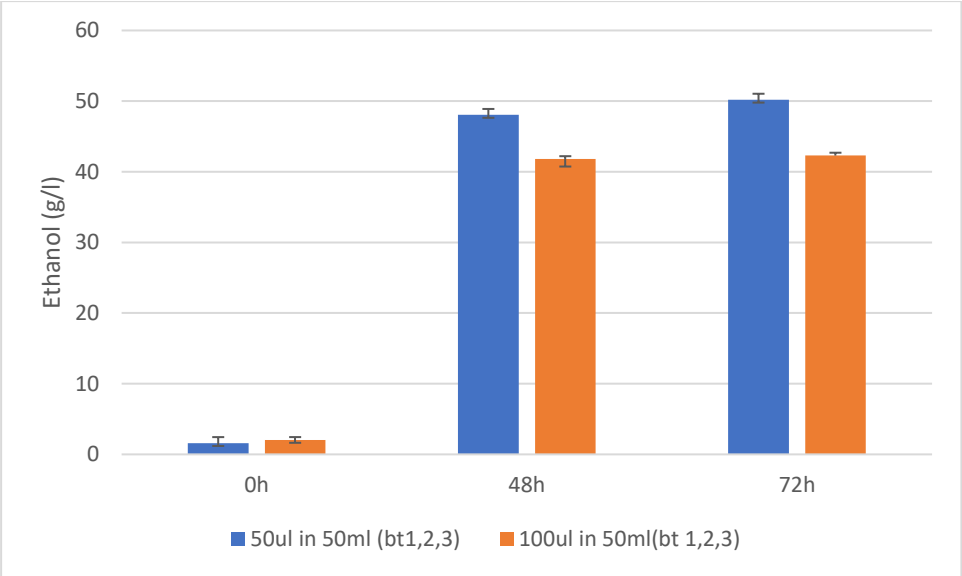

Figure 18: Validation of model data for ethanol production optimization, utilizing predicted conditions obtained from the DoE1 analysis (n=3). The targeted ethanol is 50 g/l

HPLC analysis was used to provide a more accurate and reliable assess ethanol yield and productivity. The data obtained from the experiments was used to create a contour plot and prediction plot (Figure 17 A and B) which helped predict optimal conditions for ethanol production. The contour plot suggested that for a target of 50 g/l ethanol production, the optimal conditions were 78 g/l glucose at 40 °C with 5% Potato Fruit Water (PFW) in YPG medium. Additionally, choosing robust conditions validation experiment evaluated in a triplicate (n=3), the effect of inoculum size on ethanol yield was examined by comparing two sizes: 50 µl and 100 µl. The result showed that at 48-hour (after completion of the fermentation process), the 50 µl inoculum in 50 ml (sbt 1,2,3) yielded 48.04 g/l ethanol, while the 100 µl inoculum in 50 ml (sbt 1,2,3) yielded 41.8 g/l. The comparison between 50 µl and 100 µl inoculum sizes aimed to increase yeast propagation during the aerobic phase (25 ^{o}C) for higher ethanol production during fermentation (40 ^{o}C). However, the observed results, where the 100 µl inoculum did not yield higher ethanol production than the 50 µl inoculum assuming higher inoculum consumed most of the glucose before the fermentation step, thus lower EtOH.

3.5 Immobilization of beads Ebbegarden (Y9) for ethanol production.

3.5.1 Biomass production

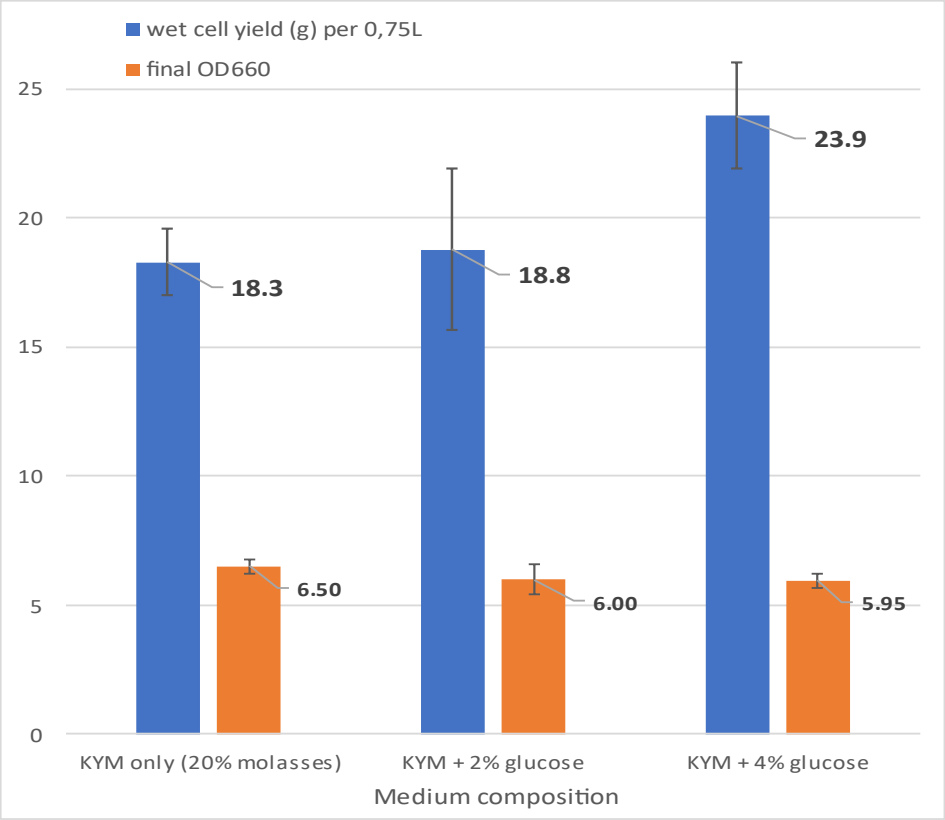

Figure 19: Result of yeast biomass production with the effect of glucose supplement in KYM on the cell yield (gram wet weight per 0.75 L), and final OD_{660} at 30 °C, 150 rpm (n=2)

Figure 19 demonstrates the positive impact of adding 2% and 4% glucose to a medium that initially contained 20% molasses on yeast growth. After incubating aerobically at 30 °C with 150 rpm for 24 hours in a shaking incubator, yields increased from 18.28 g (with 20% molasses) to 18.78 g (with 2% glucose) and further to 23.95 g (with 4% glucose) per 0.75 L medium.

3.5.2 Pilot Experiment

A pilot experiment was conducted as the initial phase of an immobilization study for ethanol production using an alginate yeast bead. The experiment aimed to evaluate the effectiveness of fermentation in yeast beads, specifically Y9 (Ebbegarden), using a minimal glucose medium without nitrogen. The yeast beads were created through the entrapment immobilization method, and the experiment was performed using a continuous flow stirred tank reactor (CFSTR) model at a temperature of 40 °C.

The pilot experiment systematically studied two significant factors: glucose concentration (10 g/l and 20 g/l) and dilution rates (0.2 /h and 0.9 /h) of the glucose feed. Sampling intervals were established at key time points, including 0h, 20, 30, 40, 120, 180, and 240 min, over 3 hours.

The pilot experiment provided crucial information on the performance of yeast beads and determined the optimal fermentation period for future runs. Additionally, the impact of yeast bead size variations on the immobilization process was assessed, offering a comprehensive evaluation of their effectiveness. This approach allowed for a better understanding of the feasibility and efficiency of yeast beads, guiding the subsequent stages of the study.

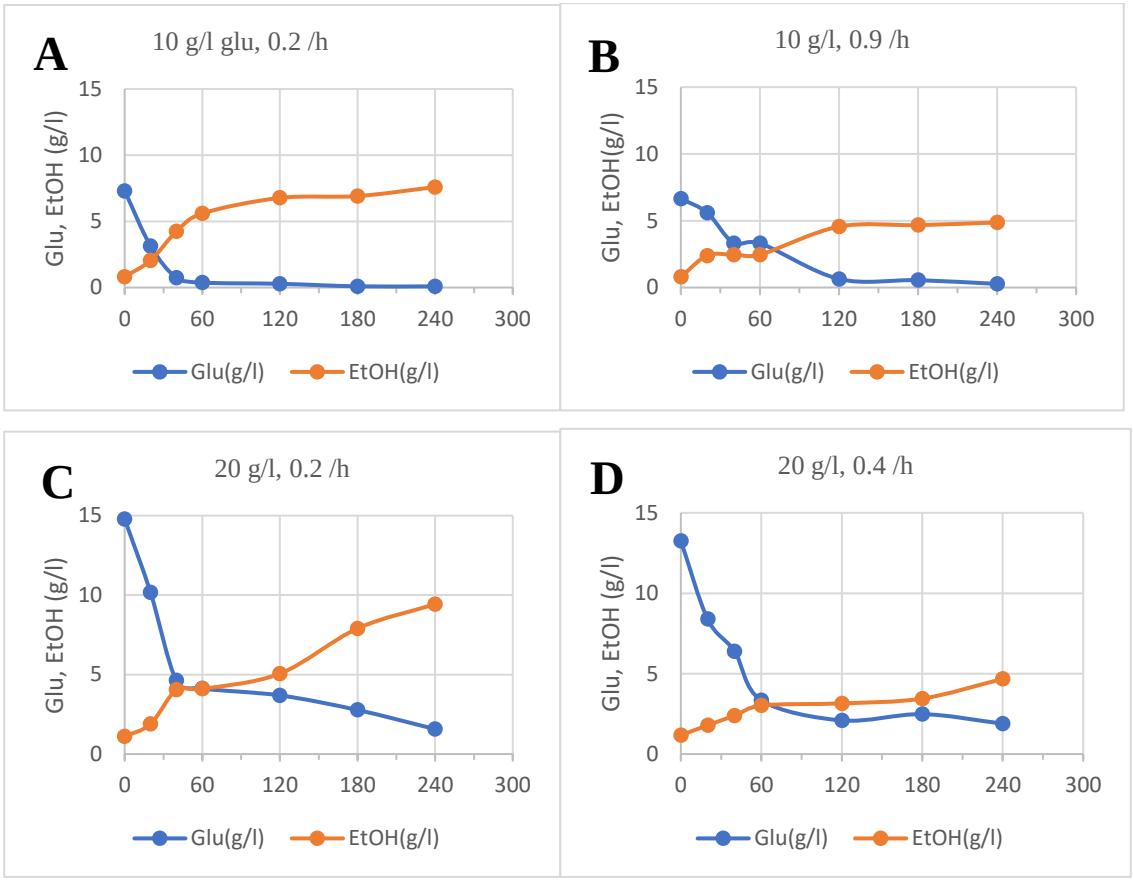

Figure 20: Pilot experiment for immobilized yeast beads using Kveik yeast Ebbegarden (Y9) with the CFSTR model at 40 °C. Panels A, B, C, and D represent the glucose consumption and ethanol production curves 0-240 minutes (n=1).

Panels A and B represent the glucose consumption and ethanol production curves with 10 g/l glucose concentration and 0.2 /h and 0.9 /h dilution rates, illustrating the impact of dilution rates on ethanol production. Panel A, with a 0.2 /h dilution rate, shows higher ethanol production with 7.59 g/l, while Panel B, having a 0.9 /h dilution rate, exhibits a distinct pattern with 4.87 g/l. In comparison, higher glucose concentrations represented by Panel C (20 g/l, 0.2/h) elevate ethanol production with 9.43 g/l, whereas Panel D (20 g/l, 0.9 /h) shows lower production with 4.67 g/l of ethanol. Panels A and B stabilize after 60 minutes, while Panels C and D have not reached equilibrium even after 240 minutes.

The results indicate that the dilution rate significantly impacts ethanol production, with lower dilution rates yielding higher ethanol production. Additionally, the results suggest that increasing glucose concentration can enhance ethanol production, but only up to a certain point. Beyond that point, it appears increasing glucose concentration does not significantly impact ethanol production, as yeast cannot efficiently convert high glucose concentrations into

ethanol in this system. These observations underscore the critical importance of carefully controlling both the dilution rate and glucose concentration when optimizing the performance of immobilized yeast beads for ethanol production.

3.6 Optimization of ethanol production using immobilized yeast bead (Y9)

Table 8: Design of experiment 2 (DOE2) including 7 sets of experiments with varying factors i.e., glucose concentration and dilution rate at 40^{o}C using CFSTR experimental setup.

Expt.no	**Glucose feed(g/l)**	**Dilution rate(/h)**	**EtOH (g/l)**	**Sugar conversion (%)**	**Yield(g/g)**	**Productivity (g/l/h)**
1	40	0.18	16.1	95.9	0.42	2.86
2	80	0.18	24.6	86.2	0.43	4.61
3	40	0.9	15.8	81.4	0.51	7.55
4	80	0.9	17.9	61.2	0.39	8.19
5	60	0.34	20.9	86.5	0.45	7.17
6	60	0.34	20.9	82.3	0.50	7.09
7	60	0.34	21.2	81.2	0.50	7.14

The Design of Experiment 2 (DOE2) focused on the immobilization of beads #Y9 for ethanol production. The design consisted of seven experiments with varying two factors, the glucose feed concentration and dilution rate. Each experiment was conducted at 40 °C using (CFSTR) experimental setup. The run order, glucose feed, and dilution rate are detailed for each experimental run.

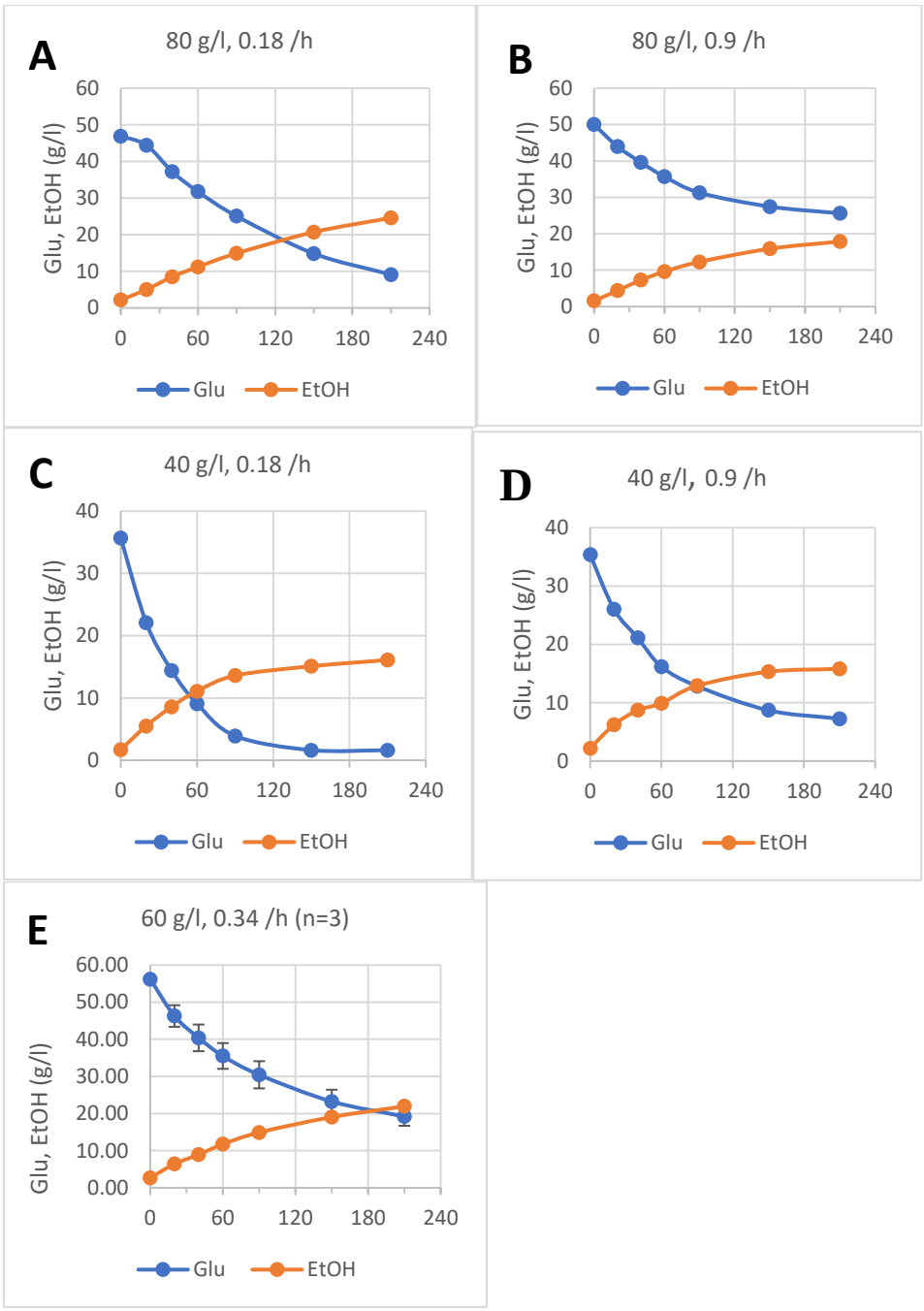

Figure 21: A graphical representation of glucose consumption and ethanol production based on a Design of Experiments (DoE2), including seven experiments. Panels A and B include 2 experiments with 80 g/l glucose at dilution rates of 0.9 /h and 0.18 /h, and panels C and D with 40 g/l glucose at a dilution rate of 0.9 /h and 0.18 /h. Panel E is the average of glucose and ethanol from 3 replicates (n=3), which was used to create a representative curve line with 60 g/l at a dilution rate of 0.34 /h. Immobilized sodium alginate yeast beads were used along with fermentation to convert glucose to ethanol. Ethanol and glucose values were measured at 0, 20, 40, 60, 90, 150, and 210 min to optimize ethanol production.

Table 9: Continuous Flow Stirred Tank Reactor (CFSTR) Representing glucose conversion, ethanol production, yield, and productivity value analyses from enzymatic method and HPLC.

Experiment number	Glucose conversion (%)		EtOH (g/l)		Yield (gEtOH/gGlu)		Productivity (gEtOH/l/h)	
	Enz	HPLC	Enz	HPLC	Enz	HPLC	Enz	HPLC
1- 80g/l, 0.9	60.8	61.0	38.0	18.0	0.95	0.39	18.6	8.19
2- 40g/l, 0.18	95.0	95.9	14.1	16.1	0.33	0.43	2.22	2.91
3- 80g/l, 0.34	80.0	86.0	34.0	24.6	0.61	0.43	6.5	4.61
4-40g/l, 0.9	76.0	81.4	13.5	15.8	0.43	0.50	5.81	7.41
5-6-7:60g/l,0.34	81.0	83.3	21.0	20.7	0.44	0.47	6.39	7.13

Table 9 presents the glucose conversion (%), ethanol level (g/l), ethanol yield (g/g), and productivity (g/l/h) of experiments investigating the effects of different glucose concentrations (40 g/l, 60 g/l, and 80 g/l) and dilution rate (0.18 /h, 0.34 /h and 0.9 /h) on fermentation parameters and ethanol production in the CFSTR model. Importantly, the HPLC analysis revealed differences between the intended and actual glucose concentrations. The observed values were 48.45 g/l for an intended concentration of 80 g/l, 42.43 g/l for 60 g/l, and 35.63 g/l for 40 g/l. These variations may be attributed to potential errors during the preparation of glucose solutions, including pipetting errors.

For experiments with an 80 g/l glucose concentration shown in Table 9, the enzymatic analysis showed glucose conversion values of 60.8% and 80% for dilution rates of 0.9 /h and 0.34 /h. The corresponding ethanol production values were 38 g/l and 34 g/l, with yields of 0.95 g/g and 0.61 g/l and productivities of 18.6 g/l/h and 6.5 g/l/h, respectively. However, HPLC analysis shows a huge difference in ethanol values, suggesting the possibility of pipetting errors during the manual performance of the enzymatic method.

For experiments with a 40 g/l glucose concentration, enzymatic analysis indicated ethanol production values of 13.5 g/l and 14.1 g/l, respectively, for dilution rates of 0.9 /h and 0.18 /h. The corresponding yields were 0.43 g/g and 0.33 g/g, and productivities were 5.81 g/l/h and

2.22 g/l/h, respectively. However, HPLC analysis showed ethanol values of 15.8 g/l and 16.1 g/l, with yields of 0.50 g/g and 0.43 g/g, and productivities of 7.41 g/l/h and 2.9 g/l/h.

For experiments with a 60 g/l glucose concentration and a dilution rate of 0.34 /h, the enzymatic analysis indicated an ethanol production value of 21 g/l, yielding 0.44 g/g and productivity of 6.39 g/l/h. HPLC analysis shows a corresponding ethanol value of 20.7 g/l, yielding 0.47 g/g and productivity of 7.13 g/l/h. Experiments 5, 6, and 7 were conducted separately, each representing the same condition of glucose concentration at 60 g/l and 0.34 /h dilution rate. The reported values are the average of three independent experiments and are indicated as triplicate for clarity.

Based on the results obtained, it appears that a lower dilution rate of 0.18 /h may be better for ethanol production, as it allows for more time for the yeast cells to convert sugar to ethanol. The ethanol yield is close to the theoretical value of 0.51 g/g, and overall sugar conversion is higher than 80%. Additionally, the triplicate (experiment 5-6-7) yields are quite good for all four responses of the contour plot (Fig 22).

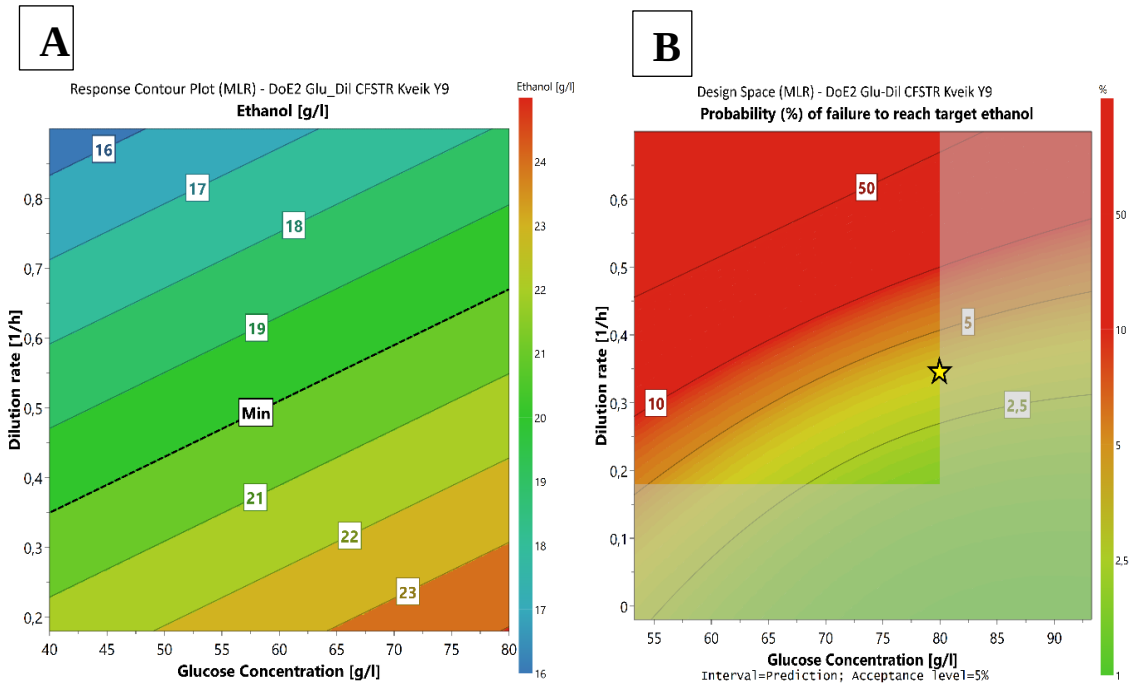

Figure 22: Response contour plot for ethanol Panel A and prediction plot panel B model with glucose (g/l), and dilution rate (/h) affecting ethanol production (g/l). The different colour indicates the heat plot (A) where warmer colour provides better results and in prediction plot (panel B) is showed with extended axes for glucose and dilution since the target area (greyish shade) is somewhere outside the lower right corner. The star in the prediction plot represents

the condition for the validation experiment, indicating less than 5% probability of failure ($p<0.05$).

A contour plot was created using MODDE to predict the optimal conditions for ethanol production by adjusting factors like glucose concentration and dilution rate using data from DoE2. The ethanol and glucose levels were tested using HPLC and enzymatic methods to calculate sugar conversion, yield, and productivity. The plot uses warmer colour to indicate areas where conditions will likely yield better results. The optimal conditions for ethanol production are represented by a heat map with a glucose concentration of 80 g/l a dilution rate of 0.34 /h, and with yield of approximately 22 g/l.

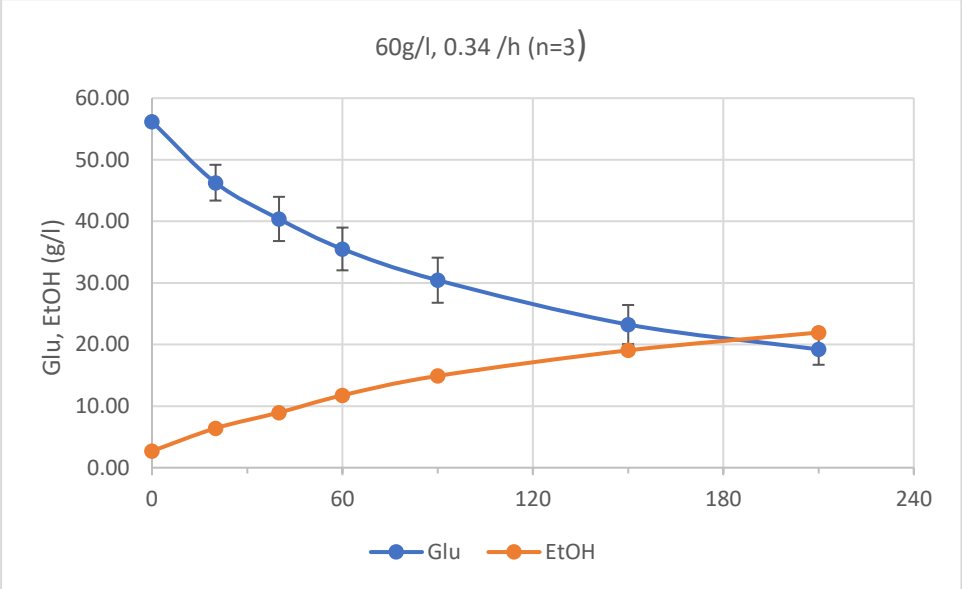

Figure 23: graphical representation of glucose consumption and ethanol production analyzed from validation of model data DoE2 (n=2). Samples were collected at various intervals during the fermentation period using the CFSTR method.

Experimental validation of the model was obtained through the Modde software. The targeted conditions, guided by the contour plot, involved 80g/l glucose concentration and a dilution rate of 0.34 per hour. Experimental samples were collected at regular intervals of 0, 20, 40, 60, 90, 150, and 210 min during the fermentation process using the Continuous Flow Stirred

Tank Reactor (CFSTR) model using an immobilized sodium alginate yeast bead. At the end of fermentation time (210 minutes), ethanol production was observed to be 21.95 g/l.

While the contour plot predicted an ethanol production of 22 g/l under the optimized conditions of 80 g/l glucose concentration and a dilution rate of 0.34 per hour, the experimental validation yielded a value of 21.95 g/l. which closely aligns with the predicted value.

3.7 The comparison between batch fermentation using free cells (Y9) and continuous batch fermentation using immobilized kveik yeast beads (Y9)

Table 10: Comparison of data obtained with batch fermentation with a free cell using the syringe piston method (n=3) and continuous fermentation using the CFSTR model at 40 ^{o}C (n=2)

Parameter	Suspended cells (Y9) (n=3)	Immobilized cells (Y9) (n=2)
Fermentation model	Batch	Continuous (CFSTR)
Reactor volume (ml)	50	150
Fermentation time	24 hours (anaerobic)	3-4 hours
Biomass load- CDW (g)	1.42	0.35
Glu conc (g/l) (initial feed)	71.75	56.2
Sugar conversion (%)	85.8	67.9
Ethanol concentration (g/l)	48.04 ± 0.85	21.95 ± 0.07
Ethanol Yield (g/g)	0.669	0.399
Productivity (g/l-h)	2.01	7.3

The table above compares the performance of batch fermentation with suspended cells (Kveik Ebbegarden) using the syringe piston method and continuous fermentation with immobilized

kveik yeast beads using the CFSTR model in terms of sugar conversion, ethanol concentration, yield, and productivity. In the batch fermentation with suspended cells, the model predicted an ethanol production of 50 g/l, and the actual result was 48.04 g/l. Similarly, in the continuous fermentation with immobilized cells, the predicted ethanol production was 22 g/l, and the actual result closely matched at 21.95 g/l. The initial sugar concentration for both processes was intended to be 80 g/L but was measured at 71.75 g/L and 56.2 g/l glucose using HPLC. While the free cell fermentation achieved a higher sugar conversion of 85.8%, the immobilized cell fermentation demonstrated a slightly lower 67.9% conversion.

Although the ethanol production and yield of immobilized cells is lower (21.95 g/L) compared to suspended cells (48.04 g/L), in continuous fermentation, where the process runs continuously, the immobilized cells have a significantly higher rate of ethanol production per hour (7.3 g/L/h) compared to suspended cells (2.01 g/L/h) with lower cell biomass (0.35 g).

4. Discussion

4.1 Experimental usefulness of the model

The pilot experiment aimed to validate a simple and efficient model for observing ethanol fermentation, focusing on significant gas development during the anaerobic fermentation phase using the syringe piston expansion method. Although ethanol measurements were not taken, the experiment tested the practicality of the syringe piston in terms of working hours, volume readings (CO_2), and testing temperature, revealing a sigmoid pattern in gas development. A study (Christensen & Strætkvern, 2018), primarily employed the syringe piston method to measure gas evolution during anaerobic digestion. Using 100 ml syringe-stoppered glass bottles, they created a sealed environment for precise gas evolution measurement. This approach ensured controlled anaerobic conditions in both small-scale (100 ml) and scaled-up (1.0 L) experiments, facilitating the assessment of biogas production, pH, and related factors. Thus, the initial phase of our study similarly focused on observing gas development during anaerobic fermentation using the syringe piston method.

Glucose consumption was assessed using glucose sticks to verify the model's functionality. The established model proved reliable for indicating ethanol production, as confirmed by subsequent analysis. Vitamin supplementation in YPG did not enhance gas yield during anaerobic fermentation. YPG served as a known glucose testing medium, while KYM, derived from molasses and other chemicals, presented challenges in determining its exact glucose concentration.

The findings demonstrate the reliability and practical utility of the syringe piston expansion method for monitoring CO_2 formation during the anaerobic phase. A similar kind of study (Østgaard et al., 2017) used the syringe expansion method and mentioned about its reliability and practicality. Our results also suggested that the technique proved effective in measuring gas production during the fermentation of various feedstocks for ethanol production, contributing to enhanced process optimization and yield. This study aimed to determine the optimal temperature for the yeast aerobic phase by comparing yeast propagation at 25 °C to 37 °C. The results (Figure 11) clearly showed that yeast propagation at 25 °C was more effective, promoting better CO_2 formation during the subsequent anaerobic phase, indicating the 37 °C propagation exhausted sugar before ethanol fermentation.

The yeast propagation conditions of 22-24 hours for the aerobic phase were identified to ensure sufficient yeast cell growth, essential for efficient glucose-to-ethanol conversion during the anaerobic fermentation phase. To monitor yeast cell growth and viability during fermentation, optical density (OD_{660}) was measured at different time intervals, providing valuable information about yeast proliferation. (Table 4). Gas formation is an equimolar proportional response to ethanol formation, which can be represented as $2CO_2 = 2EtOH$, confirmed by ethanol analysis (Fig 11).

Overall, the pilot experiment demonstrated the model's practicality and reliability for observing ethanol fermentation. Later, the model was confirmed and served in the screening and optimization phase of the study for further exploration.

4.2 Fermentation Robustness and Thermotolerance of Kveik Yeast Strains

Ale fermentations (using traditional yeasts) usually occur between 15-25 °C, with 20 °C being the standard temperature. On the other hand, lagers (using commercial yeasts) ferment at colder temperatures ranging from 6-14 °C (Bamforth, 2023). Clearly, our investigation shows the effectiveness of kveik strains in different temperature ranges, with a preference for fermentations between 32-47 °C (Fig 11). It is essential to note that there is significant variation among kveik strains. For example, strains like Eitrheim (Y14) are versatile and can ferment at a wide temperature range from 15-42 °C. On the other hand, strains like Stalljen (Y22) have a narrower temperature range preference of 8-38 °C and are used for rapid fermentation at elevated temperatures. Kveik strains are known to have better cell viability at higher temperatures, but they are not all equally thermotolerant (Foster et al., 2022b). Among the kveik strains, Voss, Ebbegarden, and Eitrheim are more resistant to temperatures of 40-42 °C than Stalljen. The more heat-tolerant strains also have better fermentation efficiency at higher temperatures than the less tolerant ones.

This study explored the ethanol fermentation robustness of kveik strains (Y1, Y9, Y14, and Y22) across temperatures from 32 °C to 47 °C. kveik strains, known for robust fermentation, particularly in warmer temperatures (Preiss et al., 2018), exhibited accelerated fermentation at

higher temperatures (37 °C, 39 °C, 40 °C and 42 °C), but a decline was observed at 45 °C and 47 °C (Figure 11, 16,18). These aligns with (Lin et al., 2012) findings, emphasizing the inhibitory effects of very high temperatures on cell growth and ethanol production.

Traditionally, the brewing process with kveik occurs around 30 °C or higher (Garshol, 2021), supporting our anticipation of optimal efficiencies at higher temperatures. In the initial 8-9 hours of the anaerobic phase, all strains exhibited significant gas formation (50% or more) at 32-42 °C but declined at 47 °C (Table 1). Similarly, Voss (Y1), Eitrheim (Y14), and Stalljen (Y22) displayed reduced gas formation (CO_2) at 47 °C, essentially slowing down fermentation after 8-9 hours. Inhibitory effects at high temperatures can arise from interrupted cellular transport activity and lead to toxin accumulation, such as organic acid, glycerol, and other alcohol (Phisalaphong et al., 2006). High temperatures can also denature ribosomes and enzymes and affect membrane fluidity. Thus, this may be the situation for kveik yeast at higher temperatures.

While considering the optimal temperature range, yeast fermentation generally favours temperatures between 30-35 °C (Hu et al., 2012). However, the study also indicates that certain yeast enzymes, like the inulinases of *S. cerevisiae* JZ1C, can exhibit practical functionality over a broader temperature range, specifically between 40 and 50 °C. Whereas, our experiment shows the maximum gas formation (CO_2) and ethanol production (g/l) between 37 °C and 42 °C with different culture mediums (YPG and KYM) (Figure 12). It was also noted that the culture medium significantly influenced the outcomes of the fermentation process. (Foster et al., 2022b; Preiss et al., 2018) found that most yeast cultures can use maltose at high temperatures. However, some strains, like Ebbegarden, have reduced maltose utilization at extreme temperatures. Our study showed that the ethanol yield in KYM was twice as high as YPG, indicating the utilization or conversion of additional sugars in molasses. Molasses contains other fermentable sugars, such as maltose, that may be consumed but are not detected as glucose.

The kveik yeast demonstrates that fermentation times are shorter and rates are faster across a wide temperature range (32-47 °C), which is consistent with the findings of (Foster et al., 2021). The differential consumption rates of sugars indicate that glucose was utilized rapidly within the first 7-8 hours at preferred temperatures, which is a characteristic of kveik strains.

Kveik yeast strains, known for their unique genetics and efficient fermentation in warm conditions, contribute distinct flavours (Garshol, 2020; Preiss et al., 2018). Overall, the study investigated four kveik yeast strains to understand how they ferment and survive in temperatures ranging from 32 °C to 47 °C. The initial studies aimed to analyse the strengths and limitations of each strain under different temperature conditions, to finalize the ideal yeast strain for ethanol production. The findings from the study indicated that Ebbegarden (Y9) and Eitrheim (Y14) were good choices due to their high temperature tolerance, fast fermentation rates, and high ethanol production compared to the other kveik strains. Notably, Ebbegarden (Y9) showed an even higher yield of ethanol in comparison to Eitrheim (Y14) strains.

4.3 Comparative Analysis of Glucose Utilization and the Crabtree Effect

Finally, Ebbegarden (Y9) was chosen as the ideal candidate over other strains for further ethanol production analysis. Chang and his team found that when sugar concentrations in the media exceeded 120 g/l, yeast growth, and viability decreased due to the osmotic effect caused by high glucose concentrations (Chang et al., 2018). Our study revealed that using Kveik yeast with glucose concentrations exceeding 80 g/l resulted in remaining unconsumed glucose, suggesting that osmotic stress might affect glucose utilization during fermentation. (Prasertwasu et al., 2014) fermenting baker’s yeast on acid- and enzyme-treated hydrolysate of Thai Mission grass found residual glucose after 48 hours It suggested that compounds like furfural and hydroxymethylfurfural inhibited the glucose conversion efficiency. However, our study differs from theirs since the lignocellulosic substrate was not used in the experimental conditions. This observation underscores that factors affecting glucose utilization in the study may differ from those observed by Prasertwasu and his team. Other factors that can slow glucose utilization include osmotic stress from high glucose and toxic compounds, such as glycerol, aldehyde, alcohol, and organic acids (Saint-Prix et al., 2004).

(Taherzadeh & Karimi, 2011) observed that elevated glucose levels can stress yeast cells, potentially causing bursting, which is influenced by the strains' osmotic stress management, affecting glucose consumption rates. In the experiment using kveik yeast Ebbegarden (Y9), the highest ethanol production occurs within 24 hours of the anaerobic fermentation phase,

followed by a rapid decline due to osmotic stress from remaining glucose, ethanol. However, surviving yeast cells adapt and efficiently utilize remaining glucose until it drops to almost 0 g/L after 48 hours (Figure 18), demonstrating robust glucose utilization kveik strain.

The appearance of ethanol at the initial phase (0h) before the aerobic phase in our experiment can be attributed to the Crabtree effect (Fig 13, 15, and 16). This effect is exhibited by Crabtree-positive yeasts, such as Kveik strains, which preferentially undergo fermentation even in aerobic conditions when there is an excess of glucose (Pfeiffer & Morley, 2014).

During the aerobic phase, Crabtree-positive yeasts may engage in fermentative metabolism, producing ethanol. The Crabtree effect is characterized by a metabolic shift towards fermentation, even in the presence of oxygen. The switch to fermentation might be triggered by factors such as high glucose concentrations (Fig 14), the need for rapid energy production, or limitations in the respiratory capacity of the yeast (Postma et al., 1989).

In our experiment, the pre-existing 2 g/l of ethanol observed at 0h before the aerobic phase could result from this Crabtree effect. Upon encountering a high glucose concentration, the yeast might have initiated fermentation, producing ethanol as a metabolic byproduct. The pre-existing ethanol could be attributed to using a 2/3-week-old inoculum, which may have experienced some fermentation during storage.

4.4 Predictive Modeling for Optimizing Ethanol Production

After testing temperature robustness and glucose conversion, the focus turned to optimizing ethanol production using Ebbegarden (Y9) yeast through a full factorial 3 -factor design, through modelling of the outcome from the screening experiment, predicted optimal conditions at 78 g/l glucose, 40 °C temperature, and 4.5% PFW, resulting in g/l ethanol were identified. The achieved result of 48.5 g/l ethanol aligned closely with this prediction. Temperature and the initial glucose concentration were identified as the most critical parameters influencing ethanol production from Kveik yeast.

Comparing our work to (Lai et al., 2019), who focused on *S. cerevisiae* S5 for ethyl acetate (EA) production using Box-Behnken Design and Response Surface Methodology (BBD-RSM), both studies aimed to enhance fermentation efficiency through advanced DOE and

contour plots. Similarly, the study (Ebrahimiaqda & Ogden, 2018), which employed a full factorial design and predictive modelling emphasized the impact of three main factors on ethanol yield efficiency during uncontrolled fermentation. Dissolved oxygen and temperature were found to significantly influence yield, recommending the creation of anaerobic conditions during yeast addition. This corresponds with our findings, where temperature improved ethanol yield.

Thus, the findings from various studies, including ours, emphasize the significance of focusing on temperature and glucose concentration, in the production of ethanol strengthening the reliability of predictive modelling for optimizing fermentation.

4.5 Enhancing Fermentation with Immobilization

Immobilizing cells in beads offers several advantages, including easy separation from the medium, cost reduction due to reusability in subsequent reaction cycles, and decreased possibility of contamination (Duarte et al., 2013). The immobilization technique using calcium alginate entrapment enhances fermentation efficiency by improving resistance to inhibitors in hydrolysates, enabling simultaneous utilization of sugars, and streamlining separation processes and yeast reusability (Chacón-Navarrete et al., 2021).

The findings from our immobilization study of kveik Y9 with calcium alginate showed that higher initial glucose levels in the feed resulted in a significant increase in ethanol production within a short experimental time (Figure 21). The study by (Najafpour et al., 2004) indicates that immobilizing *S. cerevisiae* enhances the utilization of high sugar concentrations, leading to increased ethanol productivity in a shorter fermentation time, which aligns with the findings from our study.

Yu and their colleagues (2007) conducted a study on ethanol productivity and found that the most efficient productivity occurs at a higher dilution rate of 0.3/h, which results in reduced glucose conversion. On the other hand, lower dilution rates (0.1/h) result in complete sugar utilization but decreased ethanol productivity. Our results align with Yu's findings, indicating that lower dilution rates lead to better sugar conversion but lower ethanol productivity (g/l/h), while higher dilution rates result in higher productivity (Table 5) (Yu et al., 2007).

Along with the glucose conversion and productivity, dilution rate also plays a role in ethanol yield where a lower dilution rate contributes to an elevated ethanol yield, aligning with the findings of (Kim et al., 2010), where the observed yield was close to the maximum theoretical yield of 0.51 g/g. While some previous studies, such as those conducted by (Najafpour et al., 2004; Razmovski & Vučurović, 2011; Yu et al., 2007), reported an increase in ethanol yield due to the conversion of other hexose sugar (e.g. mannose) present in hydrolysate in bioethanol or other additional sugars, our study using immobilized kveik yeast did not show any ethanol yield exceeding the theoretical limit

Mathew and his team found that higher dilution rates resulted in a decrease in glucose utilization. Our observations also support this finding, as we consistently observed a decrease in glucose consumption at increasing dilution rates, ranging from low to high (as shown in Table 9), suggesting the presence of limitations to the diffusion of metabolites within the gel-like structure, which hinders the movement of glucose through the gel matrix. This phenomenon has been previously supported by (Bringi & Dale, 2002) and further strengthened by (Mathew et al., 2013). (Nigam, 2000) illustrate the benefits of using small beads to improve solid-liquid interfacial areas per unit reactor volume. Small beads can minimize mass-transfer limitations of sugars, ethanol, and CO_2. In our experiment, beads with a diameter ranging from 3mm to 5mm were used. Further improvement in mass transfer efficiency could be achieved by focusing on even smaller beads with a 2-3 mm diameter, which would minimize limitations associated with metabolite transfer within the fermentation system.

This study found that using immobilized Kveik beads for multiple experiments can be consistent and successful in ethanol production. This reflects the increasing trend of utilizing microorganisms for recycling, which can offer advantages in terms of saving time, energy, and costs when applied effectively. Our findings are in line with previous research, such as the work of (Tesfaw & Assefa, 2014), who investigated yeast recycling multiple times.

After utilizing the same alginate-yeast beads for 9-10 consecutive experiments, the ethanol production remained stable, and the cell viability showed no significant changes throughout the short time of an experiment, confirming their suitability for repeated use. These findings align with those from similar studies, where bacterial cellulose-alginate sponge achieved 15 reuse cycles (Kirdponpattara & Phisalaphong, 2013), and very high gravity fermentation demonstrated 10 reuse cycles (Ji et al., 2012). This study's consistent and efficient reuse of

immobilized Kveik beads further underscores the practicality and potential economic benefits of this approach in industrial applications.

4.6 Comparing fermentation in batch continuous mode using immobilized yeast beads

The use of cell immobilization for alcoholic fermentation has gained attention due to its advantages, including improved substrate utilization and continuous processing feasibility (Behera et al., 2010). Calcium alginate has become a preferred material for this purpose due to its cost-effectiveness and effectiveness in protecting cells (Behera et al., 2010).

The fermentation duration played a crucial role in shaping glucose consumption and ethanol production. The results suggest that although the immobilized cells had lower ethanol levels, they produced ethanol more efficiently over time than the free cells, indicating that immobilized cells have advantages in terms of productivity, making them a promising candidate for consistent and steady ethanol production.

Studies, including (Behera et al., 2010; Mathew et al., 2013; and Singh et al., 2013), have reported lower ethanol production by free cells compared to calcium alginate-immobilized yeast. (Nigam, 2000) discovered that the ethanol productivity of immobilized cells was 11 times higher compared to free cells. Our study has shown a more than three times increase in ethanol productivity compared to suspended cells. However, it's important to note that differences in fermentation times and initial glucose feed between studies may affect these results.

Duarte and his team observed that the concentration of ethanol was higher in free cells (40 g/l) than in immobilized cells (30 g/l) with an initial glucose concentration of 100 g/l (Duarte et al., 2013). These results closely align with the findings of our study (Table 10). Our results also demonstrate an improvement over the experiment conducted by (Ghorbani et al., 2011) who reported an ethanol concentration of 19.51 g/l with a productivity of 2.39 g/l/h using sodium alginate-immobilized yeast with 150 g/l molasses. Despite the higher initial sugar concentration and prolonged fermentation cycles, the kveik yeast maintained stable ethanol production, demonstrating its robustness and efficacy compared to immobilized *S. cerevisiae*.

Additionally, it is essential to consider the benefits of immobilized cells, such as reusability, fermentation time, and potential productivity improvements with optimized conditions.

Singh and his colleagues conducted a study to investigate using immobilized *S. cerevisiae* cells encapsulated in Ca-alginate beads for ethanol production. They achieved 11.8 g/l of ethanol with an initial sugar concentration of 50 g/l but observed a decline in output after three cycles (Singh et al., 2013). Our study employed immobilized Kveik yeast and obtained a significantly higher ethanol yield of 21.95 g/l after nine cycles with a glucose concentration of 56 g/l. According to some studies conducted by (Ji et al., 2012; Kirdponpattara & Phisalaphong, 2013; Tesfaw & Assefa, 2014), ethanol production was found to decrease after 6-8 cycles. However, our experiments have shown that immobilized kveik yeast cells can be reused consistently for more than 9 cycles, further emphasizing their practicality for sustainable and cost-effective ethanol production for industrial use.

Although immobilized cells have a lower biomass than suspended cells (Table 5), they exhibit higher ethanol productivity per biomass unit. One possible reason is that immobilized cells do not have to use energy in division and growth, unlike suspended cells. Instead, they can focus only on converting substrate to ethanol, which results in a more efficient substrate conversion within a shorter fermentation time.

Willaert (2011) This article discusses the use of small-pore membranes for mechanically containing yeast cells, focusing on the need to minimize compound transfer and free cell presence. The approach to immobilization, utilizing the entrapment method with calcium alginate and kveik yeast aligns with this strategy by creating small pores in the beads to hold the yeast cells behind the membrane. This method has the potential to reduce compound transfer and prevent unwanted by-products. Overall, this immobilization method shows promise for producing high-quality yeast-based products.

Furthermore, Kveik yeast's high-temperature fermentation capability could align with the challenges of SSF in ethanol production from non-food biomass. The study results demonstrate that the kveik yeast strain can ferment at high temperatures of up to 40-42 °C, which is key in SSF, as it requires optimal conditions for both enzymatic saccharification and fermentation. (Chacón-Navarrete et al., 2021) mentions that the high-temperature fermentation capability of yeast could simplify the SSF process and enhance its efficiency in ethanol production from non-food biomass. Furthermore, the findings from our study also

suggest that, although kveik yeast may not necessarily outcompete traditional yeast strains in all applications, its unique temperature robustness makes it well-suited for specialized applications such as combining it with processes for converting cellulosic biomass. Thus, it can be stated that, by enabling fermentation at elevated temperatures, kveik yeast can simplify process conditions and enhance overall efficiency in ethanol production from non-food biomass.

In SSF, microorganisms can be reused for multiple fermentation cycles, allowing for successive rounds of fermentation using the same microorganism. This allows for consecutive rounds of fermentation with the same microorganism, which optimizes resource utilization and maximizes ethanol production capacity (Ishola et al., 2015). Despite significant mechanical damage and bursting of the kveik yeast beads after 2-3 cycles, our observations reveal that ethanol production remains unaffected. This indicates that the cells' viability and vitality is preserved throughout the fermentation process, underscoring the robustness of the kveik yeast.

5. Conclusion

Initially, pilot experiments were performed to analyse and validate batch fermentation using Y9 Kveik strains. Findings from these studies suggested the practicality of the syringe piston in terms of CO2 formation and identifying the temperature for propagation of yeast growth. The initial screening studies aimed to finalize the ideal yeast strain for ethanol production by analysing each strain under different temperatures and initial glucose concentrations (g/l). The results from this study suggested that Ebbegarden (Y9) is the ideal yeast strain due to its high-temperature tolerance, fast fermentation rates, and high ethanol production compared to the other Kveik strains.

During the studies for optimizing ethanol production by using predictive modelling, it was noted that temperature and the initial glucose concentration are the most critical parameters influencing ethanol production from Kveik yeast. Utilizing the design of experiment (DoE) and contour plot methodologies, ethanol production with kveik Ebbegarden (Y9) yeast was optimized.

The study shows that kveik yeast is well-suited for specialized applications, particularly in converting cellulosic biomass into ethanol. kveik yeast simplifies the process conditions by enabling fermentation at elevated temperatures, enhancing overall efficiency in ethanol production from non-food biomass. Additionally, the study shows the practicality and economic advantages of utilizing immobilized kveik yeast, as evidenced by its consistent and efficient reuse for over ten cycles. In employing batch and continuous fermentation methods, the study demonstrated that immobilized cells exhibited superior ethanol productivity compared to suspended cells, demonstrating that immobilized cell fermentation enhances ethanol efficiency and sustainability in industrial applications.

While the data produced here provided evidence of the potential for ethanol production using brewing kveik yeast at an industrial scale, utilizing higher sugar concentrations, and operating at high temperatures, future studies could extend this investigation to explore the utilization of lignocellulosic compounds for bioethanol production. It is important to note that this study focused on only four kveik yeast strains, with only one strain selected for optimizing ethanol production. This selection does not imply that other yeast strains cannot produce ethanol; instead, the chosen kveik yeast demonstrated optimal performance within the parameters studied. Moreover, there are several other kveik yeast strains accessible for further study and

investigation, providing opportunities to expand our knowledge of their ethanol production capabilities under varying conditions.

6. References

Aasen, N. M. (2020). Growth, metabolism, and beer brewing with kveik [Master thesis, Norwegian University of Life Sciences, Ås]. In *138*. https://nmbu.brage.unit.no/nmbu-xmlui/handle/11250/2681970

Balat, M. (2011). Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review. *Energy Conversion and Management*, *52*(2), 858–875. https://doi.org/10.1016/j.enconman.2010.08.013, 835-875

Bamforth, C. W. (2023). *Beer: Tap Into the Art and Science of Brewing*. Oxford University Press.

Bangrak, P., Limtong, S., & Phisalaphong, M. (2011). Continuous ethanol production using immobilized yeast cells entrapped in loofa-reinforced alginate carriers. *Brazilian Journal of Microbiology*, *42*, 676–684. https://doi.org/10.1590/S1517-83822011000200032

Behera, S., Kar, S., Mohanty, R. C., & Ray, R. C. (2010). Comparative study of bio-ethanol production from mahula (Madhuca latifolia L.) flowers by Saccharomyces cerevisiae cells immobilized in agar agar and Ca-alginate matrices. *Applied Energy*, *87*(1), 96–100. https://doi.org/10.1016/j.apenergy.2009.05.030

Biörnstad, A. (1972). Review of Brewing and Beer Traditions in Norway [Review of *Review of Brewing and Beer Traditions in Norway*, by O. Nordland]. *Bijdragen Tot de Taal-, Land- En Volkenkunde*, *128*(1), 182–184.

Bringi, V., & Dale, B. E. (2002, May 1). *Experimental and theoretical evidence for convective nutrient transport in an immobilized cell support* (world) [Research-article]. ACS Publications; American Chemical Society. https://doi.org/10.1021/bp00003a008, 205-209.

Cerevisiae—An overview | ScienceDirect Topics. (n.d.). Retrieved January 21, 2024, from https://www.sciencedirect.com/topics/engineering/cerevisiae

Chacón-Navarrete, H., Martín, C., & Moreno-García, J. (2021). Yeast immobilization systems for second-generation ethanol production: Actual trends and future perspectives. *Biofuels, Bioproducts and Biorefining*, *15*(5), 1549–1565. https://doi.org/10.1002/bbb.2250

Chang, Y.-H., Chang, K.-S., Chen, C.-Y., Hsu, C.-L., Chang, T.-C., & Jang, H.-D. (2018). Enhancement of the Efficiency of Bioethanol Production by Saccharomyces cerevisiae via Gradually Batch-Wise and Fed-Batch Increasing the Glucose Concentration. *Fermentation*, *4*(2), Article 2. https://doi.org/10.3390/fermentation4020045

Chibata, I., Tosa, T., & Sato, T. (1986). Biocatalysis: Immobilized cells and enzymes. *Journal of Molecular Catalysis*, *37*(1), 1–24. https://doi.org/10.1016/0304-5102(86)85134-3

Christensen, E., & Strætkvern, K. (2018, May 16). *BOOSTING BIOGAS WITH HYDROLYTIC ENZYMES - EFFECT OF CELLULASE PRETREATMENT OF WHEAT STRAW ON SUGAR RELEASE, ONSET AND ULTIMATE GAS PRODUCTION*. https://doi.org/10.5071/26thEUBCE2018-2CV.5.31

Cubillos, F. A., Gibson, B., Grijalva-Vallejos, N., Krogerus, K., & Nikulin, J. (2019). Bioprospecting for brewers: Exploiting natural diversity for naturally diverse beers. *Yeast*, *36*(6), 383–398. https://doi.org/10.1002/yea.3380

Dashko, S., Zhou, N., Compagno, C., & Piškur, J. (2014). Why, when, and how did yeast evolve alcoholic fermentation? *Fems Yeast Research*, *14*(6), 826–832. https://doi.org/10.1111/1567-1364.12161

Díaz-Montaño, D. M. (2013). Continuous Agave Juice Fermentation for Producing Bioethanol. In *Biomass Now—Sustainable Growth and Use*. IntechOpen. https://doi.org/10.5772/55923, 209-230.

Dondrup, M., Eiken, H. G., Martinussen, A. O., Haugland, L. K., Holdhus, R., Dolan, D., Grellscheid, S., Hagen, S., Elameen, A., & Myking, T. (2023). *Traditional Norwegian Kveik Yeast: An Ancient Sister Group to Domesticated Saccharomyces cerevisiae* (p. 2023.07.03.547515). bioRxiv. https://doi.org/10.1101/2023.07.03.547515

Duarte, J. C., Rodrigues, J. A. R., Moran, P. J. S., Valença, G. P., & Nunhez, J. R. (2013). Effect of immobilized cells in calcium alginate beads in alcoholic fermentation. *AMB Express*, *3*(1), 31. https://doi.org/10.1186/2191-0855-3-31, 1-8.

Ebrahimiaqda, E., & Ogden, K. L. (2018). Evaluation and Modeling of Bioethanol Yield Efficiency from Sweet Sorghum Juice. *BioEnergy Research*, *11*(2), 449–455. https://doi.org/10.1007/s12155-018-9909-0

Feedstock for bioethanol production from a technological paradigm perspective: BioResources. (n.d.). Retrieved December 6, 2023, from https://bioresources.cnr.ncsu.edu/

Foster, B., Tyrawa, C., Ozsahin, E., Lubberts, M., Krogerus, K., Preiss, R., & Van Der Merwe, G. (2021). *Kveik brewing yeasts demonstrate wide flexibility in beer fermentation temperature and flavour metabolite production and exhibit enhanced trehalose accumulation* [Preprint]. Microbiology. https://doi.org/10.1101/2021.07.26.453768, bioRxiv, 2021-07.

Foster, B., Tyrawa, C., Ozsahin, E., Lubberts, M., Krogerus, K., Preiss, R., & van der Merwe, G. (2022a). Kveik Brewing Yeasts Demonstrate Wide Flexibility in Beer Fermentation Temperature Tolerance and Exhibit Enhanced Trehalose Accumulation. *Frontiers in Microbiology*, *13*. https://www.frontiersin.org/articles/10.3389/fmicb.2022.747546

Foster, B., Tyrawa, C., Ozsahin, E., Lubberts, M., Krogerus, K., Preiss, R., & van der Merwe, G. (2022b). Kveik Brewing Yeasts Demonstrate Wide Flexibility in Beer Fermentation Temperature Tolerance and Exhibit Enhanced Trehalose Accumulation. *Frontiers in Microbiology*, *13*. https://www.frontiersin.org/articles/10.3389/fmicb.2022.747546

Garshol, L. M. (2020). *Historical Brewing Techniques: The Lost Art of Farmhouse Brewing*. Brewers Publications.

Garshol, L. M. (2021). Pitch Temperatures in Traditional Farmhouse Brewing. *Journal of the American Society of Brewing Chemists*, *79*(2), 181–186. https://doi.org/10.1080/03610470.2020.1805699

Genisheva, Z., Teixeira, J. A., & Oliveira, J. M. (2014). Immobilized cell systems for batch and continuous winemaking. *Trends in Food Science & Technology*, *40*(1), 33–47. https://doi.org/10.1016/j.tifs.2014.07.009

Ghorbani, F., Younesi, H., Esmaeili Sari, A., & Najafpour, G. (2011). Cane molasses fermentation for continuous ethanol production in an immobilized cells reactor by Saccharomyces cerevisiae. *Renewable Energy*, *36*(2), 503–509. https://doi.org/10.1016/j.renene.2010.07.016

Gibson, B. R., Lawrence, S. J., Leclaire, J. P. R., Powell, C. D., & Smart, K. A. (2007). Yeast responses to stresses associated with industrial brewery handling. *FEMS Microbiology Reviews*, *31*(5), 535–569. https://doi.org/10.1111/j.1574-6976.2007.00076.x

Habschied, K., Krstanović, V., Šarić, G., Ćosić, I., & Mastanjević, K. (2022a). Pseudo-Lager—Brewing with Lutra® Kveik Yeast. *Fermentation*, *8*(8), Article 8. https://doi.org/10.3390/fermentation8080410

Habschied, K., Krstanović, V., Šarić, G., Ćosić, I., & Mastanjević, K. (2022b). Pseudo-Lager—Brewing with Lutra® Kveik Yeast. *Fermentation*, *8*(8), Article 8. https://doi.org/10.3390/fermentation8080410

Hagman, A., Säll, T., & Piškur, J. (2014). Analysis of the yeast short-term Crabtree effect and its origin. *The FEBS Journal*, *281*(21), 4805–4814. https://doi.org/10.1111/febs.13019

Hu, N., Yuan, B., Sun, J., Wang, S.-A., & Li, F.-L. (2012). Thermotolerant Kluyveromyces marxianus and Saccharomyces cerevisiae strains representing potentials for bioethanol production from Jerusalem artichoke by consolidated bioprocessing. *Applied Microbiology and Biotechnology*, *95*(5), 1359–1368. https://doi.org/10.1007/s00253-012-4240-8

Isbell, T. (2023, July 22). *Assessment of stress tolerance and fermentation performance in traditional Norwegian Landrace (Kveik) yeast* [Thesis (University of Nottingham only)]. University of Nottingham. http://eprints.nottingham.ac.uk/72087/

Ishola, M. M., Brandberg, T., & Taherzadeh, M. J. (2015). Simultaneous glucose and xylose utilization for improved ethanol production from lignocellulosic biomass through SSFF with encapsulated yeast. *Biomass and Bioenergy*, *77*, 192–199. https://doi.org/10.1016/j.biombioe.2015.03.021

Ji, H., Yu, J., Zhang, X., & Tan, T. (2012). Characteristics of an Immobilized Yeast Cell System Using Very High Gravity for the Fermentation of Ethanol. *Applied Biochemistry and Biotechnology*, *168*(1), 21–28. https://doi.org/10.1007/s12010-011-9280-5

Jones, A. M., Thomas, K. C., & Ingledew, W. M. (2002, May 1). *Ethanolic Fermentation of Blackstrap Molasses and SugarCane Juice Using Very High Gravity Technology* (world). ACS Publications; American Chemical Society. https://doi.org/10.1021/jf00041a037, 42 (5), 1242-1246.

Kawa-Rygielska, J., Adamenko, K., Pietrzak, W., Paszkot, J., Głowacki, A., & Gasiński, A. (2022). Characteristics of New England India Pale Ale Beer Produced with the Use of Norwegian KVEIK Yeast. *Molecules*, *27*(7), Article 7. https://doi.org/10.3390/molecules27(07), 2291.

Kawa-Rygielska, J., Adamenko, K., Pietrzak, W., Paszkot, J., Głowacki, A., Gasiński, A., & Leszczyński, P. (2021). The Potential of Traditional Norwegian KVEIK Yeast for Brewing Novel Beer on the Example of Foreign Extra Stout. *Biomolecules*, *11*(12), 1778. https://doi.org/10.3390/biom11121778

Kim, Y., Hendrickson, R., Mosier, N. S., Ladisch, M. R., Bals, B., Balan, V., Dale, B. E., Dien, B. S., & Cotta, M. A. (2010). Effect of compositional variability of distillers' grains on cellulosic ethanol production. *Bioresource Technology*, *101*(14), 5385–5393. https://doi.org/10.1016/j.biortech.2010.02.054

Kirdponpattara, S., & Phisalaphong, M. (2013). Bacterial cellulose–alginate composite sponge as a yeast cell carrier for ethanol production. *Biochemical Engineering Journal*, *77*, 103–109. https://doi.org/10.1016/j.bej.2013.05.005

Klimczak, K., & Cioch-Skoneczny, M. (2022). Application of kveik yeasts for beer production ®. *Postępy Techniki Przetwórstwa Spożywczego*, *nr 1*. http://yadda.icm.edu.pl/baztech/element/bwmeta1.element.baztech-c86c86c6-f9cc-494d-b543-94e81045e599

Kosaric, N., & Velikonja, J. (1995). Liquid and gaseous fuels from biotechnology: Challenge and opportunities. *FEMS Microbiology Reviews*, *16*(2), 111–142. https://doi.org/10.1016/0168-6445(94)00049-5

Kveik: Norwegian farmhouse yeast | Larsblog. (n.d.). Retrieved September 18, 2023, from https://www.garshol.priv.no/blog/264.html

Kveik Yeastery. (n.d.). Kveik Yeastery. Retrieved January 29, 2024, from https://www.kveikyeast.com

Lai, Y.-T., Cheng, K.-C., Lai, C.-N., & Lai, Y.-J. (2019). Isolation and identification of aroma producing strain with esterification capacity from yellow water. *PLoS ONE*, *14*(2), e0211356. https://doi.org/10.1371/journal.pone.0211356

Laopaiboon, L., Thanonkeo, P., Jaisil, P., & Laopaiboon, P. (2007). Ethanol production from sweet sorghum juice in batch and fed-batch fermentations by Saccharomyces cerevisiae. *World Journal of Microbiology and Biotechnology*, *23*(10), 1497–1501. https://doi.org/10.1007/s11274-007-9383-x

Li, T., Chen, C., Chen, J.-C., Wu, Q., & Chen, G.-Q. (2014). Open and continuous fermentation: Products, conditions and bioprocess economy. *Biotechnology Journal*, *9*, 1503–1511. https://doi.org/10.1002/biot.201400084

Limayem, A., & Ricke, S. C. (2012). Lignocellulosic biomass for bioethanol production: Current perspectives, potential issues and future prospects. *Progress in Energy and Combustion Science*, *38*(4), 449–467. https://doi.org/10.1016/j.pecs.2012.03.002

Lin, Y., Zhang, W., Li, C., Sakakibara, K., Tanaka, S., & Kong, H. (2012). Factors affecting ethanol fermentation using Saccharomyces cerevisiae BY4742. *Biomass and Bioenergy*, *47*, 395–401. https://doi.org/10.1016/j.biombioe.2012.09.019

Liu, R., & Shen, F. (2008). Impacts of main factors on bioethanol fermentation from stalk juice of sweet sorghum by immobilized Saccharomyces cerevisiae (CICC 1308). *Bioresource Technology*, *99*(4), 847–854. https://doi.org/10.1016/j.biortech.2007.01.009

Maicas, S. (2020). The Role of Yeasts in Fermentation Processes. *Microorganisms*, *8*(8), 1142. https://doi.org/10.3390/microorganisms8081142

Mallouchos, A., Komaitis, M., Koutinas, A., & Kanellaki, M. (2003). Wine fermentations by immobilized and free cells at different temperatures. Effect of immobilization and temperature on volatile by-products. *Food Chemistry*, *80*(1), 109–113. https://doi.org/10.1016/S0308-8146(02)00247-9

Margaritis, A., & Merchant, F. J. A. (1987). The technology of anaerobic yeast growth. In D. R. Berry, I. Russell, & G. G. Stewart (Eds.), *Yeast Biotechnology* (pp. 231–276). Springer Netherlands. https://doi.org/10.1007/978-94-009-3119-0_8

Mathew, A. K., Crook, M., Chaney, K., & Humphries, A. C. (2013). Comparison of entrapment and biofilm mode of immobilisation for bioethanol production from oilseed rape straw using Saccharomyces cerevisiae cells. *Biomass and Bioenergy*, *52*, 1–7. https://doi.org/10.1016/j.biombioe.2013.02.028

Mohd Azhar, S. H., Abdulla, R., Jambo, S. A., Marbawi, H., Gansau, J. A., Mohd Faik, A. A., & Rodrigues, K. F. (2017). Yeasts in sustainable bioethanol production: A review. *Biochemistry and Biophysics Reports*, *10*, 52–61. https://doi.org/10.1016/j.bbrep.2017.03.003

Nadir, N., Mel, M., Karim, M. I. A., & Yunus, R. M. (2009). Comparison of sweet sorghum and cassava for ethanol production by using Saccharomyces cerevisiae. *Journal of Applied Sciences*, *9*(17), 3068–3073.

Najafpour, G., Younesi, H., & Syahidah Ku Ismail, K. (2004). Ethanol fermentation in an immobilized cell reactor using Saccharomyces cerevisiae. *Bioresource Technology*, *92*(3), 251–260. https://doi.org/10.1016/j.biortech.2003.09.009

Nigam, J. N. (2000). Continuous ethanol production from pineapple cannery waste using immobilized yeast cells. *Journal of Biotechnology*, *80*(2), 189–193. https://doi.org/10.1016/S0168-1656(00)00246-7

OECD. (2015). *OECD-FAO Agricultural Outlook 2015*. Organisation for Economic Co-operation and Development. https://www.oecd-ilibrary.org/agriculture-and-food/oecd-fao-agricultural-outlook-2015_agr_outlook-2015-en

Østgaard, K., Kowarz, V., Shuai, W., Henry, I. A., Sposob, M., Haugen, H. H., & Bakke, R. (2017). Syringe test screening of microbial gas production activity: Cases denitrification and biogas formation. *Journal of Microbiological Methods*, *132*, 119–124. https://doi.org/10.1016/j.mimet.2016.11.021

Parapouli, M., Vasileiadis, A., Afendra, A.-S., & Hatziloukas, E. (2020). Saccharomyces cerevisiae and its industrial applications. *AIMS Microbiology*, *6*(1), 1–31. https://doi.org/10.3934/microbiol.2020001

Pfeiffer, T., & Morley, A. (2014). An evolutionary perspective on the Crabtree effect. *Frontiers in Molecular Biosciences*, *1,17*. https://www.frontiersin.org/articles/10.3389/fmolb.2014.00017

Phisalaphong, M., Srirattana, N., & Tanthapanichakoon, W. (2006). Mathematical modeling to investigate temperature effect on kinetic parameters of ethanol fermentation. *Biochemical Engineering Journal*, *28*(1), 36–43. https://doi.org/10.1016/j.bej.2005.08.039

Postma, E., Verduyn, C., Scheffers, W. A., & Van Dijken, J. P. (1989). Enzymic analysis of the crabtree effect in glucose-limited chemostat cultures of Saccharomyces cerevisiae. *Applied and Environmental Microbiology*, *55*(2), 468–477. https://doi.org/10.1128/aem.55.2.468-477.1989

Prasertwasu, S., Khumsupan, D., Komolwanich, T., Chaisuwan, T., Luengnaruemitchai, A., & Wongkasemjit, S. (2014). Efficient process for ethanol production from Thai Mission grass (Pennisetum polystachion). *Bioresource Technology*, *163*, 152–159. https://doi.org/10.1016/j.biortech.2014.04.043

Preiss, R., Tyrawa, C., Krogerus, K., Garshol, L. M., & van der Merwe, G. (2018). Traditional Norwegian Kveik Are a Genetically Distinct Group of Domesticated Saccharomyces

cerevisiae Brewing Yeasts. *Frontiers in Microbiology*, *9*. https://www.frontiersin.org/articles/10.3389/fmicb.2018.02137

Pronk, J. T., Yde Steensma, H., & Van Dijken, J. P. (1996). Pyruvate Metabolism in Saccharomyces cerevisiae. *Yeast*, *12*(16), 1607–1633. https://doi.org/10.1002/(SICI)1097-0061(199612)12:16<1607::AID-YEA70>3.0.CO;2-4

Razmovski, R., & Vučurović, V. (2011). Ethanol production from sugar beet molasses by S. cerevisiae entrapped in an alginate–maize stem ground tissue matrix. *Enzyme and Microbial Technology*, *48*(4), 378–385. https://doi.org/10.1016/j.enzmictec.2010.12.015

Saint-Prix, F., Bönquist, L., & Dequin, S. (2004). Functional analysis of the ALD gene family of Saccharomyces cerevisiae during anaerobic growth on glucose: The NADP+-dependent Ald6p and Ald5p isoforms play a major role in acetate formation. *Microbiology*, *150*(7), 2209–2220. https://doi.org/10.1099/mic.0.26999-0

Sarkar, N., Ghosh, S. K., Bannerjee, S., & Aikat, K. (2012). Bioethanol production from agricultural wastes: An overview. *Renewable Energy*, *37*(1), 19–27. https://doi.org/10.1016/j.renene.2011.06.045

Silbir, S., Dagbagli, S., Yegin, S., Baysal, T., & Goksungur, Y. (2014). Levan production by Zymomonas mobilis in batch and continuous fermentation systems. *Carbohydrate Polymers*, *99*, 454–461. https://doi.org/10.1016/j.carbpol.2013.08.031

Singh, A., Sharma, P., Saran, A. K., Singh, N., & Bishnoi, N. R. (2013). Comparative study on ethanol production from pretreated sugarcane bagasse using immobilized Saccharomyces cerevisiae on various matrices. *Renewable Energy*, *50*, 488–493. https://doi.org/10.1016/j.renene.2012.07.003

Taherzadeh, M. J., & Karimi, K. (2011). Chapter 12—Fermentation Inhibitors in Ethanol Processes and Different Strategies to Reduce Their Effects. In A. Pandey, C. Larroche, S. C. Ricke, C.-G. Dussap, & E. Gnansounou (Eds.), *Biofuels* (pp. 287–311). Academic Press. https://doi.org/10.1016/B978-0-12-385099-7.00012-7

Tesfaw, A., & Assefa, F. (2014). Current Trends in Bioethanol Production by Saccharomyces cerevisiae: Substrate, Inhibitor Reduction, Growth Variables, Coculture, and Immobilization. *International Scholarly Research Notices*, *2014*, 532852. https://doi.org/10.1155/2014/532852

Torija, Ma. J., Rozès, N., Poblet, M., Guillamón, J. M., & Mas, A. (2003). Effects of fermentation temperature on the strain population of Saccharomyces cerevisiae. *International Journal of Food Microbiology*, *80*(1), 47–53. https://doi.org/10.1016/S0168-1605(02)00144-7

Tse, T. J., Wiens, D. J., & Reaney, M. J. T. (2021). Production of Bioethanol—A Review of Factors Affecting Ethanol Yield. *Fermentation*, *7*(4), Article 4. https://doi.org/10.3390/fermentation7040268

Wang, F.-S., Li, C.-C., Lin, Y.-S., & Lee, W.-C. (2013). Enhanced ethanol production by continuous fermentation in a two-tank system with cell recycling. *Process Biochemistry*, *48*(9), 1425–1428. https://doi.org/10.1016/j.procbio.2013.06.023

Willaert, R. (2011). *Cell Immobilization and Its Applications in Biotechnology* (pp. 313–367). https://doi.org/10.1201/b11490-13

Yang, Y., & Sha, M. (n.d.), 2019. *A Beginner's Guide to Bioprocess Modes – Batch, Fed- Batch, and Continuous Fermentation 408, 1-16*.

Ylitervo, P., Franzén, C. J., & Taherzadeh, M. J. (2011). Ethanol production at elevated temperatures using encapsulation of yeast. *Journal of Biotechnology*, *156*(1), 22–29. https://doi.org/10.1016/j.jbiotec.2011.07.018

Yu, J., Zhang, X., & Tan, T. (2007). An novel immobilization method of Saccharomyces cerevisiae to sorghum bagasse for ethanol production. *Journal of Biotechnology*, *129*(3), 415–420. https://doi.org/10.1016/j.jbiotec.2007.01.039

Zabed, H., Faruq, G., Sahu, J. N., Azirun, M. S., Hashim, R., & Nasrulhaq Boyce, A. (2014). Bioethanol Production from Fermentable Sugar Juice. *The Scientific World Journal*, *2014*, e957102. https://doi.org/10.1155/2014/957102

Zhou, Z., Li, G., & Li, Y. (2010). Immobilization of Saccharomyces cerevisiae alcohol dehydrogenase on hybrid alginate–chitosan beads. *International Journal of Biological Macromolecules*, *47*(1), 21–26. https://doi.org/10.1016/j.ijbiomac.2010.04.001

Appendix

Glucose D enzymatic Bioanalysis from R-Biopharm AG (Dams Stadt, Germany)

- Bottle 1 contained 2 g of powder mixture consisting of buffer with pH 7.6, NADP 110 mg, ATP 260 mg, and magnesium sulfate. The powder mixture was mixed with 45 ml of distilled water to prepare a solution.
- Bottle 2 contained 1.1 ml suspension, consisting of hexokinase (320 U) and glucose-6-phosphate dehydrogenase (160 U).
- D-glucose assay control solution for assay control purposes.

Both the bottle contents are stable at 2-8 ^{o}C. The test was conducted using a spectrophotometer (name of brand) with a wavelength of 340 nm. The assay was performed according to the manual as follows:

Pipette into cuvettes	Reagent Blank (RB)	Sample
Solution 1	1.0 ml	1.000 ml
Sample solution	-	0.100 ml
Redist water	2.0 ml	1.900 ml

The mixer solution was incubated for 3 minutes at room temperature, and absorbance was taken (A1)

Solution 1	0.020 ml	0.020

The mixer solution was incubated for 10-15 minutes at room temperature, and absorbance was taken (A2)

Calculation formulas are shown here:

Glucose concentration (g/l) = 0.864 * $\Delta A_{glucose}$

Liquid ethanol Enzymatic kit from R-Biopharm

Enzymatic UV determination of ethanol was performed according to R-biopharm manual at 340nm.

Reagent 1 containing buffer and reagent 2 containing NAD, and alcohol dehydrogenase were used as follows:

	Reagent blank (RB)	Samples/ controls
Reagent 1	2000 µl	2000 µl
Sample/control	-	100 µl
Dist. water	100 µl	-

Reagent 1 was mixed and incubated for 3 min at 37^{o}C and absorbance A1.

Reagent 2	500 µl	500 µl

Reagent 2 was mixed and incubated for 10 min at 37 ^{o}C and absorbance was taken using a spectrophotometer.

Enzymatic determination of **D-GLUCOSE**

The photometer is blanked against water. Wavelength 340 nm. Disposable cuvettes. Use the recommended dilution factor but calculate the concentrations as you proceed. Standard solution is measured as an ordinary sample and **is not diluted.** Use Used for adjusting results of unknown samples to the real assay response:
K= ($c_{std.\ nominal}$ / $c_{std.\ observed}$)

Concentration calculation: $c_{glucose}$ = 0,864 x $\Delta A_{D\text{-}glucose}$

Time (min)	A1 A2	A2-A1 = ΔA_{sample}	ΔA_{sample}- ΔA_{rbg} = $\Delta A_{D\text{-}glucose}$	Concentration in cuvette c (g l^{-1})	Dilution factor F	Corr. conc. in crude sample c' = cxFxK	*Remarks*
ROB YPG	0.098 0.099	0.001 =ΔA_{rbg}	-----	-----			*K =0.984*
StandardD-Glu	0.097 0.686	0.589	0.588	0.508= $C_{obs.}$	1	0.499	*C_{std}(g ^{1}):0.500*
T=0h	T=0h 0.441 19.02	19.19	19.18	16.57	1	16.30	
T=48h 32 °C	0.349 0.361	0.012	0.011	0.0095	1	0.0098	
37 °C	0.193 0.197	0.004	0.003	0.0025	1	0.0026	
42 °C	0.187 0.194	0.007	0.006	0.0051	1	0.0049	
47 °C	0.332 0.372	0.040	0.039	0.033	1	0.032	
ROB KYM	0.099 0.098	0.001					
Std Glu	0.097 0.686	0.589	0.588	0.508	1	0.500	
T=0h	0.087 0.274	0.187	0.186	0.161	100	15.84	
T=48h 32 °C	0.083 0.086	0.003	0.002	0.002	100	0.1968	
37 °C	0.067 0.063	0.004	0.003	0.0025	100	0.246	
42 °C	0.062 0.059	0.003	0.002	0.0017	100	0.167	
47 °C	0.090 0.092	0.002	0.001	0.001	100	0.0984	

Enzymatic determination of **ETHANOL**

The photometer is blanked against water. Wavelength 340 nm. Disposable cuvettes. Use the recommended dilution factor but calculate concentrations as you proceed. The standard solution is measured as an ordinary sample but **is not diluted.** Used for adjusting results of unknown samples to the real assay response: $K = (c_{\text{std. nominal}} / c_{\text{std. observed}})$

Concentration calculation: $c_{\text{ethanol}} = 0.095\Delta A_{\text{ethanol}}$

Time (min)		**A1**	**A2**	**A2-A1 = DA_{sample}**	**DA_{sample}-DA_{rbg} = $DA_{ethanol}$**	**Concentration in cuvette c (g l^{-1})**	**dilution**	**Corr. conc. in crude sample c' = cxFxK**	**Remarks**
Reaction background	ROB	0.001 0.192		0.191 =DA_{rbg}	-----	-----		-----	K =1.376
Standard ethanol	std	0.001 3.986		3.985	3.794	0.218			Cstd (0.300g l-1)
YPG Y9									
	T=0 h	0.005 0.243		0.238	0.047	0.004	100	0.61	
	T=48h 32 ºC	0.003 0.663		0.660	0.469	0.027	100	3.72	
	37 ºC	0.004 0.670		0.666	0.475	0.027	100	3.72	
	42 ºC	0.004 0.631		0.627	0.436	0.025	100	3.44	
	47 ºC	0.006 0.734		0.728	0.537	0.031	100	4.26	
KYM Y9									
	T=0h	0.040 0.348		0.308	0.117	0.010	100	1.44	
	T=48h 32 ºC	0.046 1.349		1.303	1.112	0.064	100	8.81	
	37 ºC	0.053 1.443		1.390	1.199	0.069	100	9.49	
	42 ºC	0.054 1.543		1.489	1.298	0.074	100	10.18	
	47 ºC	0.052 0.955		0.903	0.712	0.041	100	5.64	